UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Fitossanidade

Tese

Resistência de *Fimbristylis miliacea* (L.) Vahl aos herbicidas inibidores da enzima ALS

Carlos Eduardo Schaedler

Pelotas, 2011

CARLOS EDUARDO SCHAEDLER

RESISTÊNCIA DE *Fimbristylis miliacea* (L.) VAHL AOS HERBICIDAS INIBIDORES DA ENZIMA *ALS*

Tese apresentada ao Programa de Pós-Graduação em Fitossanidade da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área do conhecimento: Plantas Daninhas).

Orientador: José Alberto Noldin

Co-Orientadores: Dirceu Agostinetto
Nilda Roma Burgos

Pelotas, 2011

Dados de catalogação na fonte:
(Marlene Cravo Castillo – CRB-10/744)

S293r Schaedler, Carlos Eduardo
Resistência de fimbristylis miliacea(L.) Vahl aos herbicidas inibidores da enzima ALS / Carlos Eduardo Schaedler ; orientador José Alberto Noldin; co-orientador Dirceu Agostinetto. Pelotas,2011.-117f. :; il..- Tese (Doutorado) –Programa de Pós-Graduação em Fitossanidade. Faculdade de Agronomia Eliseu Maciel . Universidade Federal de Pelotas. Pelotas, 2011.

1.Planta daninha 2.Cuminho 3.Resistência a herbicidas 4.Acetolactato sintase I.Noldin, José Alberto(orientador) II. Título.

CDD 632.954

Banca examinadora:

_____
Ph.D. Aldo Merotto Junior

_____
Ph.D. Fernando A. Tcacenco

_____
Ph.D. Luis Antorio Avila

_____
Ph.D. Nilda Roma Burgos

_____
Ph.D. José Alberto Noldin
(Orientador)

Aos meus pais, Ismar e Beatriz;
Aos meus irmãos Luiz, Marcos, Márcio e minha irmã Lissandra;
A minha esposa, Fabiane.

OFEREÇO E DEDICO

"The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind."

Khalil Gibran

Agradecimentos

A Deus pela saúde e força em todos os momentos.

Ao Professor José Aberto Noldin por sua dedicada orientação, ensinamentos, incentivo e amizade.

A minha família, meus pais Ismar e Beatriz, e meus irmãos Luis, Marcos, Márcio e Lissandra, por todo o carinho, incentivo e apoio.

A minha esposa Fabiane Pinto Lamego pelo amor, estímulo na realização do curso, e também a sua família.

A Prof(a). Nilda Roma Burgos pela sua excepcional acolhida durante o doutorado sanduíche, e, também por sua dedicada e pacienciosa orientação, ensinamentos, incentivo e amizade.

Ao Prof. Dirceu Agostinetto, pelos ensinamentos, amizade e apoio durante o curso.

Aos integrantes da comissão de orientação, Professores Aldo Merotto Junior, Luis Antonio Avila, Nilda Roma Burgos e também ao pesquisador Fernando A. Tcacenco, pelas contribuições no aprimoramento deste trabalho e também pelo incentivo.

Ao Prof. Nilson Gilberto Fleck, pela amizade e incentivo na realização do curso na área de Estudo de Plantas Daninhas.

A UFPel e ao Programa de Pós-Graduação em Fitossanidade pela oportunidade de realização do curso e aos professores que contribuíram para minha formação.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro durante o curso e, também pela oportunidade de realização do doutorado-sanduíche.

Aos meus colegas da UFPel e amigos Angela Bundt, Camila Tarouco, Claudia Oliveira, Diecson da Silva, Franciele Mariani, Lisiane Fontana, Marcos Nohato, Nixon Westendorff, Rafael Rubin, Taísa Dal Magro, pela amizade, incentivo, auxílio na execução dos trabalhos e pelos momentos de convívio.

Aos meus amigos e colegas da University of Arkansas Paul Tseng, Ed Alcober, Reiofeli Salas, Leopoldo Estorninos e George Botha, pelo apoio tecnico e tambem amizade durante o Doutorado Sanduiche, e tambem Redentor Burgos, Eduardo Kawakami, Milena Quadros, Joao Meisen, Melissa Meisen e Adriano Mastrodomenico pelos momentos de descontração e convívio.

Aos estagiários e bolsistas da UFPel: Ana Langaro, Edimara Polidoro, Eduardo Mariotti, Lais Perboni, Thiago Duarte, pelo auxílio na execução dos trabalhos.

A todos os que, direta ou indiretamente, contribuíram para a realização deste trabalho.

Resumo

SCHAEDLER, Carlos Eduardo. **Resistência de *Fimbristylis miliacea* (L.) Vahl aos herbicidas inibidores da enzima ALS.** 2011. 117f. Tese (Doutorado) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas.

A evolução de muitos casos de resistência a herbicidas por diversas espécies infestantes das áreas de arroz irrigado tem causado problemas de falha de controle e redução de produtividade da área infestada. O objetivo deste trabalho foi de explorar a resistência de biótipos de *Fimbristylis miliacea* aos herbicidas inibidores da enzima ALS e investigar a habilidade competitiva relativa e a competição acima e abaixo do solo de biótipos de *Fimbristylis miliacea* resistente ou suscetível aos herbicidas inibidores da ALS com arroz, e entre os biótipos. Foram realizados quatro estudos: um em campo, dois em casa de vegetação, e um em laboratório. Foi determinada a resistência cruzada aos herbicidas inibidores da ALS em condição de campo (Artigo 1); para caracterizar o nível de resistência, experimentos de curvas de dose-resposta foram conduzidos em casa de vegetação, utilizando dois biótipos resistentes (FIMMI 10 e FIMMI 12) e um suscetível (FIMMI 13) (Artigo 2); foram comparadas habilidades competitivas e a competição pelos recursos luz e nutrientes entre os biótipos de *F. miliacea* resistente e suscetível aos herbicidas inibidores da ALS e destes com arroz irrigado (Artigo 3); e foi sequenciado o gene da enzima ALS nos biótipos estudados, a fim de desvendar o mecanismo que confere resistência aos herbicidas inibidores de ALS (Artigo 4). O experimento do artigo 1 foi conduzido em Santa Catarina, em área de arroz irrigado com populações de *F. miliacea* resistente ao herbicida pyrazosulfuron-ethyl; o experimento do artigo 2 e parte do experimento do artigo 3 foram conduzidos em casa de vegetação no Departamento de Fitossanidade da UFPel, em Capão do Leão – RS; parte dos trabalhos referentes

aos artigos 3 e 4 foram conduzidos no “Department of Crop, Soil and Environmental Sciences da University of Arkansas – Fayetteville, AR, USA”. Em condições de campo, *F. miliacea* apresenta resistência cruzada a inibidores da ALS utilizados em arroz irrigado. O biótipo FIMMI 10 apresentou resistência cruzada para três herbicidas inibidores da ALS dos grupos pyrimidinylthiobenzoates, sulfonylureas e triazolopyrimidines. O biótipo FIMMI 12 apresentou resistência cruzada para dois herbicidas inibidores da ALS dos grupos sulfonylureas e triazolopyrimidines. Para o estudo de competição, em proporções iguais de plantas, o arroz, independente do biótipo de *F. miliacea*, apresentou, em geral, maiores valores nas variáveis área foliar, estatura e matéria seca da parte aérea. A competição intraespecífica entre plantas de arroz é maior do que a competição inter-específica entre arroz e *F. miliacea.* A competição por recursos do solo tem maior efeito para *F. miliacea* e arroz. Análise das seqüências nucleotídicas e aminoácidos entre os diferentes biótipos indicaram um único ponto de mutação, timina-adenina, no biótipo FIMMI 10; a mutação encontrada resultou na substituição do aminoácido $Asp_{376}Glu$, na região F, entre os domínios C, A, D e B, E do gene da ALS.

Palavras-chave: Planta daninha, cuminho, resistência a herbicidas, acetolactato sintase.

Abstract

SCHAEDLER, Carlos Eduardo. **Resistance to ALS-inhibitor herbicides in *Fimbristylis miliacea* (L.) Vahl.** 2011. 117f. Ph.D. (dissertation) - Programa de Pós-Graduação em Fitossanidade, Departament of Plant Protection, Federal University at Pelotas, Pelotas.

Rice is one of the main foods for the most part of the world population. One of the main barriers that limit the full expression of the potential productivity of this crop is the competition by weeds. Among the methods of weed control in rice fields, herbicide is the most used. However, the evolution of many cases of resistance to these chemicals for several weed species has required farmers to adopt alternative methods of control and / or alternate herbicide mechanisms of action, which does not always result in the desired efficiency. The objective of this study was to explore the resistance of *Fimbristylis miliacea* biotypes to ALS inhibitor herbicides. Four studies were conducted: one in the field, two in greenhouse and one in the laboratory. It was determined the cross-resistance to ALS-inhibiting herbicides in field conditions (Article 1); caracterized the resistance level dose-response curve experiments were conducted in the greenhouse with two resistant biotypes (FIMM 10 FIMMI 12) and one susceptible (FIMM 13) (Article 2); it was compared the competitive ability and competition for light and nutrients resources between the biotypes of *F. miliacea* resistant and susceptible to ALS-inhibiting herbicides, and these with rice (Article 3); and it was sequenced the ALS enzyme gene from the biotypes studied to evaluate the mechanism of resistance to ALS-inhibiting herbicides (Article 4). The study 1 (Article 1) was conducted in Santa Catarina in a flooded rice field with resistant populations to pyrazosulfuron-ethyl. The study 2 (Article 2) and a part of the research of study 3 (article 3) were conducted in a greenhouse at the Departament of Plant Protection (“Fitossanidade”), UFPel in Capão do Leão – RS; part of the research of

study 3 (Article 3) and study 4 (Article 4) was conducted in the Department of Crop, Soil and Environmental Sciences at the University of Arkansas - Fayetteville, AR, USA. In field conditions, it was determined that *F. miliacea* is cross-resistant to ALS-inhibitors herbicides. The biotype FIMMI 10 showed cross-resistance to three chemical families of ALS-inhibiting herbicides (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). FIMMI 12 biotype showed cross resistance to two ALS-inhibiting herbicides (sulfonylureas and triazolopyrimidines). In equal proportions of plants, the rice, regardless of FIMMI biotype, presented, in general, higher values in the variables evaluated. Intraspecific competition is stronger among rice plants than interspecific with FIMMI biotypes. The competition for soil resources had higher effect for F*. miliaceae* and rice. Analysis of the nucleotide and amino acid sequences among the different biotypes indicated that a single point mutation, Thymine-Adenine, in the FIMMI 10 biotype; the mutation found resulted in an amino acid substitution $Asp_{376}Glu$, in the region F between C, A, D and B, E domains of the ALS gen.

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Introdução geral

O arroz e o cereal mais cultivado e principal alimento para a maior parte da população muncial juntamente com o trigo e o milho (AGOSTINETTO et al., 2001). Tanto no Brasil, como no Rio Grande do Sul (RS) e em Santa Catarina (SC), ele se posiciona entre as principais culturas. Nestes Estados, o cultivo de arroz, na safra 2010/11, foi superior a 1,2 milhão de ha. A produção desta mesma safra ficou em torno de 8,2 milhões de toneladas e a produtividade obtida no RS e SC foi 36% superior à média nacional (IBGE, 2011).

Nos últimos anos, o arroz vem se destacando, juntamente com a soja, como uma das culturas mais importantes no Sul do Brasil durante a estação estival de crescimento. A produtividade média do arroz no RS e em SC cresceu nos últimos anos. Na última safra, atingiu 7,1 t ha^{-1} (IBGE, 2011), o que decorre, especialmente, da utilização de cultivares com alto potencial produtivo e da adoção de tecnologias modernas. Porém, esta produtividade ainda está aquém daquela alcançada pela pesquisa. Este fato decorre, principalmente, do controle insatisfatório de plantas daninhas, as quais causam prejuízos quantitativos e qualitativos na produção do cereal.

Plantas daninhas interferem economicamente, de forma negativa, nas atividades humanas. A adoção do controle químico, a partir da década de 60, através do uso de herbicidas, representou um avanço nas técnicas de eliminação de plantas daninhas. Os benefícios obtidos com os herbicidas incluem: redução da competição das plantas daninhas desde o início do ciclo da cultura, rapidez de utilização, baixo custo quando comparado aos demais métodos de controle, dentre outros. Deste modo, formas alternativas para o controle das infestantes foram sendo menosprezadas, principalmente por agricultores que utilizam nível elevado de

tecnologia. No entanto, o uso de herbicidas também apresenta algumas limitações, destacando-se o surgimento de muitos casos de resistência a tais compostos por diversas espécies daninhas.

Até agosto de 2011, em nível mundial, haviam registrados 365 biótipos resistentes aos herbicidas envolvendo 200 espécies daninhas (HEAP, 2011). No Brasil, existem 18 plantas daninhas registradas como resistentes a algum grupo químico herbicida. Cerca de 60% dos casos representam resistência aos inibidores da enzima ALS (acetolactato sintase), envolvendo, principalmente, lavouras de soja, milho e arroz irrigado (HEAP, 2011).

A resistência de plantas daninhas aos herbicidas assume grande importância, principalmente em razão do limitado, ou inexistente número de herbicidas alternativos para serem usados no controle dos biótipos resistentes. O número de ingredientes ativos disponíveis para controle de algumas espécies daninhas é bastante restrito e o desenvolvimento de novas moléculas é difícil e oneroso. Portanto, o controle dos biótipos resistentes com o uso de herbicidas fica comprometido, o que restringe esta prática a outros métodos, muitas vezes, menos eficiente.

Herbicidas inibidores da enzima ALS incluem-se entre aqueles mais utilizados, constituindo importante grupo de herbicidas, devido à utilização em doses reduzidas, elevada seletividade para as principais culturas, ao grande espectro de infestantes controladas e ao perfil toxicológico favorável, devido à ausência desta enzima em animais.

Dentre as plantas daninhas resistentes mais problemáticas na cultura do arroz no Sul do Brasil há uma ciperácea, o cuminho (*Fimbristylis miliacea*). O controle seletivo desta espécie se dá unicamente através do uso de herbicidas seletivos, inibidores da enzima ALS. Em SC, existe relato de que esta espécie apresenta resistência a alguns grupos químicos de herbicidas inibidores da ALS (EBERHARDT; NOLDIN, 2004). Estes autores observaram resistência cruzada para os grupos sulfoniluréias e pyrimidinyl thiobenzoato em uma das populações de *F. miliacea* testadas na pesquisa.

Deste modo, visando obterem-se maiores informações sobre nível da resistência; habilidade competitiva; e determinação da base molecular entre biótipos resistentes e suscetíveis de *F. miliacea* aos herbicidas inibidores de ALS, o presente trabalho propõem-se a explorar estes temas.

O presente trabalho teve como hipóteses gerais: certos biótipos de *F. miliacea* apresentam resistência aos herbicidas inibidores da enzima ALS quando comparados a biótipos suscetíveis, aos diferentes grupos químicos que inibem a ALS; biótipos de *F. miliacea* resistentes e suscetíveis aos inibidores da ALS demonstram diferenças quanto a habilidade comeptitiva entre biótipos e entre a cultura do arroz; O mecanismo responsável pela resistência aos inibidores da ALS em certos biótipos de *F. miliacea* é o local de ação alterado;

Os objetivos do trabalho foram: Avaliar níveis de resistência em biótipos de FIMMI aos herbicidas inibidores da ALS, em resposta à aplicação de herbicidas pertencentes a quatro grupos químicos de inibidores da enzima; comparar, através de experimentos em série de substituição e de compatimentalização dos recursos luz e solo, habilidade competitiva dos biótipos de *F. miliacea* resistentes e suscetíveis aos inibidores da ALS; e isolar e sequenciar o gene da enzima ALS, em biótipos resistentes e suscetíveis de *F. miliacea*, associando-os ao mecanismo de resistência.

Revisão de literatura

Entre os produtos agrícolas que alimentam a população humana, o arroz (*Oryza sativa*) é utilizado como o principal alimento, constituindo-se, juntamente com o trigo e o milho, nos cereais mais produzidos no mundo. No Brasil, na safra 2009/10, a produção de arroz alcançou mais de 12 milhões de toneladas (t), o que garantiu ao País a nona posição entre os países produtores de arroz (FAO, 2008).

Devido às condições edafoclimáticas adequadas ao desenvolvimento dessa cultura na Região Sul do País, os Estados do Rio Grande do Sul (RS) e Santa Catarina (SC) respondem por 72% do total de arroz produzido no Brasil, alcançando produtividade média de 7,1 t ha^{-1} (IBGE, 2011). No entanto, esta produtividade está abaixo do seu potencial que é de 12 t ha^{-1} em lavouras experimentais (FAGUNDES et al., 2007). Um dos fatores negativos que se destaca como limitante ao preenchimento desta "lacuna" de produtividade é a alta infestação da maioria das lavouras com plantas daninhas, o que causa elevada redução na produtividade de grãos.

As plantas daninhas são responsáveis por significativas perdas nos cultivos agrícolas. Desta forma, o controle destas espécies torna-se indispensável no sentido de se evitarem prejuízos à produtividade de grãos. O emprego de herbicidas como forma de controle das plantas daninhas, aliado ao desenvolvimento de novas tecnologias de aplicação, representou um avanço para a agricultura moderna. Diversos herbicidas foram lançados no mercado, atuando por diferentes mecanismos de ação, tanto em pré como em pós-emergência.

O controle químico de plantas daninhas através de herbicidas justifica-se por reduzir a competição desde o início do ciclo da cultura, controlar as infestantes em

épocas chuvosas, causar poucos danos às raízes e folhas das culturas, rapidez de utilização e baixo custo, quando comparado com outros métodos de controle (VIDAL; MEROTTO JR., 2001).

Plantas daninhas, assim como outras espécies, estão em constante evolução, adaptando-se a perturbações ambientais provocadas pela natureza e pelo homem. Assim, as aplicações constantes de herbicidas têm proporcionado seleção da flora, resultando em populações resistentes.

Os primeiros registros de biótipos de infestantes resistentes incluíram *Commelina difusa* nos Estados Unidos e *Daucus carota* (cenoura-silvestre) no Canadá, em 1957, ambas resistentes aos herbicidas pertencentes ao grupo herbicida das auxinas sintéticas (WHITEHEAD; SWITZER, 1967; HEAP, 2011). Posteriormente, em 1970, nos Estados Unidos, constatou-se biótipos de *Senecio vulgaris* resistentes ao herbicida simazine, uma triazina pertencente ao grupo dos herbicidas inibidores do fotossistema II (FS2) (RYAN, 1970).

A evolução da resistência em uma área depende do fluxo gênico e da dispersão de pólen e de sementes no ambiente. O fluxo gênico, mediado pela dispersão de pólen, depende do movimento do pólen da planta resistente até a sensível e posterior formação de sementes viáveis (STALLINGS et al., 1995). A transferência do pólen entre plantas resistentes e sensíveis permite a dispersão da resistência principalmente em plantas com alta taxa de fecundação cruzada (SAARI; COTTERMAN; THILL, 1994).

O diagnóstico de resistência aos herbicidas pode ser realizado de duas maneiras: através de estudos *in vivo* ou *in vitro*. Os testes *in vivo*, podem ser realizados diretamente no campo ou em casa-de-vegetação. No campo, tem-se a desvantagem de não haver testemunha suscetível e de não ser prudente utilizar doses muito além daquelas indicadas no rótulo dos produtos. Para os testes em casa de vegetação, sementes de plantas suspeitas de resistência e também de plantas conhecidamente não resistentes são coletadas no campo. Após emergência das plantas, testa-se um conjunto de doses, comparando-se, então, o desempenho do herbicida nas populações sob teste (GAZZIERO et al., 1998; VIDAL; MEROTTO JR., 1999; BECKIE et al., 2000).

Estudos realizados *in vivo* em diversos locais no Brasil (GAZZIERO et al., 1998; VIDAL; MEROTTO JR., 1999; GAZZIERO et al., 2000) e no mundo (ITOH; WANG; OHBA, 1999; PRATLEY et al., 1999; ELEFTHEROHORINOS;

VASILAKOGLOU; DHIMA, 2000; TUESCA; NISENSOHN, 2001) foram eficazes na constatação de resistência de plantas aos herbicidas. No entanto, estes estudos apresentam, como desvantagem, a demora na obtenção dos resultados, a necessidade das sementes não estarem dormentes e a elevada utilização de mão-de-obra e de material (SAARI; COTTERMAN; THILL, 1994).

Considerando que a otimização de tempo seja importante, uma das grandes vantagens de métodos-diagnósticos, baseados no DNA, é que mutações geradas por genes recessivos também podem ser detectadas, assim como aquelas dominantes. Isto não ocorre em ensaios com plantas heterozigotas, uma vez que a aplicação herbicida elimina o resistente recessivo da população. Na espécie *Ambrosia trifida*, dois fragmentos de DNA isolados mostraram que a mutação de triptofano para leucina na posição 574 do gene de ALS (conforme ALS de *Arabidopsis thaliana*), foi a responsável pela resistência ao herbicida cloransulam. A partir desta informação, *primers* específicos foram desenhados para promoverem amplificações na região onde se identificou a mutação (PATZOLDT; TRANEL, 2002). Um conjunto de seis *primers*, baseados em seqüências de ALS depositadas no GenBank, permitiu obter-se a seqüência completa do gene de ALS para *Amaranthus retroflexus*. Neste caso, observou-se que as mutações de Ala_{122} para Thr, Ala_{205} para Val e Trp_{574} para Leu foram responsáveis por diferentes padrões de resistência dos biótipos aos herbicidas (McNAUGHTON et al., 2005). Trabalho mais recente, que utilizou PCR e cromatografia líquida de alta performance, demonstrou sucesso na detecção de mutações no gene de ALS (SIMINSZKY; COLEMAN; NAVEED, 2005).

Em muitos casos, a resistência de herbicidas inibidores de ALS resultou em alteração na enzima ALS, reduzindo a sensibilidade aos herbicidas (CHRISTOFFERS et al., 2006; LAMEGO et al., 2009; SCARABEL et al., 2010; MASSA et al., 2011); entretanto, aumento na taxa do metabolismo do herbicida também foi registrado (CHRISTOPHER, et al., 1991, CHRISTOPHER; POWLES; HOLTUM, 1992; VELDHUIS, et al., 2000).

Atualmente, já foram identificados um total de 17 diferentes substituições de aminoácidos selecionados intencionalmente em plantas, leveduras, bactérias, algas verdes e biótipos naturais selecionados (DUGGLEBY; PANG, 2000). Entretanto, 8 destes locais, sendo alanina 122 (Ala122), prolina 197 (Pro197), alanina 205

(Ala205), asparagina 376 (Asp376) arginina 377 (Arg377) triptofano 574 (Trp574), serina 653 (Ser 653) e glicina 654 (Gly654), foram confirmados em locais de ação em biótipos de plantas daninhas resistentes aos inibidores da ALS que foram investigados (TRANEL; WRIGHT, 2002; TRANEL.; WRIGHT; HEAP, 2011).

A resistência de espécies daninhas aos herbicidas inibidores da enzima ALS podem apresentar diferencas em valores adaptativos quando relacionado ao efeito de mutações observadas na enzima (VILA-AIUB; NEVE; POWLES, 2009). Como os herbicidas inibidores da ALS não se assemelham ao substrato envolvido na produção dos aminoácidos essenciais, e o domínio de ligação ser separado por um sítio catalizador, e provável que resistência devido a mutações tem um efeito desprezível sobre a funcionalidade da enzima ALS, enquanto outras mutações poderão alterar a funcionalidade ou ter efeitos pleiotrópicos (negativos) na planta. Deste modo, estudos mostram que determinados biótipos apresentam redução (EBERLEIN et al., 1997, EBERLEIN et al., 1999; ASHINGH; TARDIF, 2007), aumento (BOUTSALIS; KAROTAM; POWLES, 1999; YU et al., 2007; YU et al., 2003), ou inaltera a atividade da enzima ALS (BOUTSALIS; KAROTAM; POWLES, 1999; PRESTON et al., 2006).

Recentemente estudos foram realizados para avaliar habilidade competitiva entre biótipo resistente e suscetível a herbicidas inibidores da ALS. Um biótipo de picão-preto resistente aos herbicidas inibidores da ALS devido a mutação Trp574Leu no gene ALS (LAMEGO et al., 2009) foi comparado em experimento em série de substituição. Neste trabalho, os autores observaram que, em geral, não há diferença na habilidade competitiva entre os biótipos resistente e suscetível de picão-preto (LAMEGO et al., 2011). Neste mesmo sentido, estudos de habilidade competitiva entre biótipos de *Cyperus difformis* resistente e suscetível a herbicidas inibidores da ALS e destes com arroz irrigado, mostraram que os biótipos de *C. difformis* apresentam habilidade competitiva equivalente, por outro lado, a cultura do arroz mostrou habilidade competitiva superior aos biótipos (DAL MAGRO et al., 2011). Em estudo de competição por recursos do solo e radiação solar entre biótipos de *F. miliacea* resistente e suscetível aos herbicidas inibidores da ALS e destes com a cultura do arroz, o biótipo resistente foi menos competitivo com a cultura do que o biótipo suscetível (SCHAEDLER et al., 2011), porém, não foi possível afirmar que a

diferença na capacidade competitiva é causada por uma penalidade fisiológica devido a resistência, porque as populações estudadas nao sao isolinhas.

Biótipos de *Amaranthus retroflexus* (CONARD; RADOSEVICH, 1979), e *Chenopodium album* (PARKS et al., 1996) sensíveis a herbicidas triazinas apresentaram maior área foliar, altura e produção de sementes, em comparação ao resistente. No entanto, não foram detectadas diferenças na capacidade competitiva de *Abutilon theophrasti* resistente a triazina em relação ao biótipo sensível (GRAY; STOLTENBERG; BALKE, 1995). Em condições isentas de competição, genótipos de *Kochia scoparia*, resistentes e sensíveis a sulfoniluréias, apresentaram crescimento e produção de sementes similares; contudo, em condições de competição, o comportamento dos genótipos resistentes diferiu (THOMPSON; THILLD; SHAFFI, 1994).

ALS é a primeira enzima da rota biossintética dos aminoácidos de cadeia ramificada, valina, leucina e isoleucina (DURNER; GAILUS; BOGER, 1991). A primeira espécie resistente aos herbicidas inibidores da enzima ALS foi *Lolium rigidum*, no ano de 1982, na Austrália. Desde então, 109 espécies de plantas daninhas, incluindo 67 magnoliopsidas e 42 liliopsidas foram documentadas apresentando resistência simples ou cruzada aos herbicidas inibidores da ALS (HEAP, 2011). Neste sentido, devido à ocorrência de espécies daninhas resistentes aos herbicidas inibidores da ALS que se estende em muitas regiões agrícolas, esta enzima recebeu atenção considerável da comunidade científica envolvida com plantas daninhas. No Brasil, o primeiro caso de resistência foi registrado para a espécie *Euphorbia heterophylla* aos herbicidas inibidores da ALS, no ano de 1992. No Brazil, até julho de 2011, foram registradas 16 de 34 espécies como resistentes a este mecanismo de ação e 5 espécies de 6 estão registradas em lavouras de arroz (HEAP, 2011).

Na cultura do arroz irrigado, várias espécies daninhas ciperáceas a infestam, dentre elas, as do gênero *Fimbristylis*. *Fimbristylis miliacea*, conhecida popularmente por cuminho, é uma planta nativa que ocorre frequentemente na América Tropical. No Brasil, ela é mais freqüente na faixa litorânea da Região Sul, mas também é encontrada em outras regiões, como na Amazônia (KISSMANN, 1999). Esta espécie é uma importante infestante em lavouras de arroz irrigado em SC, podendo causar severas perdas quando não controlada. Para controlar *F. miliacea* nas lavouras de

arroz, os herbicidas inibidores da ALS incluem-se entre os mais utilizados. Entretanto, no Brasil, após anos seguidos de utilização deste mecanismo de ação, indivíduos de *F. miliacea* passaram a não mais responder a estes herbicidas. O primeiro caso de *F. miliacea* resistente a inibidores de ALS foi registrado em 2001, em SC (NOLDIN; EBERHARDT; RAMPELOTTI, 2002). Desde então, novos casos de *F. miliacea* resistente vêm surgindo com resistência a diferentes grupos químicos de inibidores da ALS.

A presença de plantas daninhas resistentes aos herbicidas nas lavouras causa incremento no custo de controle e, conseqüentemente, no custo final da produção. O manejo preventivo, ou seja, a utilização da rotação de mecanismos de ação herbicida para o controle de infestantes representa uma forma de se evitar que biótipos venham a ser selecionados para resistência. Entretanto, uma vez que haja suspeita de plantas daninhas resistentes numa área, é prudente obter-se, de forma rápida e eficiente, confirmação da resistência, de forma a serem adotadas as medidas de manejo adequadas, prevenindo-se a produção de sementes e sua disseminação na área.

Os herbicidas que compõem o grupo dos inibidores da ALS estão disponíveis no mercado mundial desde meados da década de 70. O primeiro inibidor de ALS registrado para a cultura do arroz no Brasil foi o herbicida Sirius (pyrazosulfuron-ethyl), no inicio da década de 90. Os inibidores da ALS dividem-se em cinco grupos químicos: sulfuniluréias, imidazolinonas, triazolopirimidinas sulfonanilidas, pirimidiniltiobenzoatos e sulfonilamina carboniltriazolinonas (TRANEL; WRIGHT, 2002). Estes herbicidas, de elevada eficácia e baixa toxicidade, apresentam um mecanismo de ação bastante específico, sendo utilizados em doses baixas. Entretanto, vários exemplos do aparecimento de resistência a tais herbicidas já foram relatados em nível mundial.

Casos de resistência cruzada ocorreram entre as classes de herbicidas inibidores da enzima ALS, dependendo da posição do aminoácido na ALS afetada e a substituição específica (SHANER, 1999). Geralmente, os padrões de resistência cruzada em plantas daninhas associam-se com alteração na ALS, podendo ser classificados como resistência aos grupos sulfoniluréias e triazolopirimidina sulfonanilidas, imidazolinonas e pirimidinil tiobenzoatos, ou ainda, resistência aos quatro grupos da enzima ALS (TRANEL; WRIGHT, 2002).

Em caso de detecção da resistência, necessitam-se estudos posteriores para verificar quais os mecanismos envolvidos na resistência, como suporte na tomada de decisão em estratégias de controle da espécie. Estes estudos incluem confirmação da resistência em biótipos suspeitos, definição da dose herbicida necessária para controlar 50% da população, realização de comparações biológicas, quantificações de absorção e translocação do herbicida e da atividade enzimática sequenciamento da enzima, em biótipos resistentes e sensíveis.

Artigo 01 - Revista Planta Daninha

CROSS RESISTANCE TO ALS-INHIBITOR HERBICIDES IN GLOBE FINGERUSH (*Fimbristylis miliacea*) UNDER FIELD CONDITIONS IN SOUTHERN BRAZIL

CROSS RESISTANCE TO ALS-INHIBITOR HERBICIDES IN GLOBE FRINGERUSH (*Fimbristylis miliacea*) UNDER FIELD CONDITIONS IN SOUTHERN BRAZIL[1]

ABSTRACT – ALS-inhibiting herbicides are highly effective, applied in low rates, have low toxicity to animals and wide weed control spectrum. Some of these herbicide have long soil persistence. Usually, the weeds control in flooded rice fields is achieved with these herbicides. Among the most problematic resistant weeds in flooded rice in Southern Brazil, is globe fringerush (*Fimbristylis miliacea* (L.) Vahl) belonging to the Cyperaceae family. This species used to be selectively controlled by ALS-inhibiting herbicides in rice. However, after consecutive years of use, *F. miliacea* biotypes began to show resistance to ALS inhibitors. The objective of this research was to investigate cross resistance to ALS inhibitors in *F. miliacea* biotype, under field conditions. A field experiment was conducted in a rice field naturally infested with *F. miliacea ALS*-resistant biotype, located in Santa Catarina, Southern Brazil, in 2008/09 and 2009/10. The experiment was a randomized complete block design, with five replicates, consisting of two factors (herbicide and rate) in a 4 x 5 factorial arrangement. The ALS herbicides were bispyribac-sodium, ethoxysulfuron, pyrazosulfuron-ethyl and penoxsulam. Plants at the six-leaf stage were sprayed with herbicide equivalent to 0, 0.5, 1, 2 and 4x the regular rates, using a sprayer calibrated to deliver 200 L ha^{-1} at 200 kPa. The variables evaluated in rice were culm number, filled and sterile grains, plant height, dry shoot biomass and grain yield. *F. miliacea* control was evaluated at 14, 28, and 70 days after herbicide application (DAA); Shoot biomass was harvested at 70 DAT and the dry weight recorded. The data were analyzed using ANOVA ($p \leq 0.05$) and fitted with regression models. A significant reduction was observed in culm number and yield of rice in competition with *F. miliacea*. *F. miliacea* biotype present in the experimental area showed to be resistant to all herbicides evaluated. Penoxsulam showed the highest level of activity among treatments at 28 and 70 DAT, but weed control level was only 50% and 43%, respectively. This was not enough to prevent crop yield loss. Therefore, *F. miliacea* in Santa Catarina is cross-resistant to ALS inhibitors used in rice. Alternative herbicides are necessary, to avoid yield losses in rice fields infested with ALS-resistant biotypes of *F. miliacea*.

[1] Recebido para publicação em __/__/__e na forma revisada em __/__/

Key Words: *F. miliacea*, herbicide resistance, sulfonylureas, pyrimidinyloxybenzoates, triazolopyrimidines.

RESUMO – Herbicidas inibidores da ALS são altamente eficazes, aplicados em baixas doses, apresentam baixa toxicidade para animais, amplo espectro de controle e alguns deles, são persistentes no solo. Esses herbicidas tem sido os mais comumente utilizados no controle de plantas daninhas em lavouras de arroz irrigado. O cuminho (*Fimbristylis miliacea* (L.) Vahl), pertence à família Cyperaceae e está entre as plantas daninhas problemáticas nas lavouras de arroz irirgado em Santa Catarina. Os herbicidas inibidores da enzima ALS tem sido utilizados para controlar seletivamente esta espécie. No entanto, após anos consecutivos de uso de inibidores da ALS, biótipos *F. miliacea* mostraram resistência aos inibidores da ALS. O objetivo deste trabalho foi investigar a resistência cruzada aos inibidores da ALS em biótipos de *F. miliacea*, em condições de campo. Os experimentos de campo foram realizados em lavoura de arroz naturalmente infestados por biótipo de *F. miliacea* resistente a ALS, localizada no municipio de Forquilhinha, Estado de Santa Catarina, Brasil, nos anos 2008/09 e 2009/10. O delineamento experimental foi em blocos casualizados, com cinco repetições, constituído de dois fatores (herbicida e dose) em arranjo fatorial 4 x 5. Os herbicidas foram bispyribac-sodium, ethoxysulfuron, pyrazosulfuron-ethyl e penoxsulam. Plantas no estádio de ate seis folhas, foram pulverizadas com herbicidas nas doses equivalentes a 0, 0,5, 1, 2 e 4X, sendo X=dose de referência recomendada, através de um pulverizador de pressão contante calibrado para uma vazão de 200 L ha^{-1} com pressão de 200 kPa. As variáveis avaliadas foram: número de colmos de arroz, número de espiguetas cheias e estéreis, estatura de planta, biomassa seca da parte aérea e produtividade. O controle de *F. miliacea* foi avaliada visualmente aos 14, 28 e 70 dias após aplicação de herbicida (DAA), utilizando a escala percentual de zero a 100%. A biomassa seca da parte aérea foi colhida aos 70 DAA e do peso seco registrado. Os dados foram analisados por ANOVA ($p \leq 0,05$) com modelos de regressão. Uma redução significativa foi observada no número de colmos e na produtividade do arroz em competição com *F. miliacea*. O biótipo presente na área experimental foi resistente a todos os herbicidas avaliados. O penoxsulam apresentou maior nivel de atividade entre os tratamentos aos 28 e 70 DAT, mas somente em 50 e 43%, respectivamente, foram controladas pelo herbicida. Esse nível de controle não foi suficiente para evitar a perda de produtividade da cultura. Portanto, *F. miliacea* apresenta resistência cruzada a inibidores da ALS utilizados em arroz irrigado. Herbicidas alternativos são necessários, para evitar perdas

de produtividade em lavouras de arroz infestadass por biótipos resistentes de *F. miliacea* aos inibidores da ALS.

Palavras-chave: *F. miliacea*, resistência a herbicidas, sulfoniluréas, pirimidinilthiobenzoates, triazolopirimidinas.

INTRODUCTION

Flooded rice ranks third among the major cash crops in the states of Rio Grande do Sul (RS) and Santa Catarina (SC), Brazil. In these states, the cultivation of flooded rice in the 2010/2011 season was more than 1.2 million ha. The production of rice in these states was around 9.2 million ton which was about 35% higher than the national average (IBGE, 2011).

In recent years, rice production has been increasing, along with soybeans, as one of the most important crops in southern Brazil during the summer growing season. The average yield of rice in RS and SC has grown in recent years. In 2010/11 crop season, the average yield was 7.1 t ha^{-1} (IBGE, 2011), which is the result of the use of cultivars with high yield potential and the adoption of new technologies. However, this productivity is still lower than the yield potential observed in experimental areas or the productivity obtained by many producers. In some regions, yields up to two times above average (12-14 t ha^{-1}) has been observed. Losses in productivity are due to several factors such as the unsatisfactory control of weeds, which cause quantitative and qualitative yield losses in flooded rice production.

The most frequent weed control method in rice fields in southern Brazil is with herbicides. However, the use of herbicides has limitations due to the increasing problems of weed resistance to several herbicidal compounds.

Currently, there are over 365 biotypes resistant to herbicides worldwide, involving approximately 200 weed species (Heap, 2011). In Brazil, there are 18 weed species recorded as resistant to herbicides and about 60% of cases involve resistance to ALS (acetolactate synthase) inhibitors used in soybean, maize and rice (Heap, 2011).

ALS-inhibiting herbicides are among those most used in rice in Southern Brazil. These, herbicides are used at low rates, are highly selective for the crop, has broad spectrum of weed control and have favorable toxicity profile, due to the absence of the ALS enzyme in animals (Vidal and Merotto Jr., 2001).

Among the most problematic resistant weeds in flooded rice in southern Brazil is globe fringerush (*Fimbristylis miliacea* (L.) Vahl), belonging to the Cyperaceae family and is present in the coastal area of southern Brazil (Kissmann, 2007; SOSBAI, 2010). Globe fringerush is a competitive weed in moist areas such as wetlands and shallow canals and rice farming. It produces large shoot biomass, causing lodging of rice plants, making it difficult to harvest rice (Embrapa, 2005). Selective control of this species is achieved through the use of herbicides including ALS inhibitors. In Santa Catarina State, there are reports of *F. miliacea* with cross-resistance to sulfonylureas and pyrimidinylthiobenzoates, both ALS inhibitors (Noldin et al., 2002).

The ALS enzyme is the primary target site of five classes of herbicides, namely sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB), and sulfonylamino-carbonyl-triazolinones (SCT) (Fischer et al., 2000). In Brazil is possible used four classes of ALS inhibitors are used in conventional rice field such as, SU, TP, PTB or IMI, the latter is used only by farmers growing Clearfield® rice.

Sulfunylurea herbicides such as pyrazosulfuron-ethyl plays an important role in rice weed control in Santa Catarina. The advantages for these herbicides are the low use rates, high selectivity for rice, broad spectrum and low toxicity. On the other hand, repeated use of the same herbicide, results in weed resistance (Fischer et al., 2000). Bispyribac-sodium, penoxsulam and ethoxysulfuron herbicides are also ALS inhibitors and commonly used in rice fields in Brazil. However, these herbicides are still important tools for controlling *F. miliacea* populations resistant to pyrazosulfuron-ethyl. Evaluating cross-resistance patterns to ALS inhibitors in *F. miliacea* under field condition is essential to determine the effectiveness of other herbicides with the same mode of action for controlling this weed and to define rational herbicide use programs to delay or slow the evolution of resistance to ALS inhibitors.

The hypothesis for this research is that some *F. miliacea* populations have resistance to different families of ALS herbicides under field conditions in Santa Catarina, Brazil. Thus, the experiment aimed to investigate cross resistance to ALS inhibitors in a *Fimbristylis miliacea* population, under field conditions.

MATERIALS AND METHODS

Two experiments were conducted in a rice field located in the county of Forquilhinha (28º 79' S, 49º 44' W), Santa Catarina, Brazil, from October 2008 to March 2009 and November 2009 to April 2010. The soil in the experimental area is classified as INCEPTISOL (Soil Survey Staff, 2003).

The experimental design used was randomized complete block in a factorial arrangement of herbicides and rates as a factors, with five replicates in the first year and four replicates in the second year. The herbicides evaluated were: pyrazosulfuron-ethyl (Sirius 250 SC, Iharabras), penoxulam (Ricer, Dow AgroSciences) + Iharol (760 EC), bispyribac-sodium (Nominee 400 SC, Iharabras) + Veget oil (760 EC), ethoxysulfuron (Gladium WG, Bayer) and bentazon (Basagran 600 CS, BASF) + carfentrazone-ethyl (Aurora 400 EC, FMC), added as a standard treatment. Herbicides rates were 0, 0.5, 1, 2 and 4X the regular field use rate. The regular field rates for ALS-inhibitor herbicides are: 17.5, 30, 50 and 80 g a.i ha^{-1} for pyrazosulfuron-ethyl, penoxulam, bispyribac-sodium and ethoxysulfuron, respectively. The regular field rate for bentazon + carfentrazone-ethyl herbicides were 720 and 40 g a.i ha^{-1}, respectively. Each experimental unit measured 10 m^2 (2 x 5 m). The rice cultivar used in both experiments was Epagri 109. Rice seeds were pre-germinated and broadcast-planted in a flooded field at a density of 400 viable seed m^{-2}. Rice was planted on October 23rd in 2008 and November 2nd in 2009, respectively. Soil fertilization and water management followed the recommended practices for rice production (SOSBAI, 2010). Nitrogen (N) fertilizer application was split: at the beginning of tillering and at the onset of stem elongation. *Echinochloa* spp. was controlled using cyhalofop-butyl (Clincher 180 EC, Dow AgroScience). Herbicides were applied with *F. miliacea* at six-leaf stage and rice at five-leaf stage, using a backpack sprayer calibrated to deliver 200 L ha^{-1} at 200 kPa.

The stand of rice and *F. miliacea* was monitored by counting emerged seedlings in 0.25m x 0.25m (0.125m^2), two samples per plot, at 27 days after sowing (DAS). These allowed estimating plant populations established for each species.

The variables evaluated for rice were: injury, culm number, filled and unfilled grains per panicle, plant height, dry biomass and grain yield. For *F. miliacea*, data collected included weed control and shoot dry mass. The percentage of crop injury and weed control were evaluated visually at 14, 28, 70, 91 and 107 days after treatment (DAT), on a scale of 0-100%. Total mortality of plants was recorded as 100% control. The culm number was counted at 107 DAT in 0.125m^2 area at two sites per plot. Immediately after counting, above-ground plant material was harvested from the same area. Plants were separated (rice and *F. miliacea*) and oven-dried for 72h at 60ºC and the dry weights were recorded. The weight of seeds was

obtained by weighing 500 grains randomly separated from grains harvested from each plot. Rice panicle were harvested 134 DAA from 4.25 m^2 per plot. After weighing the grains, the moisture was recorded and the weight was adjusted to 13% moisture, and the yield expressed in kg ha^{-1}.

Data were tested for homogeneity of variance and analyzed by ANOVA ($p \leq 0.05$) to determine herbicide x dose interactions, using Statistical Analysis System (SAS Institute, Cary, NC, USA) software. When a significant difference was detected, the treatment means were compared by applying Dunnet`s test (for culm number) or by Fisher`s test at the 5% level of probability (for unfilled grains). There were no significant ($p>0.05$) experiment effects, thus data from repeated experiments were pooled. The interaction of herbicide and dose was significant ($p<0.05$).

Weed control (%), shoot biomass and yield loss (%) data were fitted with the sigmoidal loglogistic model:

Equation 1: $$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where: Y corresponds to control (%), shoot biomass or yield loss (%), X is the herbicide application rate in g a.i ha^{-1}, *a* is the maximum asymptote, X_0 is the herbicide dose required to kill 50% of individuals or to reduce the shoot biomass or yield loss by 50% and *b* is the slope of the curve around X_0. Sigma Plot 10.0 was used for the regression analysis and curve fitting.

RESULTS AND DISCUSSION

The results reported below are derived from the field experiment involving the response of rice and the weed *F. miliacea* resistant to ALS-inhibiting herbicides. *F. miliacea* control at 28 and 70 DAT showed resistance to ALS-inhibitor herbicides there was a significant interaction between herbicide x rate ($p \leq 0.05$). Control data were fitted with a sigmoidal model (Equation 1), and the values of the parameters of the equation are shown in Table 1.

Table 1. Regrassion equation[a] and ED_{50} in *Fimbristylis miliacea* resistant to ALS inhibitors herbicides. Forquilhinha – SC, 2009/2010.

Herbicides	DAA[b]	a	b	ED_{50}[c]	R^2
pyrazosulfuron-ethyl	28	17.10	-2.31	> 4x	0.97
	70	22.79	-0.95	> 4x	0.99
bispyribac-sodium	28	28.64	0.96	> 4x	0.98
	70	5.62	-1.00	> 4x	0.99
penoxsulam	28	69.33	-1.44	> 4x	0.99
	70	91.35	-1.00	> 4x	0.99
ethoxysulfuron	28	26.95	-3.73	> 4x	0.99
	70	11.27	-2.48	> 4x	0.99

[a] Log-logistic equation: $Y = a/(1 + (X\text{-}X0)^b)$. ($p < 0.001$).
[b] Days after herbicide application.
[c] Herbicide dose responsible that would control 50% of the *F. miliacea*.

The level of control increased with herbicide rate but the herbicides did not provide adequate *F. miliacea* control at the recommended rate under field condition. The average control was less than or equal to 25%. At the first evaluation (28 DAT) 50% control was achieved only with the 4X dose of penoxsulam (120 g a.i. ha^{-1}) (Figure 1). At 70 DAT, weed control was similar to the first evaluation; for penoxsulam (43%), but other herbicides were only 4-10% effective (Figure 1).

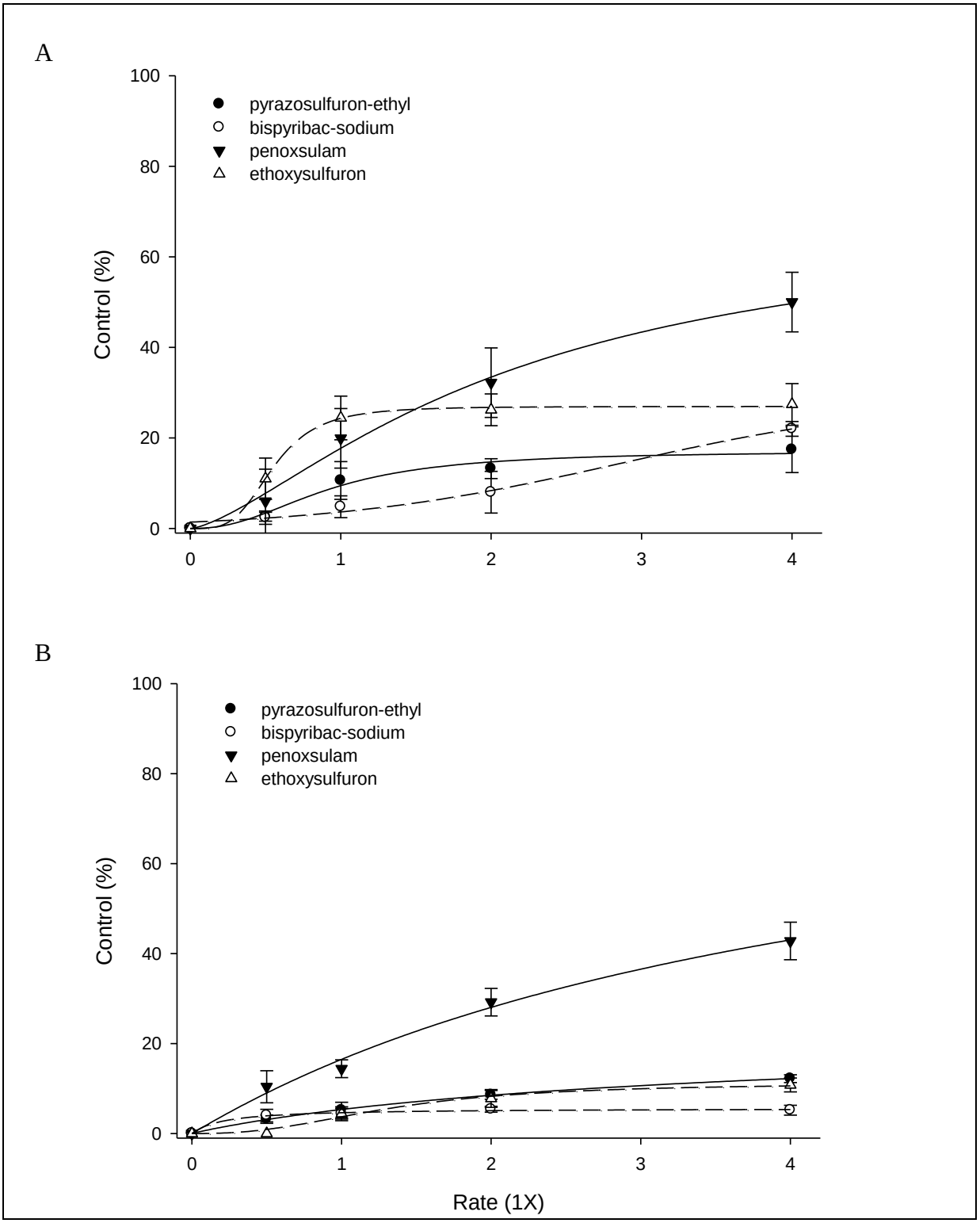

Figure 1. Control at 28 (A) and 70 DAT (B) of *Fimbristylis miliacea* resistant to ALS inhibitors herbicides following POST applications of pyrazosulfuron-ethyl (●), bispyribac-sodium (○), penoxsulam (▼) and ethoxysulfuron (Δ) herbicides. The vertical bars represent the confidence interval ($p \leq 0.05$). Forquilhinha – SC, 2009/2010.

The values of the parameters of the equation for Dry weight biomass are shown in Table 2. *F. miliacea* shoot dry weight at 91 DAA was reduced with increasing herbicide rate (Figure 2). The reduction caused by penoxsulam was higher than others herbicides at the maximum rate of 120 g a.i. ha^{-1} (Figure 2). The recommended rate of bispyribac-sodium (50 g a.i. ha^{-1}), reduced the dry weight of *F. miliacea* only 13% compared to the check plot. Herbicides pyrazosulfuron-ethyl and ethoxysulfuron showed similar reduction of shoot dry weight, about 32% at the lower rates of 17.5 and 80 g a.i. ha^{-1}, respectively. Penoxsulam provided the best control of *F. miliacea* in this study. *F. miliacea* shoot dry weight decreased more than 50% with the half rate of penoxsulam (15 g a.i. ha^{-1}), but did not differ from the efficacy obtained with rates of 30, 60 and 120 g a.i. ha^{-1}. The largest reduction in *F. miliacea* shoot dry weight (80%) was at the maximum rate of penoxsulam (Figure 2).

Table 2. Regration equation[a] and GR_{50} in *Fimbristylis miliacea* resistant to ALS-inhibitor herbicides at 107 days after herbicide application. Forquilhinha – SC, 2009/2010.

Herbicides	a	b	GR_{50}[b]	R^2
pyrazosulfuron-ethyl	99.9	0.86	3.12	0.97
bispyribac-sodium	101.8	1.39	3.42	0.99
penoxsulam	100.0	0.56	0.28	0.99
ethoxysulfuron	101.7	0.83	3.22	0.99

[a] Log-logistic equation: $Y = a/(1 + (X\text{-}X0)^b)$. ($p < 0.001$).
[b] Herbicide dose responsible for reducing growth rate of *F. miliacea* by 50%.

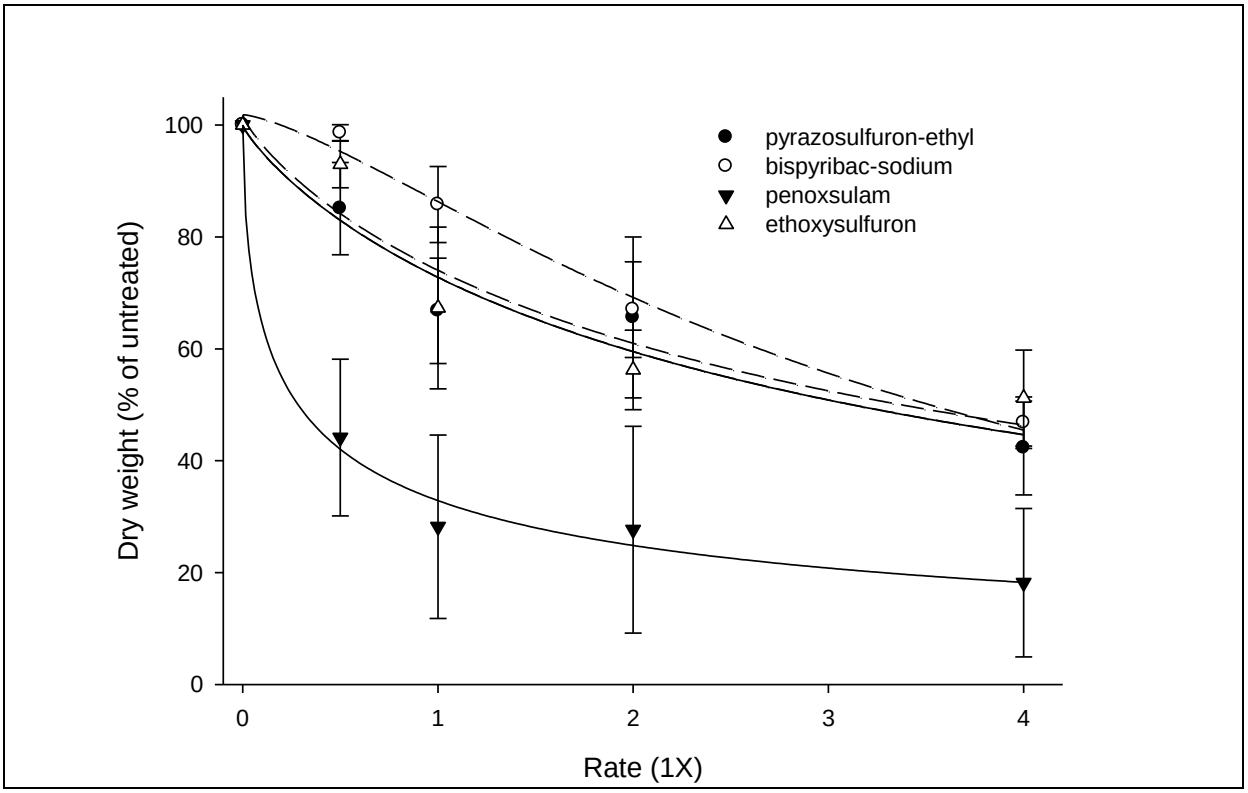

Figure 2. Dry weight biomass of *Fimbristylis miliacea* resistant to ALS inhibitors herbicides, at 70 DAA, following POST applications of pyrazosulfuron-ethyl (●), bispyribac-sodium (○), penoxsulam (▼) and ethoxysulfuron (Δ) herbicides. The vertical bars represent the confidence interval ($p \leq 0.05$). Forquilhinha – SC, 2009/2010.

Overall, the herbicides caused minimal injury on rice plants. At 28 DAA, the percentage of injury ranged from zero (check) to 6.6% (bentazon+carfentrazone-ethyl) (Table 3). Injury symptoms from the tank mixture of bentazon + carfentrazone-ethyl were characterized by initial reduction in plant growth and damage on leaves. However, at 70, 91 and 107 DAA there were no differences in crop injury among all treatments (data not shown).

Table 3. Injury of rice evaluated 28 days after herbicide application at different rates and ALS-inhibiting herbicides with comparative check (untreated and bentazon + carfentrazone-ethyl) in competition with *Fimbristylis miliacea* resistant to ALS-inhibiting herbicides

Treatments	Rates (X)	Rice injury (%)
pyrazosulfuron-ethyl	0.5	3.2 def
	1	4.2 de
	2	3.2 edf
	4	4.0 ed
bispyribac-sodium	0.5	0.6 ih
	1	0.6 ih
	2	3.0 defg
	4	6.2 bc
penoxsulam	0.5	2.2 fgh
	1	4.0 ed
	2	4.6 cd
	4	4.6 cd
ethoxysulfuron	0.5	1.4 ghi
	1	2.6 efg
	2	6.6 b
	4	6.2 bc
bentazon + carfentrazone-ethyl	0.75 + 0.83	9.4 a
Check (untreatead)		0.0 i
Means		3.7
CV		36.0

* Means within column with the same uppercase letters are not significantly different by Fischer's test ($p \leq 0.05$).

There were no differences among herbicide treatments for the number of filled grains, plant height and dry biomass of rice (Table 4). Penoxsulam tended to have higher dry biomass and taller plant compared to other ALS-inhibiting herbicides and tended to elevate the weight of rice filled grains. There was no interaction effect between herbicides and herbicide doses for unfilled grains the effect on the crop at 107 DAA had differences in herbicides treatments (Table 4). Plants sprayed with bentazon + carfentrazone-ethyl had less unfilled grains than with the others treatments.

Table 4. Filled and unfilled grains per panicle, plant height and dry biomass of rice at 107 DAA, Forquilhinha-SC, 2008/09 and 2009/10.

Treatments	Filled grains (panicle^{-1})	Unfilled grains (panicle^{-1})	Plant height (cm)	Dry biomass (g 0.25m^{-2})
pyrazosulfuron-ethyl	76^{ns}	16 a *	96^{ns}	164^{ns}
bispyribac-sodium	76	17 a	97	168
penoxsulam	79	16 a	97	183
ethoxysulfuron	78	18 a	97	159
bentazon + carfentrazone-ethyl	84	12 b	97	186
non-treated	75	16 a	99	178
Means	78	16	97	173
LSD	19	2.3	6	47

^{ns} Not significantly different by F test ($p \leq 0.05$). * Means within column with the same lowercase letters are not significantly different by Fischer's test ($p \leq 0.05$).

Culm number for rice was recorded at 107 DAA (Table 5). Rice culm number was reduced by all herbicide treatments compared with the weed-free plot (bentazon + carfentrazone-ethyl). However, there were no differences among herbicides and herbicide doses.

Table 5. Number of culm (m^{-2}) of rice evaluated at 107 days after herbicide application in competition with *Fimbristylis miliacea* resistant to ALS-inhibiting herbicides, Forquilhinha, 2008/09 and 2009/10.

Doses	pyrazosulfuron-ethyl	bispyribac-sodium	penoxsulam	ethoxysulfuron
0,5	528*	592*	560*	576*
1	536*	552*	520*	552*
2	520*	616*	600*	504*
4	576*	496*	568*	568*
Weed free		784		
LSD		152		

* Significant difference compared with the check (Weed free) by Dunnet's test ($p \leq 0.05$).

The highest rice yield, averaged over two years (9880 kg ha^{-1}) was obtained with the tank mixture of bentazon + cafentrazone-ethyl (720 + 40 g a.i. ha^{-1}), with 100% control of *F. miliacea.* This was the weed-free standard. All ALS herbicides reduced rice yield (Figure 3).

The values of the parameters of the equation for yield loss of rice are shown in Table 6. There was a significant interaction effect of herbicide x dose ($p \leq 0.05$) on rice yield. The lowest herbicide dose resulted in the highest yield loss, ranging from 33 to 44% for penoxsulam and ethoxysulfuron, respectively. At the maximum dose (4X) of pyrazosulfuron-ethyl, bispyribac-sodium and ethoxysulfuron, rice yield loss was 34% on average. The highest dose of penoxsulam showed the lowest yield loss of about 11%.

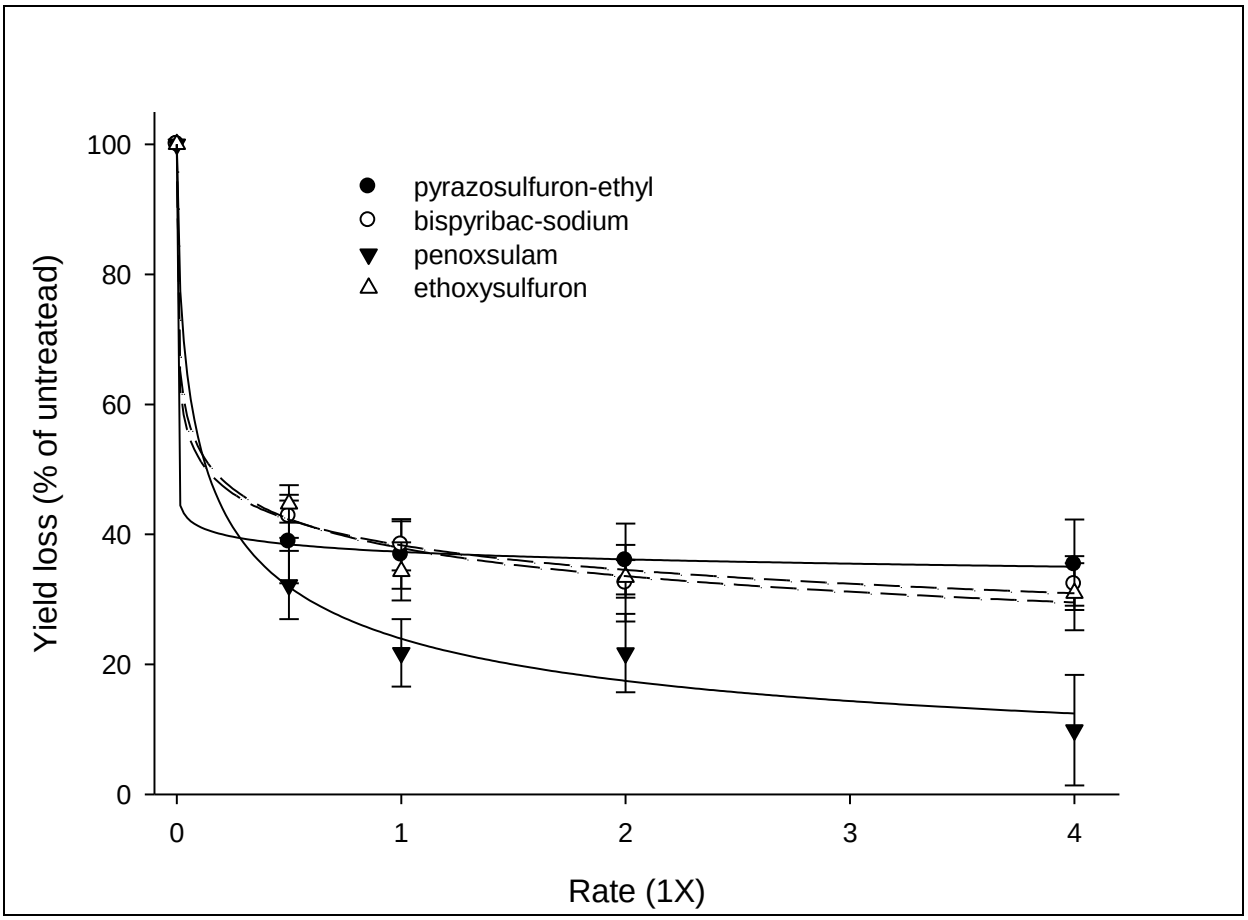

Figure 3. Yield loss of rice at 107 DAA following POST applications of pyrazosulfuron-ethyl (●), bispyribac-sodium (○), penoxsulam (▼) and ethoxysulfuron (Δ) herbicides. The vertical bars represent the confidence interval ($p<0.05$). Forquilhinha – SC, 2009/2010.

This experiment was conducted with the aim of investigating cross-resistance of the weedy species *F. miliacea* to ALS inhibitors under field conditions. There is evidence that the population infesting the experimental area (Noldin, et al., 2002) has cross-resistance to three chemical groups of ALS inhibitors: sulfonylurea, pyrimidylthiobenzoates and triazolopyrimidines (Figure 1 and 2). The occurrence of cross-resistance to different families of ALS inhibitors is common such as in ALS-resistant *Amaranthus palmeri* (Burgos et al.

2001), *C. difformis* (Galon et al. 2008), *Bidens subalternans* (Lamego et al. 2009), *Cyperus difformis* (Merotto et al. 2009) and many others. It is important to note that imidazolinone herbicides are also labeled for rice in Brazil. This herbicide family was not included in this study because the rice cultivar planted was not an imidazolinone-resistant (Clearfield^{TM} rice).

Table 6. Regression equation[a] to determine the herbicide dose necessary to obtain 50% yield loss of rice at 107 days after herbicide application. Forquilhinha – SC, 2009/2010.

Herbicides	a	b	YL_{50}	R^2
pyrazosulfuron-ethyl	100.0	0.07	< 0.01	0.99
bispyribac-sodium	100.1	0.23	0.13	0.99
penoxsulam	99.9	0.57	0.13	0.99
ethoxysulfuron	100.1	0.27	0.16	0.99

[a] Log-logistic equation: $Y = a/(1 + (X\text{-}X0)^b)$. ($p < 0.001$).
[b] Herbicide dose responsible for reducing yield loss of rice by 50%.

The dose-response curves were generated using the sigmoidal log-logistic model. This is the model that best describes plant response to increasing doses of herbicides (Seefeldt et al. 1995). In this experiment, the resistance factor to various herbicides could not be determined because there was no susceptible population; however, the experiment demonstrated cross-resistance to different families of ALS inhibitors. Weed control was lower than 50% in the first and second evaluation regardless of the ALS herbicide used. Furthermore, *F. miliacea* control declined at 28 and 70 DAA, indicating recovery from herbicide damage.

Pyrazosulfuron-ethyl and bispyribac-sodium were effective in controlling *F. miliacea*, but pyrazosulfuron caused some phytotoxic effects on rice (Begum et al., 2008). Noldin (1997) observed that bispyribac-sodium showed good activity on a number of damaging weeds, including grasses (*Echinochloa crusgalli, E. colonum*), broadleaves (*Sagitaria montevidensis, Ludwigia* spp) and sedges (*F. miliacea*) and was highly selective to rice.

In relation with grains per panicle, plant height and dry biomass there are no different between herbicides treatments. Different results were founded for grains per panicle and plant height (Begum et al., 2008). The authors observed shortest plants in plots with pyrazosulfuron-ethyl. This treatment was not different from the non-treated check. While the herbicide pyrazosulfuron-ethyl affecteds negatively the rice plant height. In this same study,

the authors observed that bispyribac-sodium increased, and pyrazosulfuron-ethyl reduced the number of grains per panicle (Begum et al., 2008). Results of this study showed that all herbicide treatments failed to increase the culm number due to the competition of *F.miliacea*. On an ALS-susceptible population, treatments with bentazon and pyrazosulfuron-ethyl also produced higher total culm numbers m^{-2} than bispyribac-sodium and the nontreated check as these treatments effectively controlled the weed (Begum et al. 2008). The ALS herbicides did not reduce the number of filled grains in the current study nor in previous related research (Begum et al. 2003).

Penoxsulam showed the best control of *F. miliacea* at 28 and 70 DAT (Fig. 1) and the highest reduction of dry biomass at 70 DAT (Fig. 2). In the area where these studies were conducted, rice was planted successively every year and pyrazosulfuron-ethyl was applied for at least ten years. The practice of rotating herbicides of the same mechanism of action such as bispyribac-sodium, penoxsulam and ethoxysulfuron cannot control the resistant *F. miliacea*. Thus, the utilization of herbicides such as bentazon, carfentrazone-ethyl or a mixture of these two chemicals can be an alternative to control F. *miliacea*. It is important to mention that a biotype of *Sagittaria montevidensis* was reported to have multiple resistance to ALS inhibitors and PSII inhibitors in Santa Catarina (Eberhardt and Noldin, 2011).

This study, supported the hypothesis that there was cross resistance to ALS-inhibitor herbicides pyrazosulfuron-ethyl (SU), penoxsulam (TP) bispyribac-sodium (PTB) and ethoxysulfuron (SU) in the population of *F. miliacea*. Alternative methods of control should be adopted to prevent the continued evolution of resistance of *F. miliacea* to ALS inhibitors in flooded rice areas in Santa Catarina.

ACKNOWLEDGEMENTS

We are grateful to the CNPq for the scholarship to Carlos Eduardo Schaedler. We are grateful to farmer Mr. Marcelino Schmitt for allowing using the area in his farm for this research.

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Artigo 02 - Revista Planta Daninha

CROSS-RESISTANCE PATTERNS OF ACETOLACTATE SYNTHASE (ALS) INHIBITOR-RESISTANT GLOBE FRINGERUSH (*Fimbristylis miliacea*) BIOTYPES IN SOUTHERN BRAZIL

CROSS-RESISTANCE PATTERNS OF ACETOLACTATE SYNTHASE (ALS) INHIBITOR-RESISTANT GLOBE FRINGERUSH (*Fimbristylis miliacea*) BIOTYPES IN SOUTHERN BRAZIL[2]

ABSTRACT – Weeds resistant to herbicides are widespread worldwide. *Fimbristylis miliacea* (L.) Vahl is one of the most troublesome weeds in water-seeded rice fields in Santa Catarina, Southern Brazil. Acetolactate synthase (ALS)-inhibiting herbicides are widely used to control weeds in rice. The continuous use of ALS-inhibiting herbicides has led to the evolution of herbicide-resistant *F. miliacea* populations. The objective of this research was to characterize resistance patterns of ALS inhibitor-resistant globe fringerush biotypes using whole-plant dose-response assays. To confirm the resistance of *F. miliacea* to ALS inhibitors, whole-plant bioassays were conducted in 2008 and 2009 at Federal University at Pelotas, RS, Brazil. The experiment was a randomized complete block design, with four replicates consisting of three factors (biotype, herbicide and rate) in a 3 x 4 x 7 factorial arrangement. The ALS herbicides were bispyribac-sodium, pyrazosulfuron-ethyl, penoxsulam and (imazethapyr+imazapic). A standard herbicide treatment, bentazon, was also included. Plants at the six-leaf stage were sprayed with herbicide equivalent to 0, 1, 2, 4, 8, 16 and 32X the label rate, for the resistant biotypes (FIMMI 10 and FIMMI 12) and 0, 1/32, 1/16, 1/8, 1/4, 1/2 and 1X for the susceptible biotype (FIMMI 13). Herbicide treatments were applied using a backpack sprayer calibrated to deliver 150 L ha^{-1} at 200 kPa. The efficacy of the herbicides treatments on *F. miliacea* plants was visually evaluated as percentage control at 7, 14, 21 and 28 days after treatment (DAT) and dry biomass weight at 28 DAT. FIMMI 10 biotype showed cross resistance to three chemical families of ALS-inhibiting herbicides (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). FIMMI 12 biotype showed cross resistance to two ALS-inhibiting herbicides (sulfonylureas and triazolopyrimidines). Bentazon is still an effective alternative to control *F. miliacea* in areas with ALS herbicide resistance populations. None of the biotypes showed cross resistance to imidazolinones, i.e. the premix formulation of (imazethapyr+imazapic).

Key Words: flooded rice, weed resistance, mechanism of resistance, herbicide mode of action.

[2] Recebido para publicação em ___/___/___e na forma revisada em ___/___/

RESUMO – Plantas daninhas resistentes a herbicidas estão mundialmente difundidas. *Fimbristylis miliacea* L. (Vahl) é uma das plantas daninhas problemáticas em lavouras de arroz irrigado em Santa Catarina.. Herbicidas inibidores da enzima acetolactato sintase (ALS) são amplamente utilizados para controlar plantas daninhas em arroz. O uso contínuo de herbicidas inibidores da ALS levou à evolução de resistência de populações de *F. miliacea* aos herbicidas inibidores da ALS. O objetivo desta pesquisa foi caracterizar os niveis de resistência de biótipos de *F. miliacea* "a inibidores da ALS, utilizando experimentos de curva-dose resposta. Para confirmar a resistência de *F. miliacea* aos inibidores da ALS, experimentos foram conduzidos em 2008 e 2009 na Universidade Federal de Pelotas. O delineamento experimental foi em blocos casualizados, com quatro repetições em arranjo factorial 4 x 3 x 7 (biótipo x herbicida x dose). Os herbicidas inibidores da ALS utilizados foram: bispyribac-sódio, pyrazosulfuron-ethyl, penoxsulam e (imazethapyr+imazapic). Também foi incluído um tratamento herbicida padrão, bentazon. Plantas em estádio de seis folhas foram pulverizadas com herbicidas nas doses equivalentes a 0, 1, 2, 4, 8, 16 e 32X a dose de rótulo para o biótipos resistentes (FIMMI 10 e FIMMI 12) e 0, 1/32, 1/16, 1/8, 1/4, 1/2 e 1X para o biótipo suscetível (FIMMI 13). Os tratamentos herbicidas foram aplicados utilizando pulverizador costal calibrado para aplicar 150 L ha^{-1} na pressão de 200 kPa. O controle de *F. miliacea* foi avaliado visualmente, utilizandso a escala percentual, de zero a 100%, aos 7, 14, 21 e 28 dias após o tratamento (DAT) e o peso de biomassa seca foi determinado aos 28 DAT. O biótipo FIMMI 10 apresentou resistência cruzada para três grupos de herbicidas inibidores da ALS (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). O biótipo FIMMI 12 apresentou resistência cruzada para dois grupos de herbicidas inibidores da ALS (sulfonylureas and triazolopyrimidines). Bentazon ainda é uma alternativa eficaz para controlar poluções de *F. miliacea* em áreas com resistência à herbicidas inibidores da ALS. Nenhum do biótipos avaliados mostrou resistência cruzada a imidazolinonas, como por exemplo, a formulação de (imazethapyr+imazapic).

Palavras-chave: arroz irrigado, plantas daninhas resistentes, mecanismo de resistência, modo de ação de herbicidas.

INTRODUCTION

Weed resistance became one of the most important limitations for weed management in many crops. There are 358 resistant (R) weed biotypes reported to date, belonging to about 197 weed species worldwide (Heap, 2011) and the list is growing (Volenberg et al., 2001; Christoffers et al., 2006; Hamouzová et al., 2010). The repeated use of herbicides with the same mode of action, in the same area and for consecutive years, has resulted in the development of weed resistance. The use of herbicides that inhibit the acetolactate synthase (ALS) enzyme has resulted in the highest number and most widespread cases of R-weeds worldwide (Heap, 2011). Currently, there are 67 dicotyledonous and 42 monocotyledonous weed species resistant to ALS-inhibiting herbicides around the world. In Brazil, 11 of the 18 weed species reported to be resistant are to ALS-inhibiting herbicides and 5 of the 6 weed species are infesting rice fields (Heap, 2011).

Herbicide resistance can be attributed to non-target site or target site alterations. Non-target site-based resistance is caused by mechanisms that reduce the amount of active herbicide that reaches the target enzyme or binding domain. ALS resistance has been attributed to an enhanced ability to metabolize the herbicides (Christopher et al., 1992; Veldhuis et al., 2000). Target site-based resistance in many cases is due to single mutations in the amino acid sequence resulting in conformational changes to the herbicide binding site of the target enzyme (Christoffers et al., 2006; Lamego et al., 2009; Scarabel et al., 2010; Massa et al., 2011). Acetolactate synthase (also referred Acetohydroxiacid synthase – AHAS, EC 2.2.1.6) is a plastidic enzyme that is found in bacteria, fungi and plants (Duggleby and Pang, 2000). AHAS catalysis the decarboxylation of pyruvate, and its condensation with another pyruvate produces acetolactate, the precursor of valine and leucine, while with an alfa-ketobutyrate the reaction produces acetohydroxy butyrate leading to isoleucine synthesis (Chipman et al., 1998).

ALS enzyme is the target site of many families of commercial herbicides including the sulfonylureas (SUs) (Chaleff and Mauvais, 1984), imidazolinones (IMIs) (Shaner et al., 1984), pyrimidinyloxybenzoates (PTBs) (Stidham, 1991); triazolopyrimidines (TPs) (Gerwick et al., 1990) and sulfonylaminocarbonyltriazolinones (SCTs) (Santel et al., 1999). As result of the intensive use of ALS-inhibitors herbicides, 109 weed species have evolved resistance (Heap, 2011). ALS-inhibiting herbicides affect growing plants two hours after treatment, before any effect on other processes such as the photosynthetic reaction, aerobic respiration or synthesis of RNA and proteins (Vidal and Merotto Jr., 2001). These herbicides have been

extensively used in the last decades due to their high efficacy at low concentrations, low impact on non-target organisms and good selectivity in several crops (Mazur and Falco, 1989; Vidal and Merotto Jr., 2001).

In Brazil, rice (*Oryza sativa* L.) ranks as a major crop mainly in Rio Grande do Sul and Santa Catarina states. Yield loss is due to several factors, highlighting the unsatisfactory control of weeds, which cause quantitative and qualitative losses in rice production. ALS inhibitors are the main herbicides used to control weeds; however, resistances to these herbicides are widespread in rice fields in Southern Brazil. A *Cyperus difformis* biotype showed cross-resistance to ALS inhibitors (sulfonylureas and pyrimidinyloxybenzoates) (Galon et al., 2008). Resistance of *C. difformis* biotype to pyrazosulfuron-ethyl results from the insensitivity of the ALS enzyme to herbicide (Dal Magro et al., 2010). Also, red rice (*Oryza sativa* L.) populations have survived treatments with ALS-inhibitor (imazethapyr+imazapic) in rice fields under Clearfield system (Menezes et al., 2009). *Sagitaria montevidensis* (SAGMO) populations resistant to ALS-inhibitors are widespread in rice fields Santa Catarina. Merotto Jr. et al. (2010) showed SAGMO resistant in a rice field in Southern Brazil treated at least for five years with pyrazosulfuron-ethyl.

Fimbristylis miliacea (L.) Vahl is currently considered a troublesome weed in flooded rice production in Santa Catarina (Noldin et al., 2002). *F. miliacea* belongs to the Cyperaceae family and is present on the coastal area of Southern Brazil and is among the species that infest rice fields (Kissmann, 2007). This weed is a summer-germinating annual sedge, often present in very high densities and has evolved resistance to pyrazosulfuron-ethyl, a ALS-inhibitors herbicide (Noldin et al., 2002).

The diagnosis of weed resistance to herbicides has been done by different types of studies (Vidal et al., 2006). Whole plant assays carried out in greenhouses in dose-response curves evaluating the growth plant are classics to confirm cases of resistance to herbicides (Vidal and Trezzi, 1999; Gazziero et al., 2000; Merotto Jr. et al., 2009; Hamouzová et al., 2011).

The hypothesis for this research is that *ALS*-resistant *F. miliacea* biotypes have cross resistance to different families of ALS-inhibitors herbicides. Thus, the objectives for this research were: 1) to confirm the resistance to ALS-inhibiting herbicides in two biotypes of *F. miliacea* and, 2) to characterize resistance and cross-resistance to ALS-inhibiting herbicides in the resistance *F. miliacea* biotypes using whole-plant dose response assays.

MATERIALS AND METHODS

Plant materials

Seeds of two globe fringerush resistant biotypes (FIMMI 10 and FIMMI 12) and one susceptible (FIMMI 13) to ALS-inhibiting herbicides were collected from rice fields in Santa Catarina, Southern Brazil (Table 1). ALS-inhibiting herbicides were applied in these areas for at least 10 consecutive years.

Table 1. Location of *Fimbristylis miliacea* resistant and susceptible biotypes to ALS-inhibitors herbicides from GPS data.

Biotype	Longitude	Latitude
FIMMI 10	49º 26’ 528’’W	28º 47’ 912’’ S
FIMMI 12	49º 33’ 971’’ W	28º 47’ 881’’ S
FIMMI 13	49º 44’ 776’’ W	27º 16’ 987’’ S

Whole-plant bioassay

Dose response experiments were performed in 2008 and 2009, using greenhouse-grown seedlings. Seeds of globe fringerush susceptible (FIMMI 13) and two resistant biotypes (FIMMI 10 and FIMMI 12) were planted in 500 mL pots filled with soil derived from rice fields and placed in a greenhouse at the Federal University at Pelotas-RS. The growth conditions were 35/25°C day/night temperature (± 5°C), with a photoperiod of 14h. Plants were treated with pyrazosulfuron-ethyl (Sirius 250 SC, Iharabras), bispyribac-sodium (Nominee 400 SC; Iharabras), penoxulam (Ricer, Dow AgroSciences), (imazethapyr+imazapic) (Only®, BASF) and bentazon (Basagran 600, BASF). The herbicide rate for S and R biotypes were equivalent to 0, 1/32, 1/16 1/8, 1/4, 1/2, 1X, and 0, 1, 2, 4, 8, 16, 32X the regular field used rate, respectively. The rates evaluated were: 17.5, 30, 50, (75+25), and 960 g a.i ha^{-1} for pyrazosulfuron-ethyl, penoxsulam, bispyribac-sodium, imazethapyr+imazapic, and bentazon, respectively (Table 2). Herbicides were applied at the six-leaf stage plants with a non-ionic surfactant (0.5% v/v), using a backpack sprayer delivering 150 L ha^{-1} at 200kPa.

Table 2. Herbicides and doses to obtain the dose response curve in *Fimbristylis miliacea* biotypes resistant (FIMMI 10 and FIMMI 12) and susceptible (FIMMI 13) to ALS-inhibitors. UFPel, Capão do Leão – RS, 2008-2009.

Herbicide dose (g a.i. ha^{-1})				
pyrazosulfuron-ethyl[a]	bispyribac-sodium[b]	penoxsulam[c]	(imazethapyr+imazapic)[d]	bentazon[e]
FIMMI 10 and FIMMI 12				
0,0	0,0	0,0	0,0	0,0
17.5*	**50**	**30**	**75+25**	**960**
35	100	60	150+50	1920
70	200	120	300+100	3840
140	400	240	600+200	7680
208	800	480	1200+200	15360
560	1600	960	2400+800	30720
FIMMI 13				
0,0	0,0	0,0	0,0	0,0
0.55	1.563	0.94	2.34+0.78	30
1.09	3.13	1.88	4.69+1.56	60
2.19	6.25	3.75	9.38+3.13	120
4.37	12.5	7.5	18.75+6.25	240
8.75	25	15	37.5+12.5	480
17.5	**50**	**30**	**75+25**	**960**

[a] Sirius 250 SC, Iharabras;
[b] Nominee 400 SC (Iharabras) + Iharol 0.5% v/v;
[c] Ricer 240 SC (DowAgroScience) + Veget Oil 0.5% v/v,;
[d] Only® CS (BASF) + Dash 0.5% v/v,;
[e] Basagran 600 (BASF) + Assist 0.5% v/v.
[*] Numbers in bold represent the regular rate used in the field.

The experiment was a randomized complete block design consisting of three factors (biotype, herbicide and rate) in a 3 x 4 x 7 factorial hierarchical arrangement. The treatments containing one plant per pot were replicated four times. Weed control was visually rated at 7, 14, 21 and 28 days after treatment (DAT), using the percentual scale (0 to 100%). Total mortality of plants was recorded as 100% control. At 28 DAT, above-ground plant material was cut and oven-dried for 72h at 60°C and biomass dry weights recorded.

GR_{50} values (the rate necessary to provide 50% reduction of control and dry matter) of resistant and susceptible biotypes were obtained from mathematical models adjusted.

Statistical analysis

Data were tested for homogeneity of variance and analyzed using ANOVA ($p \leq 0.05$) to determine biotype (rate) x herbicide interactions, using Statistical Analysis System (SAS) software. When significant difference was detected, the data were tested by sigmoidal loglogistic model:

Equation 1:
$$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

were: Y corresponds to control (%) or shoot biomass; X is the herbicide rate in g a.i ha^{-1}; *a* is the maximum asymptote; X_0 is the herbicide rate required to kill 50% of the individuals or to reduce the shoot biomass or yield loss (acordding to the variable) and *b* is the slope of the curve around X_0. Weed control and shoot biomass data were fitted with the sigmoidal loglogistic model (Seefeldt et al., 1995) using the herbicide rate as an independent variable. Sigma Plot 10.0 (Sigma Plot, 2004) was used for the regression analysis and curve fitting.

The rate of herbicide that would control 50% of the individuals (C_{50}) or reduce above-ground dry weight 50% (GR_{50}) were calculated from the regression equations. The resistance index (RI) was calculated by dividing the C_{50} or GR_{50} values of each R biotype by the corresponding values of the S biotype.

RESULTS AND DISCUSSION

F. miliacea control at 28 DAA showed resistance to ALS-inhibitors herbicides there was a significant interaction between herbicide x rate ($p \leq 0.05$). The whole-plant bioassay confirmed that FIMMI 10 and FIMMI 12 were insensitive to three and two chemical families of ALS herbicides, respectively (Tables 3 and 4). In both experiments (2008 and 2009), the highest rate evaluated (32X) of pyrazosulfuron-ethyl was not enough to achieve the the GR_{50} for biotype FIMMI 12. The sigmoidal model used to fit the weed control and dry weight data allowed the estimation of the resistance factor. For dry weight variable, a dose response curve indicates reduction in all biotypes due to the increased rate of the herbicide. However, the susceptible biotype showed higher reduction than the resistant biotypes.

Table 3. Estimated parameters a, b and C_{50} by non-linear regression equation[a], based on control (%) in whole-plant bioassays for two resistant and one susceptible biotype of *Fimbristylis miliacea*, affected by ALS-inhibiting herbicides, 4 weeks after treatment, UFPel, Capão do Leão – RS, 2008-2009.

Herbicide	Biotype	a	b	C_{50} (g a.i. ha^{-1})	RI[b]
pyrazosulfuron-ethyl	FIMMI 10	106.4	-1.32	58.4 ± 7.37	116.8
	FIMMI 12	67.1	-2.51	217.7 ± 19.17	435.4
	FIMMI 13	96.4	-2.38	0.5 ± 0.02	-
bispyribac-sodium	FIMMI 10	102.9	-1.91	134.9 ± 7.9	275.3
	FIMMI 12	94.4	-11.8	10.8 ± 3.2	22.0
	FIMMI 13	99.0	-1.68	0.49 ± 0.13	-
penoxsulam	FIMMI 10	109.4	-1.28	111.1 ± 14.7	185.2
	FIMMI 12	100.2	-2.03	47.2 ± 3.2	78.7
	FIMMI 13	99.6	-1.50	0.6 ± 0.02	-
(imazethapyr+imazapic) [c]	FIMMI 10	99.9	-0.91	7.9 ± 0.25	3.29
	FIMMI 12	101.0	-0.99	7.9 ± 0.15	3.29
	FIMMI 13	96.3	-0.92	2.4 ± 0.18	-

[a]Log-logistic equation: Y = a/(1+(X/X0/b). ($p < 0.001$).
[b]Resistance index = $C_{50}R/C_{50}S$
[c] The somation of imazethapyr + imazapic.

Table 4. Estimated parameters a, b and GR_{50} by non-linear regression equation[a], based on dry weight (g) in whole-plant bioassays for two resistant and one susceptible biotype of *Fimbristylis miliacea*, affected by ALS-inhibiting herbicides 4 weeks after treatment, 2008 – 2009

Herbicide	Biotype	a	b	GR_{50}	RI[b]
pyrazosulfuron-ethyl	FIMMI 10	99.3	1.50	56.4 ± 2.93	46.2
	FIMMI 12	101.1	0.60	690.1 ± 186.9	565.6
	FIMMI 13	105.7	1.81	1.22 ± 0.11	-
bispyribac-sodium	FIMMI 10	98.2	1.36	161.4 ± 7.6	192.1
	FIMMI 12	102.8	1.07	20.1 ± 1.01	23.9
	FIMMI 13	99.9	0.93	0.84 ± 0.06	-
penoxsulam	FIMMI 10	99.9	1.30	66.1 ± 3.4	275.4
	FIMMI 12	102.4	0.83	28.6 ± 2.6	119.2
	FIMMI 13	100.1	0.45	0.24 ± 0.07	-
(Imazethapyr + imazapic) [c]	FIMMI 10	102.4	1.26	32.1 ± 1.83	9.90
	FIMMI 12	101.7	1.06	63.3 ± 4.25	19.5
	FIMMI 13	99.8	0.86	3.24 ± 0.25	-

[a]Log-logistic equation: Y = a/(1+(X/X0/b). ($p < 0.001$).
[b]Resistance Index = $GR_{50}R/GR_{50}S$.
[c] The somation of imazethapyr + imazapic.

For the control variable, the dose response curve on plant growth indicates that for all biotypes, the level of control increased with herbicide rate (Figure 1). But, for FIMMI 10 and FIMMI 12, the control level was lower and more gradual when compared with the susceptible FIMMI 13. Biotype FIMMI 10 was 46-, 192- and 271-fold more resistant to pyrazosulfuron-ethyl, bispyribac-sodium and penoxsulam, respectively, than FIMMI 13.

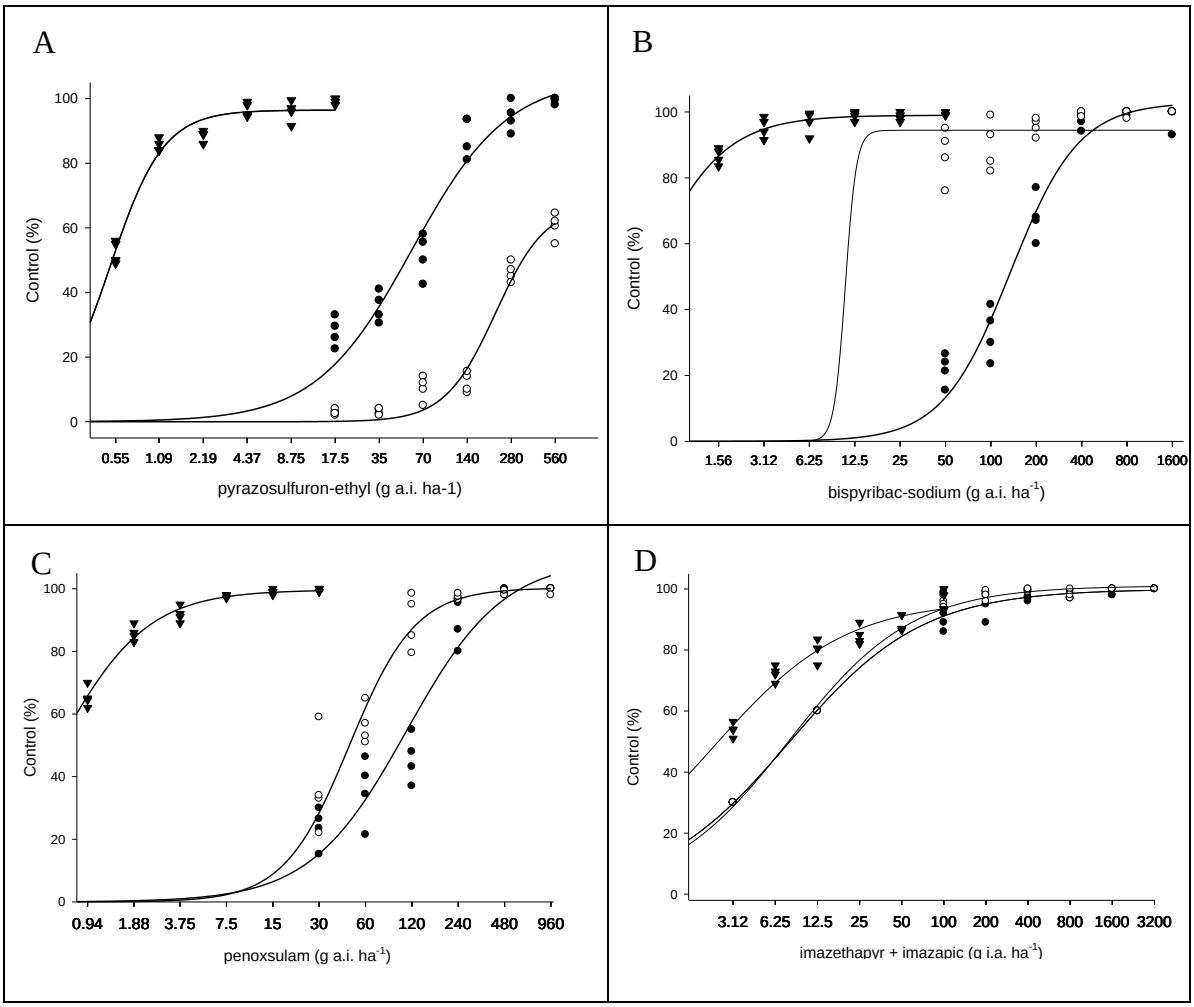

Figure 1. Observed and fitted values of weed control at 28 DAA of pyrazosulfuron-ethyl (A), bispyribac-sodium (B), penoxsulam (C) and (imazethapyr+imazapic) (D) herbicides above ground control (%) with susceptible (▼ FIMMI 13) and resistant (● FIMMI 10 and ○FIMMI 12) biotypes of *Fimbristylis miliacea*.

Moreover, based on GR_{50} values, FIMMI 12 was 566- and 114-fold more resistant to pyrazosulfuron-ethyl and penoxsulam, respectively, than FIMMI 13 biotype (Table 4). It was necessary to use 32 and 3.8 times the regular rates of pyrazosulfuron-ethyl and penoxsulam, respectively, to reduce the above-ground biomass of FIMMI 12 by 50% (Figure 2). On the other hand, for FIMMI 10 it was required to use 2.6, 3.8 and 9 times the regular rates of pyrazosulfuron-ethyl, bispyribac-sodium and penoxsulam, respectively, to reduce the above ground biomass by 50% (Figure 2).

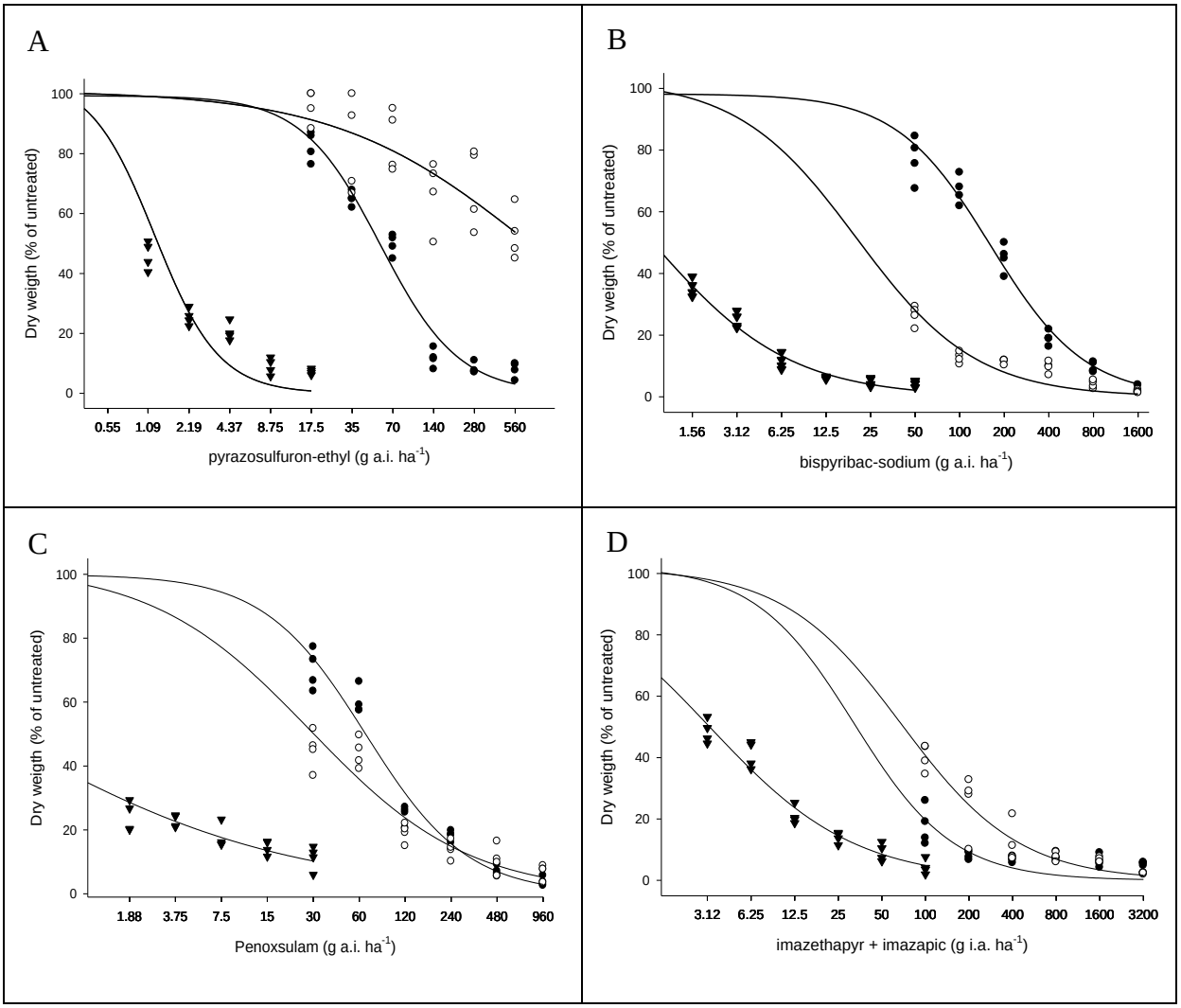

Figure 2. Observed and fitted values of dry weight at 28 DAA of pyrazosulfuron-ethyl (A), bispyribac-sodium (B), penoxsulam (C) and (imazethapyr+imazapic) (D) herbicides above ground dry weight (g) with susceptible (▼ FIMMI 13) and resistant (● FIMMI 10 and ○FIMMI 12) biotypes of *Fimbristylis miliacea*.

High level of resistance was observed in FIMMI 12 for pyrazosulfuron-ethyl, being required rate above 217 g a.i. ha^{-1} to get a 50% control, which was equivalent to 25X the regular used rate of 17.5 g a.i. ha^{-1} (Table 3). For penoxsulam, it was necessary rate above 78 g a.i. ha^{-1} to control 50% in FIMMI 12, which is equivalent to 2.6X the recommended rate of 30 g a.i. ha^{-1}. Resistance factor of 116, 275 and 185 were observed for pyrazosulfuron-ethyl, bispyribac-sodium and penoxsulam, respectively, for FIMMI 10 biotype. In this biotype, the ED_{50} were 6.6, 5.5 and 6.1X the recommended rate, respectively.

FIMMI 13, the susceptible biotype, was controlled with all tested herbicides at the recommended rate. For the resistant biotypes (FIMMI 10 and FIMMI 12), all herbicides at the maximum X rate provided 100% control, except for pyrazosulfuron-ethyl in FIMMI 12

(Figures 1A and 2A). The recommended rate of (imazethapyr+imazapic - 75+25 g a.i. ha^{-1}) reduced the dry weight of all biotypes (Figures 1 and 2). FIMMI 13 biotype was controlled above 95% with all herbicides at the recommended rates, whereas the highest control level for both resistant biotypes was above 80% with the 32X rate, except for FIMMI 12, resistant to pyrazosulfuron-ethyl where the highest control rate was 60% (Figure 1A).

FIMMI biotypes showed different behaviour to the herbicides tested (Figure 1 and 2). The rate of 560 g a.i. ha^{-1} of pyrazosulfuron-ethyl reduced FIMMI 10 dry weight by 93%. However, FIMMI 12 biotype dry weight was reduced only by 45% with the highest tested rate of pyrazosulfuron-ethyl (Figure 2A). The results obtained in these experiment show that FIMMI 12 biotype presents high level of resistance for pyrazosulfuron-ethyl and it is also cross-resistant to penoxsulam. Similar results were observed in *Cyperus difformis* (Galon et al, 2008). Those researches reported that susceptible biotype was effectively controlled by pyrazosulfuron-ethyl; however the resistant one was not controlled even with the 16X the recommended rate. *C. difformis* resistant to pyrazosulfuron-ethyl is due to the insensibility of the ALS enzyme to the herbicide, however, without penalty to K_M and V_{max} kinetic parameters of the ALS enzyme (Dal Magro et al., 2010).

These results show also that FIMMI 10 biotype presents cross-resistance to pyrazosulfuron-ethyl, bispyribac-sodium and penoxsulam herbicides (Tables 1 and 2). Pyrazosulfuron-ethyl at the 4X rate was enough to control and to reduce the dry weight of the R-biotype around 50% when compared to the check (Figure 1A and 2A).

Bispyribac-sodium at 3 or 4X rate controlled approximately 50% of FIMMI 10 and the dry weight was also reduced by 50% (Figures 1B and 2B). For penoxsulam, it was the opposite being necessary around 4X and 3X the recommended rate, respectively, to control and to reduce the dry weight in 50%.

Weed biotype resistant to ALS-inhibitor herbicides also may be less resistant or susceptible to another chemical belonging to the same mechanism of action (Kissmann, 2003). Moreover, resistant biotype may show different levels of resistance to herbicides of the same mechanism of action (Gazziero et al., 2006; Vargas et al., 2007).

It was observed in this study that imidazolinone herbicides showed control near to 100% for both resistant biotypes using the recommended rate (1X) (Figure 1D). Thus, the ALS-inhibiting resistant biotypes (FIMMI 10 and FIMMI 12) did not show cross-resistance to imidazolinones, i.e. the premix formulation of (imazethapyr+imazapic).

Bentazon controlled all *F. miliacea* biotypes at the recommended rate (data not shown). This herbicide presents medium risk for resistance development (Coutinho et al.,

2005) and can be an important alternative to control broadleaf and sedges resistant to ALS inhibitors in flooded rice (Eberhardt and Noldin, 2011; Concenço et al., 2007). However, it has been reported two cases of herbicides resistance with photosystem inhibitors II (PS II), in Southern Brazil. In the state of Paraná, beggarticks (*Bidens subalternans*) was reported to be resistant to triazines and also ALS-inhibitors (Gazziero et al., 2007). More recently, California arrowhead (*Sagittaria montevidensis*) population was reported to be multiple-resistant to ALS and PSII (bentazon) in rice fields of Santa Catariana (Eberhardt and Noldin, 2011). After at least 10 consecutive years using ALS inhibitors herbicides in Santa Catarina it was found resistant populations of *F. miliacea*.

Visible injury on FIMMI 13 typical for ALS-inhibiting herbicides was first observed 14 days after application for all tested herbicides. Although FIMMI 12 showed to be resistant to two different families of ALS-inhibitors herbicides, it was the biotype that survived the highest rate of pyrazosulfuron-ethyl (32X). FIMMI 10 showed cross resistance to three chemical families (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). Moreover, it is important to mention that in the untreated check, FIMMI 12 showed the highest dry weight, compared to FIMMI 10 and FIMMI 13. In this case, it would be interesting to investigate in future studies the fitness penalty and competitive ability for these biotypes.

FIMMI 10 biotype showed cross resistance to three chemical families of ALS-inhibiting herbicides (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). FIMMI 12 biotype showed cross resistance to two ALS-inhibiting herbicides (sulfonylureas and triazolopyrimidines). Imidazolinone herbicide (imazethapyr+imazapic) controlled all *F. miliacea* biotypes in this study, however, growers must be careful using the Clearfield system in where *F. miliacea* populations is infesting the fields in order to avoid the evolution of resistance. Bentazon is still an effective alternative to control *F. miliacea* in areas with ALS herbicide resistance. Additionally, alternative methods of weed management should be adopted by the growers to avoid the evolution and expansion of new cases of ALS resistance *F. miliacea* in rice fields.

ACKNOWLEDGEMENTS

We are grateful to the CNPq for the scholarship to Carlos Eduardo Schaedler.

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Artigo 03 - Revista Planta Daninha

COMPETITIVE ABILITY BETWEEN RICE AND *Fimbristylis miliacea* (L.) VAHL BIOTYPES RESISTANT TO ALS-INHIBITOR HERBICIDES

COMPETITIVE ABILITY BETWEEN RICE AND *Fimbristylis miliacea* (L.) VAHL BIOTYPES RESISTANT TO ALS-INHIBITOR HERBICIDES[3]

ABSTRACT – The aims of this study were to investigate the relative competitive ability and evaluate the above- and below-ground competition of ALS-resistant or susceptible *Fimbristylis miliacea* with rice, and among biotypes. The first study was conducted in a greenhouse at Federal University at Pelotas, using a completely randomized design with four replications and treatments arranged in a replacement series. The proportions of plants of rice crop and *F. miliacea* biotypes were: 100:0, 75:25, 50:50, 25:75 and 0:100, with a total population equivalent to 840 plants m^{-2}. The second study was conducted at the University of Arkansas, USA using a split-plot experimental design with four replications. The main plots were arranged in a combination of species (rice x biotype resistant, rice x susceptible biotype, resistant x susceptible biotypes) and the subplot competition partitioning (competition for soil and solar radiation, no competition, competition for soil resources, and competition for solar radiation). The variables evaluated were leaf area, plant height and shoots dry matter, in both studies. Additionally, in the second experiment, it was evaluated the number of tillers in rice. Rice cultivar was more competitive than the resistant and susceptible *F. miliacea*, without significative differences between R and S plants. In equal proportions of rice plants with *F. miliacea*, regardless of the biotype, generally increased the values in the variables evaluated. The interspecific competition for rice and intraspecific competition for *F. miliacea* were the most advantageous. The ALS-resistant biotype was less competitive with rice than the susceptible. Intraspecific competition is stronger among rice plants than interspecific with *F. miliacea*. The competition for below-ground was the most effective among rice and *F. miliacea* biotypes.

Key Words: *Oryza sativa*, weed competition, globe fringerush, herbicide resistance.

RESUMO – Os objetivos deste estudo foram investigar a habilidade competitiva relativa e a competição acima e abaixo do solo de biótipos de *Fimbristylis miliacea* resistente ou suscetível aos herbicidas inibidores da ALS com arroz, e entre os biótipos. O primeiro estudo foi realizado em casa de vegetação na Universidade Federal de Pelotas, utilizando delineamento experimental completamente casualizado, com quatro repetições, sendo os

[3] Recebido para publicação em __/__/__e na forma revisada em __/__/

tratamentos arranjados em série de substituição. As proporções de plantas de arroz e dos biótipos foram, respectivamente: 100:0, 75:25, 50:50, 25:75 e 0:100, com população total equivalente a 840 plantas m^{-2}. O segundo estudo foi realizado na University of Arkansas, Fayeteville, Estados Unidos, utilizando delineamento experimental com parcelas subdivididas, com quatro repetições, sendo que na parcela principal foi arranjada a combinação das espécies (arroz x biótipo resistente; arroz x biótipo suscetível, biótipo resistente x biótipo suscetível) e na subparcela, a competição particionada (competição por solo e radiação solar; aus***ê***ncia de competição; competição pelos recursos do solo; competição por radiação solar). As variáveis estudadas foram área foliar, estatura e matéria seca da parte aérea, em ambos os estudos. Adicionalmente, no segundo experimento, foi avaliado o número de afilhos no arroz. A cultivar de arroz apresentau superioridade competitiva em relação aos biótipos de *F. miliacea* resistente e suscetível e estes não diferiram entre si. Em proporções iguais de plantas nas associações, o arroz, independente do biótipo de *F. miliacea*, apresentou, em geral, maiores valores nas variáveis avaliadas. A competição inter-específica para o arroz e, intraespecífica para biótipos de *F. miliacea* foram as mais vantajosas. O biótipo de *F. miliacea* resistente apresentou menor competitividade com arroz do que o biótipo suscetível. A competição intraespecífica entre plantas de arroz é maior do que a competição inter-específica entre arroz e *F. miliacea.* A competição por recursos do solo tem maior efeito para *F. miliacea* e arroz.

Palavras-chave: *Oryza sativa*, competição de plantas daninhas, cuminho, resist***ê***ncia à herbicidas.

INTRODUCTION

Species or populations within one species, living close in the same environment, interact with each other and these interactions, called interference can cause positive or negative response (Radosevich et al., 1997). Among the interactions with negative effects, the competition is considered the most important type of interference (Wilson, 1998). In the early stages of the crop development, before closing the canopy, soil resources competition is comparatively more important than that occurs for solar radiation, since there is no detrimental limitation of solar radiation to plant growth (Semere and Froud-Williams, 2001). However, at later stages, competition for light is more pronounced (Marvel et al., 1992;

Fofana and Rauber, 2000). Plant traits as leaf area index, plant height and number of tillers are often referred to as those more related to the ability of crops to compete with weeds (Callaway, 1992). In rice, for example, rapid root growth is associated with increased competitiveness in the early stages of development. However, in weeds, the involvement of plant canopy in competition increases with time, becoming more important in advanced development stages (Fofana and Rauber, 2000).

Fimbristylis miliacea is one of the most troublesome weeds in flooded rice fields in Santa Catarina, Southern Brazil. Traditionally, this weed is controlled with herbicides. However, the use of herbicides as the main method of control, associated with absence of rotation mechanism of action has resulted in the development of herbicide-resistant weed populations (Gressel and Segel, 1990). *F. miliacea* is competitive in rice fields because of its prolific seed production, which allows it to become widespread after the entry into any rice production area. The competition from this weed in rice caused yield reduction of 9.43% (Pons and Utomo, 1985 apud Begum et al., 2008). In addition, the species has a unique ability to have seedlings emerging in the field during the whole period of rice crop (Watanabe et al., 1997).

Yield loss in rice due to weed interference depends on their populations, area distribution, plant height and relative time of emergency (Parker and Murdoch, 1996). The competition studies are important to evaluate the proportion among plant species involved and not just the effect of the populations in the competitive process. To determine the competitive interactions between weeds and crops, the most frequently used method has been the replacement series, which allows evaluating interspecific and intraspecific competition (Radosevich, 1987). These experiments aim to indicate which species or genotype is more competitive (Cousens, 1991). Replacement series experiments have demonstrated that crops are usually more competitive than weeds, because the effect of weeds is not due uniquely to greater competitive ability of them individually, but their infestation degree (Vilà et al., 2004).

The resources below and above the soil surface, such as water, nutrients and solar radiation are very important in the competition process under field conditions. Competitions studies for resources above and below ground are performed under controlled conditions, due to the easier separation, particularly for dry root biomass with soil, compared to field conditions. For studies under controlled conditions, are used basically three methods: divided vase, plant rows and target plant (McPhee and Aarssen, 2001). In the choice of the technique, it should be considered their limitations (McPhee and Aarssen, 2001). The method of dividing

pots and the row of plants separate the competition among plant species; however, within the species occurs overall competition.

The knowledge of the biological characteristics between resistant and susceptible biotypes is a crucial assumption to define the competitiveness and may thereby help to choose the best management method. *Lolium multiflorum* (ryegrass) susceptible to glyphosate herbicide (Vargas et al., 2005) and *Lactuca serriola* (wild lettuce) susceptible to sulfonylurea herbicides (Alcocer-Ruthling et al., 1992) produced higher shoot dry matter than the resistant biotypes.

A major question associated with herbicide resistance is that resistant plants may exhibit different fitness compared to the susceptible counterparts, depending on the physiological mechanisms involved in acquiring resistance. Therefore, the aims of this study were to investigate the relative competitive ability and evaluate the above- and below-ground competition of ALS-resistant or susceptible *F. miliacea* with rice, and among biotypes.

MATERIALS AND METHODS

Experiment 1: Monoculture experiment

A preliminar experiment was conducted in plastic pots that were placed in a greenhouse at the Faculdade de Agronomia, Federal University at Pelotas. Plastic pots of 10 cm diameter and 9 cm length with 1L capacity were filled with topsoil, Planosol Hydromorphic Euthrophic solodic soil of the Pelotas Mapping Unit, franc-sandy textured (Sosbai, 2010). The soil characteristics were: pH water (1:1) = 5.1; CTC pH 7 = 5.4 cmolc dm^{-3}; organic matter = 1.2%; clay = 15%, texture = 4; Ca = 1.8 cmolc dm^{-3}, Mg = 1 cmolc dm^{-3}; Al = 0.2 cmolc dm^{-3}; P = 4.3 mg dm^{-3} and K = 30 mg dm^{-3}. The irrigation was carried out by capillarity and the pots, with holes in the bottom, were maintained in plastic trays with water and without pudding.

The plant population, in which the final production of shoot dry matter became constant, was obtained through preliminary experiment with a susceptible biotype of *F. miliacea* and rice. The *F. miliacea* population was 2, 4, 8, 12, 16, and 24 plants pot^{-1} (equivalent to 177, 353, 707, 1060, 1413, 2120 plants m^{-2}). Rice cultivar populations were 1, 2, 4, 8, 12, 16, and 24 plants pot^{-1} (equivalent to 88, 177, 353, 707, 1060, 1413, 2120 plants m^{-2}).

The experiment was conducted in a completely randomized design with four replications. *F. miliacea* seeds were sown in trays and seven days after emergence, the seedlings were transplanted in the respective populations. Rice seeds, cultivar Epagri 109 were soaked in distilled water for 24 hours and subsequently placed in a growth chamber at 28°C for the same period in the absence of light, for pre-germination. The day temperature recorded inside the greenhouse was 28-34°C. At 38 days after transplanting, it was evaluated the shoot dry biomass (SDB), quantified after weighing the shoot parts after drying at 60°C for 72 hours.

The statistical analysis was done using the SAS Statistical Analysis System (SAS) version 9.1 (SAS Institute, 2004), and the means were fitted using an Exponential Rise to Max model with two parameters. Sigma Plot (Sigma Plot, 2004) software was used for the regression analysis and curve fitting.

Experiment 2: Replacement series

Three experiments were conducted in a greenhouse at Faculdade de Agronomia, Federal University at Pelotas, in Capão do Leão - RS, in the growing seasons 2008/2009 and 2009/2010. The experimental units were placed in plastic pots with a volume capacity of 1 L, filled with the same soil described in experiment 1. The experimental design was a completely randomized design, with four replications.

The replacement series experiments included different combinations of rice cultivar Epagri 109, resistant (FIMMI 10) and susceptible (FIMMI 13) ALS-inhibiting resistant *F. miliacea* biotypes, varying the relative proportions of plants per pot (100:0, 75:25, 50: 50, 25:75 and 0:100), maintaining the total plant population. To establish the desired populations in each treatment, *F. miliacea* seeds were sown in a growth chamber with a photoperiod of 14/10 hours day/night in a constant temperature of 30°C. Rice seeds were soaked in distilled water for 24 hours and subsequently placed in a growth chamber for the same period in the absence of light for pre-germination. For germination to occur simultaneously, *F. miliacea* biotypes seeds were sown in the chamber four days before rice. Pre-germinated rice seeds were planted 1 cm depth, and *F. miliacea* seedlings placed on the top of soil surface.

At 38 days after transplanting, it was evaluated: leaf area (LA), plant height (PH) and shoot dry biomass (SDB). LA was determined using a leaf area LI-COR model Area Meter 3100, the PH was evaluated taking its length from ground level to the tip of the longest leaf, and the SDB was quantified weighing the shoot parts after drying at 60°C for 72 hours.

For analysis of the parameters LA, PH, and SDB, it was used the graphical method of relative yield (Radosevich, 1987). This procedure involved the elaboration of the diagram based on the relative yield (RY) and total relative yield (RYT) with the respective plant proportion. If the RY result in a straight line, it is consider that there is no effect of a species (or biotype) to interfere each other, and mean that the skills of the species are equivalent. When the RY result in a concave line, it was defined that there was a loss in growth of one or both species. When the line suggested by RY assumed a convex line, there was a benefit in the growth of one or both species. RYT was equal to the unity (straight line), mean that there was competition for the same sources being greater than one (convex line), it was assumed that competition did not occur when it is less than one (concave line) mean that there was antagonism, with negative interference to the mutual growth of both species (Radosevich, 1987).

In addition to the comparisons between RY and RYT, the results were also subjected to analysis of variance. When the F test indicated significance ($p \leq 0.05$), the treatment means were compared by Dunnett's test ($p \leq 0.05$), considering the monocultures of the species. It was also calculated the relative competitiveness index (CR), the coefficients for relative clustering (K) and competitiveness (C) in proportions of 50% of plant competition. The CR represented the comparative growth of rice cultivar in relation to the *F. miliacea* biotypes (competitor), K indicated the relative dominance of one genotype over another, and point C, the genotype which manifests itself more competitive (Cousens, 1991). Rice is more competitive than *F. miliacea* biotypes when CR> 1, KA> KB and C> 0 (Hoffman and Buhler, 2002). The calculations were performed using the following equations proposed by Cousens and O'Neill (1993): CR = PRA / PRB, PRA = KA / (1 - PRA), PRB = KB / (1 - PRB), A = PRA - PRB. For comparison, rice and *F. miliacea* biotypes are KA and KB, respectively, while for the comparison between the *F. miliacea* biotypes, becomes resistant to KA and KB remains susceptible.

To analyze statistically relative yield, firstly it was calculated the differences for the values of RY obtained in proportions of 25, 50 and 75% of plants in relation to the values belonging to the hypothetical straight lines obtained in the respective proportions. The test't' ($p \leq 0.05$) was used to test for differences in the indices studied in relation to the hypothetical straight (Hoffman and Buhler, 2002). The null hypotheses used to test the mean differences were: (H0 = 0), PRT and CR (H 0 = 1) and K [H0 = (KA KB) = 0].

Experiment 3: Competition partitioning

The experiment was conducted in plastic pots placed in the field at the University of Arkansas, Fayetteville, in the summer of 2010, arranged in a split-plot design, with four replicates. The treatments tested were the combinations of the three species (*F. miliacea* resistant and susceptible biotypes, and rice) as the main-factor, and four competition conditions (no competition, competition for soil resources and solar radiation, competition for soil resources alone, and competition for solar radiation alone), as sub-factor (Figures 1 and 2). The rice cultivar used in this experiment was ”Well”. Plastic pots rectangular of 27.5 cm length x 22.5 cm width and 22 cm depth were filled with commercial substrate mixture (Sunshine Mix, Canada) plus top soil from Vegetable Station, at Kibler, AR. Soil and substrate were homogenized in a mixer about 10 minutes (1:1 v/v) and the characteristics were: pH water (1:1) = 6.6; CTC pH 7 = 19.1 cmolc dm^{-3}; Ca = 2217mg kg^{-1}, Mg = 589 mg kg^{-1}; P = 110 mg kg^{-1} and K = 220 mg kg^{-1}. Soil fertility was corrected according to official research recommendations for the rice crop (Sosbai, 2010). The pots were rotated to new positions every two days to minimize the environmental effect and border effects within the experiment.

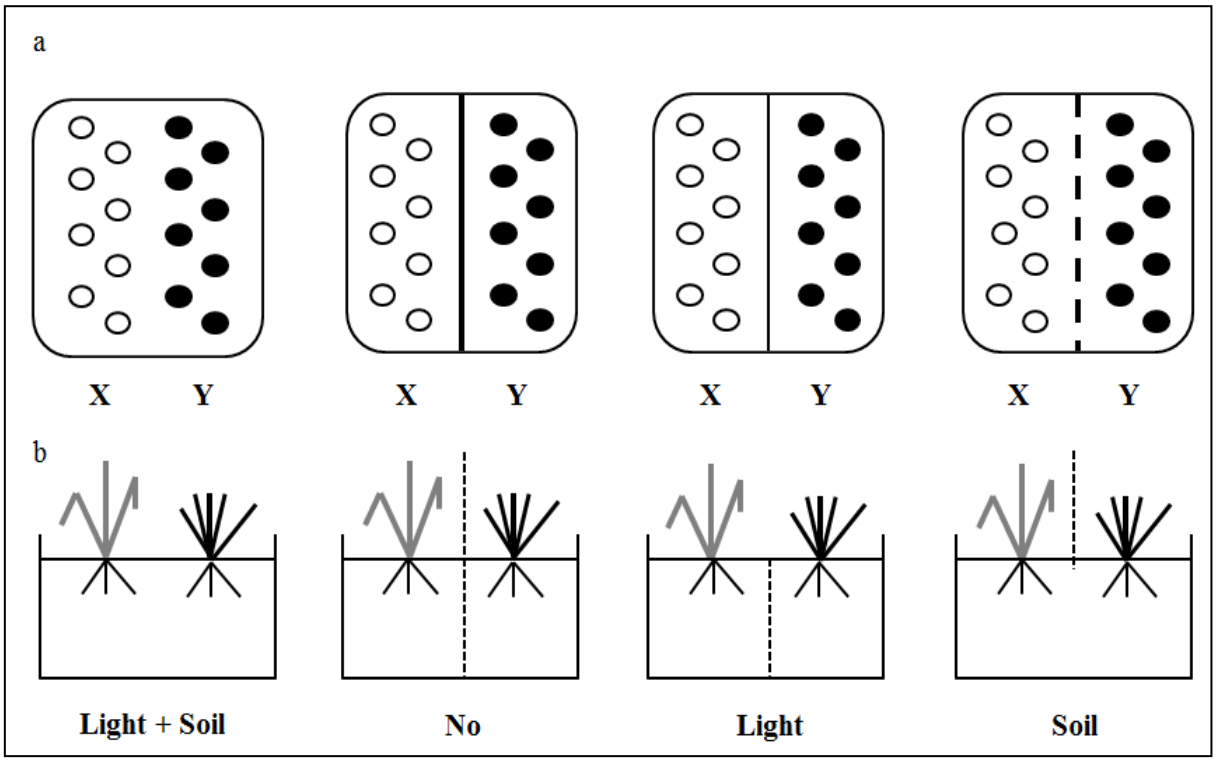

Figure 1. Schematic diagram of potted plants (a) and placement of the divider (dashed line) in different treatments (b). The letter "X" represents Rice, and the letter "Y", the *F. miliacea* biotypes.

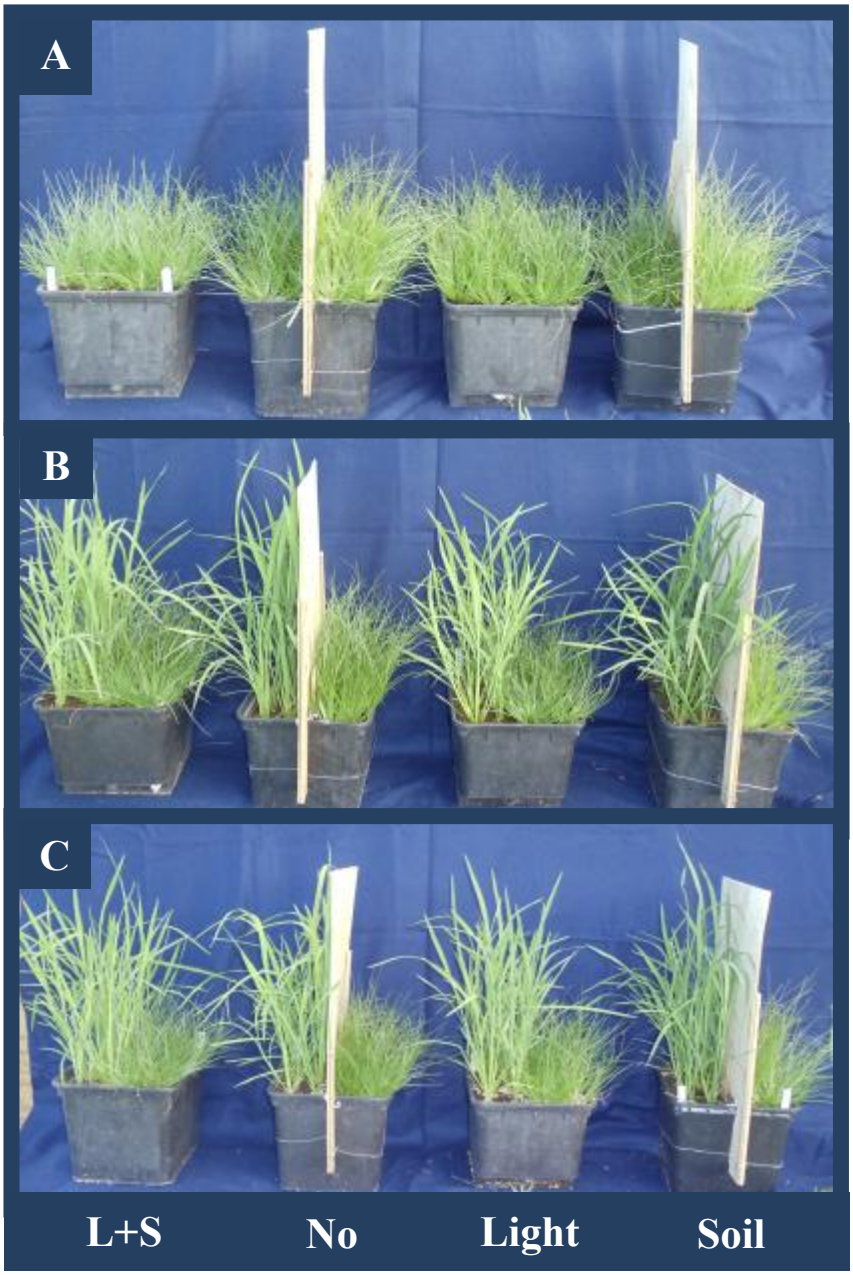

Figure 2. Representative treatments 36 days after planting: competition for soil and solar radiation (L+S), no competition (No), competition for solar radiation (Light) and competition for soil resources (Soil). A = *F. miliacea* (R) x *F. miliacea* (S); B = rice x *F. miliacea* (S); C = rice x *F. miliacea* (R). University of Arkansas, Fayetteville / AR, 2010.

Rice and *F. miliacea* seeds were sown in plastic trays in the greenhouse with a photoperiod of 14/10 hours day / night in a constant temperature of 30°C. For germination to occur simultaneously between biotypes and rice, *F. miliacea* seeds were sown six days earlier. The irrigation was daily and 14 days after transplanting the water level maintained 2 cm depth. The plant density was equivalent to 260 plants m^{-2}.

The response variables evaluated were leaf area, shoot dry biomass, root dry biomass and plant height. Rice tillers number was also recorded. All variables were evaluated at 36 days after planting. The data were analyzed using ANOVA ($\alpha \leq 0.05$) and when significant, Fischer`s test was used to compare treatment means.

RESULTS AND DISCUSSION

The results reported are derived from the two greenhouse and one field experiment involving rice and *F. miliacea* biotypes resistant and susceptible to ALS-inhibiting herbicides.

Experiment 1: Monoculture experiment

The first experiment with rice and *F. miliacea* in monoculture aimed to determine the plant population (m^{-2}) which the SDB per unit area (g m^{-2}) becomes independent of the population, according to the "constant final yield law" (Radosevich, et al., 1997). The assumption is that the total pot population is enough to capture all the resources available for growth.

For the shoot dry biomass expressed as grams pot^{-1} evaluated at 38 days after treatment (DAT), data were fitted to the Exponential Rice to Max model. The curve indicated increasing of dry biomass with the increase of population (Figure 3 A and B). For rice, the higher SDB occurred with 16 plants pot^{-1}; however, it was not different with 12 or 24 plants. On the other hand, *F. miliacea* showed the higher SDB with 24 plants pot^{-1}, and was not different when compared with 8 plants.

According to the results obtained in this study, the authors opted for the choice of 12 plants pot^{-1}, which it was supposed to be the population to capture all available resources for growth and lead to the replacement series experiments.

Experiment 2: Replacement series

For the experiments in the replacement series it was used the population of 12 plants pot^{-1} (1060 plants m^{-2}), determined in the experiment 1. The graphical analysis of the results obtained for the RY for the variables LA, PH, and SDB showed convex line for rice. This showed that rice was more competitive than *F. miliacea* biotypes (Figures 4, 5 and 6, Table 1). In general, for rice at the highest plant proportion there were no differences between lines observed and hypothetical, for LA, PH and SDB variables, independent of biotypes to be competitive, except for LA compared with the resistant biotype (Table 1).

DRY of resistant and susceptible biotypes were lower in all plant proportion and plant variables evaluated when in competition with rice, except PH for FIMMI 13 competing with rice in the lower plant proportion (Table 1). For the equivalent proportions in all variables, there were no differences for rice in competition with the resistant biotype (FIMMI 10), whereas, with the susceptible biotype, it was observed an increase in DRY for the crop (Table 1). Rice in the lowest plant proportion showed differences between the observed and hypothetical straight in all variables, independent of biotypes, showing the superiority of the crop even a lower plant proportion.

The RYT for LA, PH and SDB showed no differences in relation to the hypothetical straight, except in the proportion 25:75 (crop : susceptible biotype) to LA and SDB in the proportion of 75:25 (crop : resistant biotype) (Table 1).

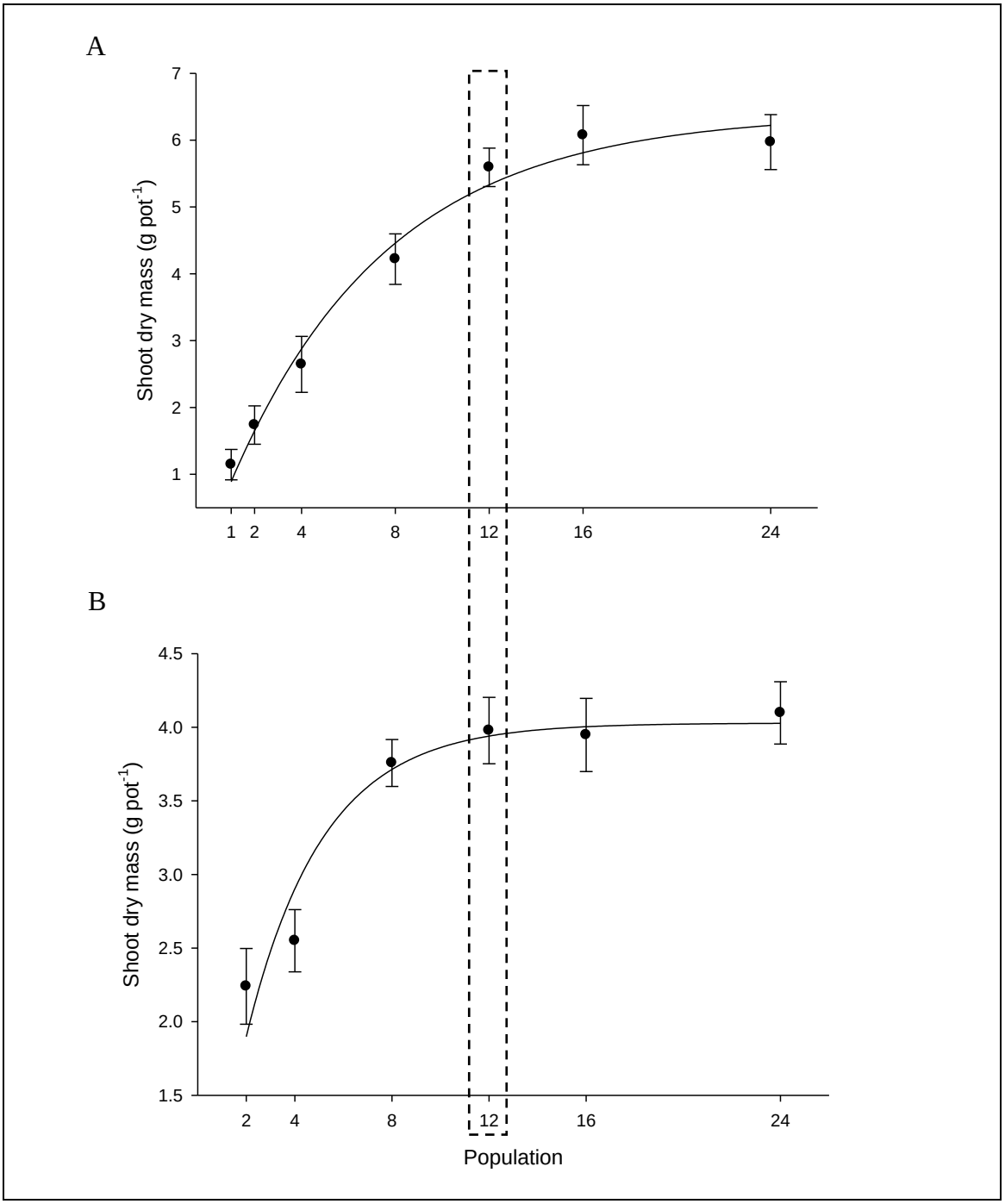

Figure 3. Effect of population on the shoot dry biomass of rice (A) and *Fimbristylis miliacea* (B) at 38 days after transplanting**.** Federal University at Pelotas, Capão do Leão / RS, 2008/09. The error bar denotes ± standard error.

The comparison between resistant and susceptible biotype showed difference only for the resistant one at the higher plant proportion. At the 50:50 ratio, there was no difference between the biotypes for the variables studied, except for SDB for the susceptible biotype. When the susceptible population participated with the highest plants proportion, there were differences for LA and SDB, whereas for the resistant biotype difference was found only for SDB.

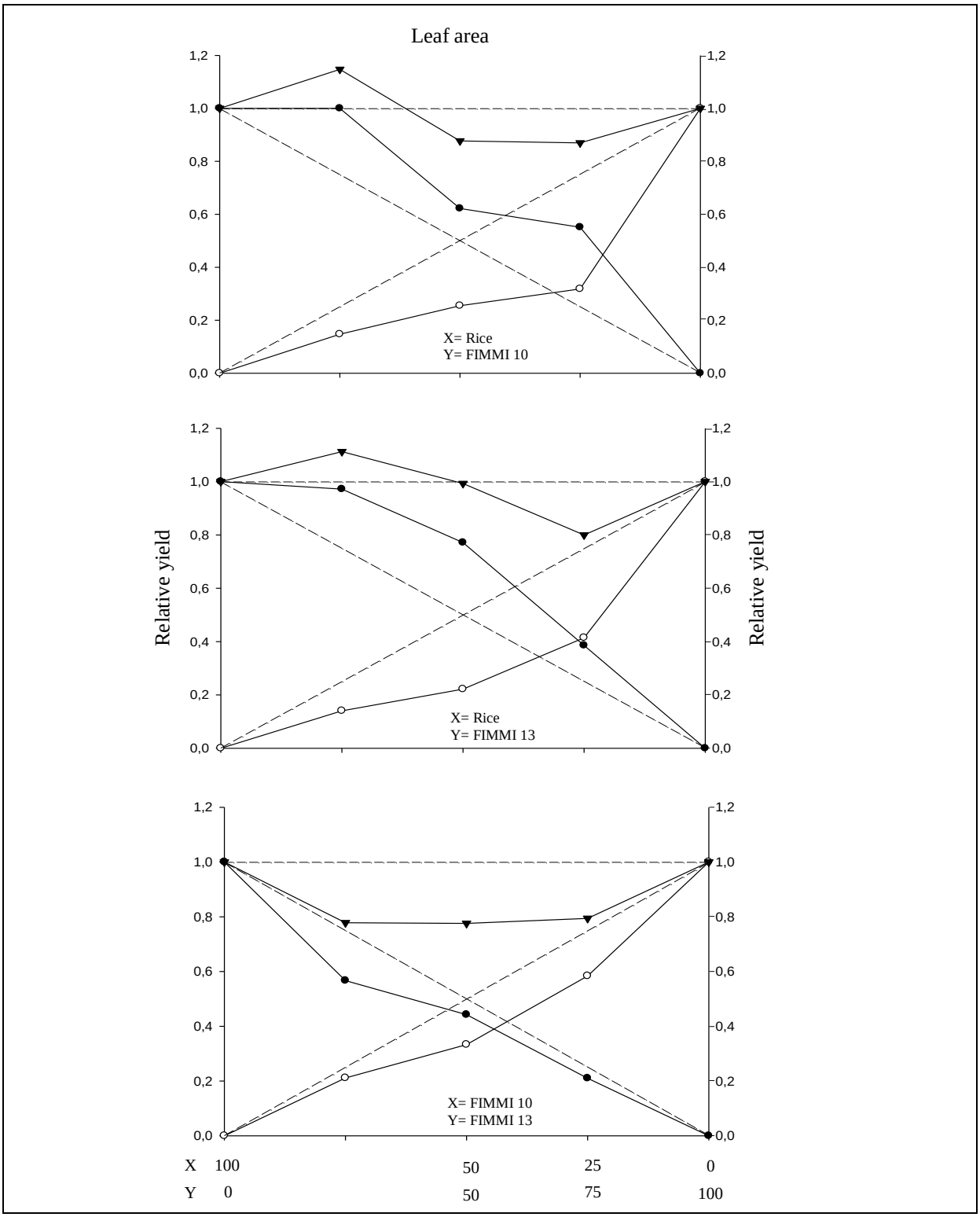

Figure 4 - Relative yield (RY) and relative yield total (RYT) to leaf area (LA) of rice and *Fimbristylis miliacea* biotypes (FIMMI 10 or FIMMI 13), Federal University at Pelotas, Capão do Leão / RS, 2008/09. Filled circles (●) and empty (○) represent the RY of LA to rice and biotypes, respectively, (▼) indicate the RYT. The dashed lines refer to hypothetical RY, when there is no interference on the cultivar or biotype and one over another.

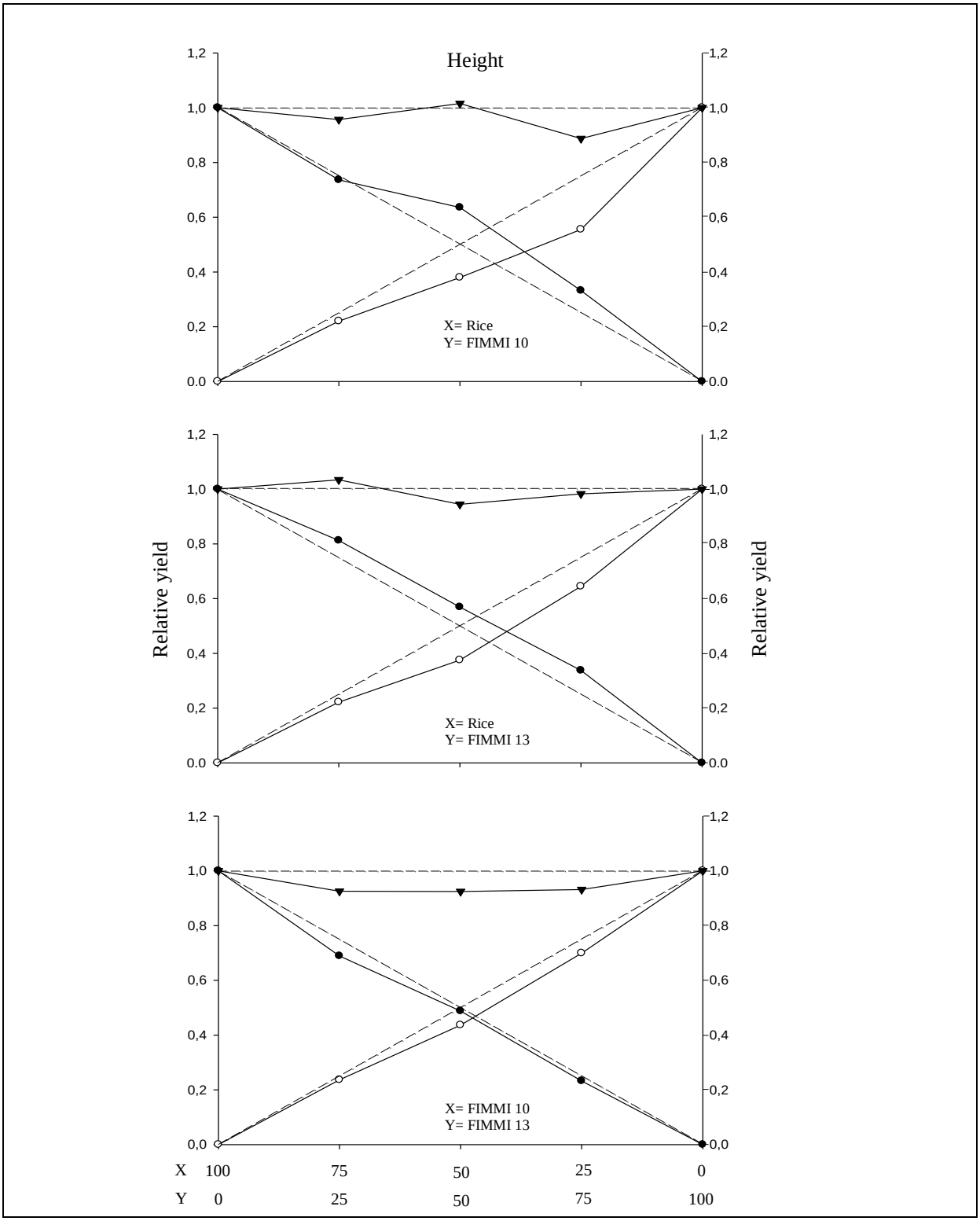

Figure 5 - Relative yield (RY) and relative yield total (RYT) to height plant (HP) of rice and *Fimbristylis miliacea* biotypes (FIMMI 10 or FIMMI 13), Federal University at Pelotas, Capão do Leão / RS, 2008/09. Filled circles (●) and empty (○) represent the RY of HP to rice and biotypes, respectively, (▼) indicate the RYT. The dashed lines refer to hypothetical RY, when there is no interference on the cultivar or biotype and one over another.

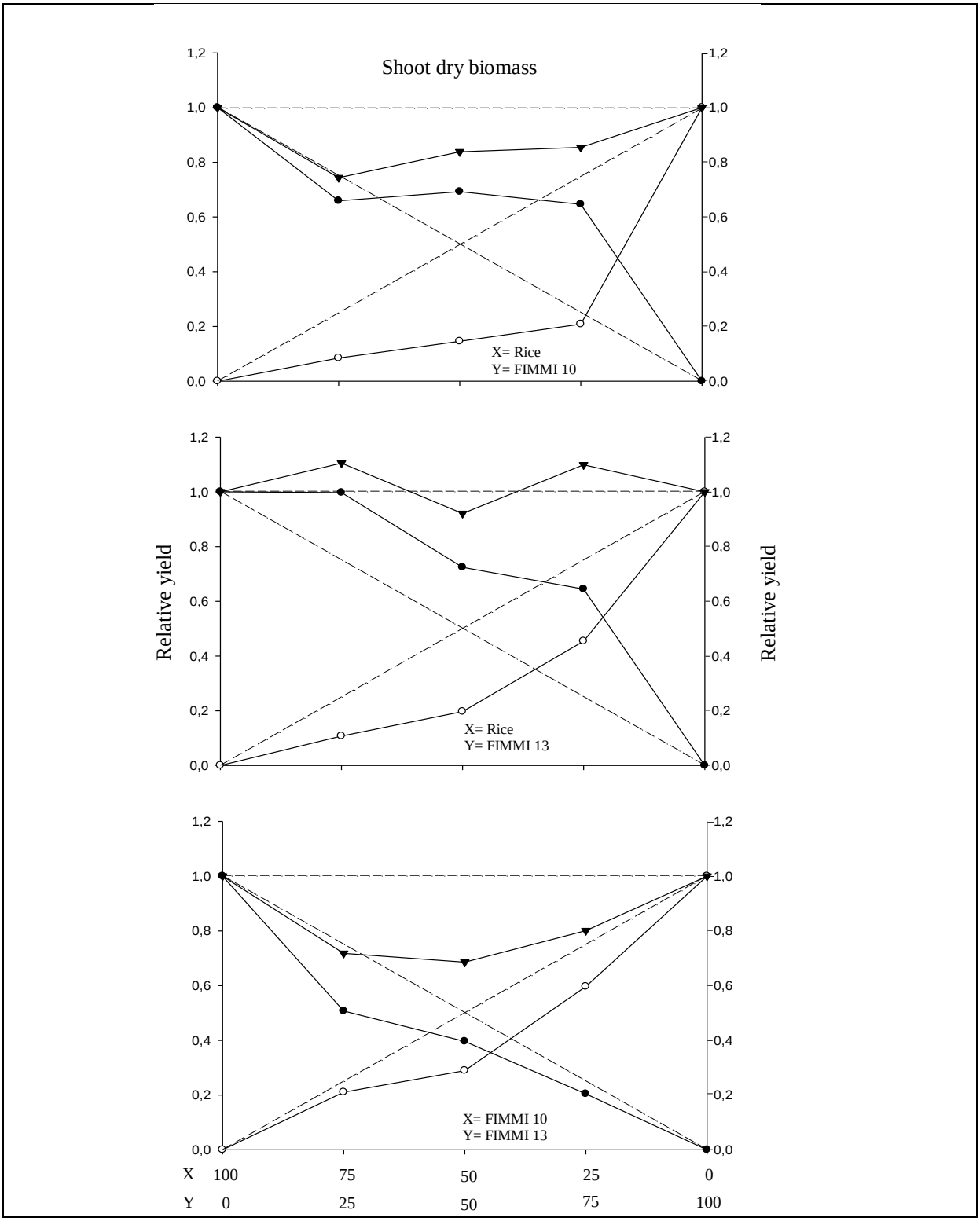

Figure 6 - Relative yield (RY) and relative yield total (RYT) to shoot dry biomass (SDB) of rice and *Fimbristylis miliacea* biotypes (FIMMI 10 or FIMMI 13), UFPel, Capão do Leão/RS, 2008/2009. Filled circles (●) and empty (○) represent the RY of HP to rice and biotypes, respectively, (▼) indicate the RYT. The dashed lines refer to hypothetical RY, when there is no interference on the cultivar or biotype and one over another.

Table 1 - Differences in relative yield (DRY) and relative yield total (RYT) to leaf area, height and shoot dry biomass of rice in competition with *Fimbristylis miliacea* biotypes (FIMMI 10 and FIMMI 13), Federal University at Pelotas, Capão do Leão / RS, 2008/09.

	Proportions of plants (Rice:competitor)		
	75:25	50:50	25:75
		Leaf area	
DRY Rice	0,25 (±0,07)*	0,12 (±0,08)^{ns}	0,30 (±0,10)*
DRY FIMMI 10	-0,10 (±0,01)*	-0,25 (±0,02)*	-0,43 (±0,04)*
RYT	1,15 (±0,07)^{ns}	0,88 (±0,09)^{ns}	0,87 (±0,14)^{ns}
DRY Rice	0,22 (±0,09)^{ns}	0,27 (±0,04)*	0,14 (±0,02)*
DRY FIMMI 13	-0,11 (±0,02)*	-0,28 (±0,03)*	-0,34 (±0,06)*
RYT	1,11 (±0,10)^{ns}	0,99 (±0,07)^{ns}	0,80 (±0,05)*
DRY FIMMI 10	-0,18 (±0,03)*	-0,06 (±0,03)^{ns}	-0,04 (±0,02)^{ns}
DRY FIMMI 13	-0,04 (±0,02)^{ns}	-0,17 (±0,06)^{ns}	-0,17 (±0,04)*
RYT	0,78 (±0,04)*	0,78 (±0,07)*	0,79 (±0,04)^{ns}
		Height	
DRY Rice	-0,01 (±0,02)^{ns}	0,14 (±0,06)^{ns}	0,08 (±0,01)*
DRY FIMMI 10	-0,03 (±0,00)*	-0,12 (±0,01)*	-0,19 (±0,05)*
RYT	0,96 (±0,02)^{ns}	1,02 (±0,06)^{ns}	0,89 (±0,06)^{ns}
DRY Rice	0,06 (±0,03)^{ns}	0,07 (±0,01)*	0,09 (±0,00)*
DRY FIMMI 13	-0,03 (±0,01)^{ns}	-0,12 (±0,03)*	-0,11 (±0,03)*
RYT	1,03 (±0,02)^{ns}	0,94 (±0,04)^{ns}	0,98 (±0,04)^{ns}
DRY FIMMI 10	-0,06 (±0,00)*	-0,01 (±0,01)^{ns}	-0,02 (±0,01)^{ns}
DRY FIMMI 13	-0,01 (±0,01)^{ns}	-0,06 (±0,02)^{ns}	-0,05 (±0,03)^{ns}
RYT	0,93 (±0,00)*	0,92 (±0,02)*	0,93 (±0,03)^{ns}
		Shoot dry biomass	
DRY Rice	-0,09 (±0,05)^{ns}	0,19 (±0,10)^{ns}	0,40 (±0,12)*
DRY FIMMI 10	-0,17 (±0,01)*	-0,35 (±0,02)*	-0,54 (±0,03)*
RYT	0,74 (±0,06)*	0,84 (±0,12)^{ns}	0,85 (±0,15)^{ns}
DRY Rice	0,25 (±0,10)^{ns}	0,22 (±0,04)*	0,39 (±0,03)*
DRY FIMMI 13	-0,14 (±0,03)*	-0,30 (±0,04)*	-0,30 (±0,05)*
RYT	1,11 (±0,09)^{ns}	0,92 (±0,07)^{ns}	1,10 (±0,05)^{ns}
DRY FIMMI 10	-0,24 (±0,03)*	0,10 (±0,04)^{ns}	0,05 (±0,01)*
DRY FIMMI 13	-0,04 (±0,03)^{ns}	-0,21 (±0,05)*	-0,15 (±0,04)*
RYT	0,72 (±0,05)*	0,69 (±0,04)*	0,80 (±0,03)*

$^{(ns)}$ Non-significant and $^{(*)}$ significant difference ($p \leq 0,05$) by test "t". Values in parentheses represent the standard error of mean.

The RYT biotypes combination showed differences for variables LA, PH, and SDB, except PH and LA in the higher proportion of the susceptible biotype. For all variables, the RYT was less than 1, which shows that there was damage to mutual growth, i.e, competition between *F. miliacea* resistant and susceptible occurred for the same resources (Table 1).

It is considered that a certain genotype is more competitive than a another when CR> 1, KA> KB and C> 0 (Hoffman and Buhler, 2002) and adopting as a criterion to prove the occurrence of competitive superiority in at least significant differences in two indices

(Bianchi et al., 2006), it was found that rice was more competitive than F. *miliacea* biotypes in all variables except for PH when competed with the resistant biotype. The biotypes did not differ from each other in competitive ability (Table 2). This shows that rice is more competitive compared to the biotypes and they do not differ. Similarly, Dal Magro et al, (2011) observed that *Cyperus difformis* biotypes ALS resistant and susceptible biotypes usually had equal competitive ability and rice showed to have superior competitive ability compared to the weed. ALS-susceptible and resistant greater beggarticks (*Bidens subalternans*) biotypes were equally competitive (Lamego et al., 2011).

Table 2 – Competitiveness index between rice and *Fimbristylis miliacea* biotypes (FIMMI 10 and FIMMI 13), expressed as relative competitiveness (RC) and clustering coefficients for (K) and competitiveness (C). Federal University at Pelotas, Capão do Leão / RS, 2008/09.

Variable	CR	K_A (rice)	K_B (biotype)	C
		Leaf area		
Rice x Resistant	2,47 (±0,26)*	2,20 (±0,95)^{ns}	0,35 (±0,04)	0,37 (±0,07)*
Rice x Susceptible	3,69 (±0,49)*	3,90 (±1,07)*	0,29 (±0,05)	0,55 (±0,03)*
Resistant x Susceptible	1,49 (±0,31)^{ns}	0,81 (±0,09)^{ns}	0,54 (±0,14)	0,11 (±0,07)^{ns}
		Height		
Rice x Resistente	1,68 (±0,15)*	2,03 (±0,62)^{ns}	0,61 (±0,04)	0,26 (±0,06)^{ns}
Rice x Susceptible	1,53 (±0,08)*	1,32 (±0,06)*	0,61 (±0,06)	0,19 (±0,02)*
Resistant x Susceptible	1,13 (±0,08)^{ns}	0,95 (±0,03)^{ns}	0,78 (±0,07)	0,05 (±0,03)^{ns}
		Shoot dry biomass		
Rice x Resistant	4,81 (±0,14)*	7,89 (±6,32)^{ns}	0,17 (±0,03)	0,55 (±0,07)*
Rice x Susceptible	4,00 (±0,59)*	2,81 (±0,50)*	0,25 (±0,05)	0,53 (±0,02)*
Resistant x Susceptible	1,55 (±0,37)^{ns}	0,67 (±0,10)^{ns}	0,43 (±0,10)	0,11 (±0,08)^{ns}

$^{(ns)}$ Non-significant and $^{(*)}$ significant difference ($p \leq 0,05$) by test "t". Values in parentheses represent the standard error of mean.

In studies carried out comparing red rice and rice, it was found that the weed was more competitive, although they are very close in morph-physiological characteristics (Fleck et al., 2008). Using an index to define competitiveness, Hoffman & Buhler (2002) found that *Sorghum* crop was more competitive than *Sorghum halepense*, and Bianchi et al. (2006) reported that radish was more competitive than soybean genotypes.

Generally, crop is more competitive than weeds because their effect is not only due to higher individual competitive ability, but mainly to the total population of plants (Vilà et al., 2004). Moreover, because rice and *F. miliacea* belong to the different botanical family, competition for resources from the environment was emphasized in this study. Thus, it was

observed changes in competitiveness between rice plants and *F. miliacea* resistant and susceptible biotypes, depending on plants proportions.

The crop and the resistant biotype coexistence increased LA, SDB and PH in the proportion 25:75 and PH in the proportion 50:50 (Table 3). On the other hand, the resistant biotype showed reduction in all proportions when compared to the monoculture, except in proportion 25:75 to the PH variable. For rice and susceptible biotype coexistence, rice plants showed higher average values in the ratio of 50:50 in all variables tested. Moreover, in the proportion 25:75, the crop was different for the variables PH and SDB. The susceptible biotype reduced SDB and PH in the proportion of 50:50 and LA and SDB in 25:75.

There were no differences in any variable and proportion of plant between resistant and susceptible *F. miliacea* biotypes, except for SDB variable in equal proportion (50:50) (Table 3). Rice in equal proportions of plant associations, independent of biotype, had, in general, increase in the values of the morphological traits, while biotypes showed, in general, reduction in the variables studied. These results demonstrate that interspecific competition for rice and intraspecific for the resistant biotype was the most advantageous. In replacement series study between rice and *C. difformis* resistant and susceptible to ALS-inhibiting herbicides, the intraspecifc competition was more important for rice while then for weed biotypes; the most important was interspecific competition (Dal Magro et al., 2011).

When rice competed with *F. miliacea* biotypes, it was not possible to identify the ways by which both competed. It is assumed that the interference occurred mainly for light, but cannot be ignored the fact that rice was able to capture nutrients more efficiently, since water was not limiting. Fischer et al. (1997) reported that the LA of rice was the characteristic most associated with competitiveness compared to PH. Additionally, a comparative study between rice cultivars showed that the accumulation of LA influenced the ability of plants to shade its neighbors and with high tillering capacity, although short height, could compete very well with red rice (Estorninos Jr. et al., 2002; Fleck et al., 2003).

In general, it is evident that the morph- physiological differences between rice plants in relation to resistant and susceptible *F. miliacea* biotypes were enough to demonstrate in this study differential competition for resources and the environment, thus characterizing significant variations in competitiveness between rice and *F. miliacea* biotypes.

Table 3 – Variation in morphological characteristics of rice plants and the competitors (*Fimbristylis miliacea*) resistant and susceptible in different plant proportion, Federal University at Pelotas, Capão do Leão / RS, 2008/09.

Plants proportion	Leaf area (cm^2 $plant^{-1}$)	Height (cm)	Shoot dry biomass (g $plant^{-1}$)
		Rice	
100:0 (T)	12,1	17,7	0,087
75:25	16,2^{ns}	17,4^{ns}	0,071^{ns}
50:50	15,1^{ns}	22,5*	0,112^{ns}
25:75	26,7*	23,5*	0,210*
CV (%)	29,4	11,6	36,7
		Resistant biotype	
100:0 (T)	10,4	12,5	0,080
75:25	4,4*	9,2*	0,022*
50:50	5,3*	9,5*	0,023*
25:75	6,1*	11,0^{ns}	0,024*
CV (%)	16,6	8,9	26,6
		Rice	
100:0 (T)	10,5	18,1	0,078
75:25	13,6^{ns}	19,6^{ns}	0,104^{ns}
50:50	16,2*	20,6*	0,114*
25:75	13,3^{ns}	24,4*	0,203*
CV (%)	14,2	4,8	13,9
		Susceptible biotype	
100:0 (T)	6,3	12,6	0,070
75:25	4,9^{ns}	10,8^{ns}	0,043^{ns}
50:50	6,1^{ns}	9,5*	0,028*
25:75	11,1*	11,2^{ns}	0,030*
CV (%)	32,4	9,8	36,6
		Resistant biotype	
100:0 (T)	13,2^{NS}	12,8^{NS}	0,099^{NS}
75:25	10,0	11,8	0,067
50:50	11,7	12,5	0,079
25:75	11,1	11,9	0,081
CV (%)	15,2	5,4	14,5
		Susceptible biotype	
100:0 (T)	11,6^{NS}	12,6^{NS}	0,082
75:25	9,0	11,7	0,065^{ns}
50:50	7,7	11,0	0,047*
25:75	9,8	11,9	0,069^{ns}
CV (%)	20,7	13,3	20,6

* significant and ^{ns} not significant, respectively, compared to the control treatment (T), by Dunnett`s test ($p \leq 0,05$). ^{NS} not significant by F test ($p \leq 0,05$).

Experiment 3: Competition partitioning

There was a significant interaction effect of species combination and competition partitioning on root dry biomass of resistant and susceptible *F. miliacea* (Table 4). When the crop was the competitor, FIMMI 10 had higher root dry weight in the light + soil treatment. On the other hand, the FIMMI 13 biotype had higher value in competition conditions for resources from the ground.

Table 4. The effect of competition partitioning and species combination on the root dry weight of FIMMI biotypes. University of Arkansas, Fayetteville / AR, 2010.

		Competition			
Biotype	Competitor	Light + Soil	Null	Light	Soil
FIMMI 10	Rice	A 1.6 b	B 0.9 a	AB 1.2 ab	B 1.0 b
FIMMI 13	Rice	B 1.5 b	B 1.1 a	B 0.9 b	A 2.3 a
FIMMI 10	FIMMI 13	A 2.5 a	B 0.7 a	A 1.7 a	A 1.0 b
FIMMI 13	FIMMI 10	A 2.9 a	B 1.2 a	B 1.3 ab	B 0.9 b

Means within rows (capital letters) and columns (lowercase letters) with the same letters are not significantly different at $p \leq 0.05$ (Fischer`s test).

FIMMI 10 showed higher values of root dry weight when in competition with FIMMI 13 in light + soil, light or soil treatments alone. The susceptible FIMMI 13 in competition with FIMMI 10 (resistant) showed the largest root dry weight and occurred when they were in total competition (light + soil). For the effect of species combination, *F. miliacea* biotypes showed decrease in root dry weight when in light + soil, in competition with rice plants. In soil resources conditions of competition, FIMMI 13 biotype was higher in this variable (Table 4).

The variables plant height and dry biomass were not affected by the interaction of *F. miliacea* combination and competition partitioning (Figure 7). *F. miliacea* biotypes showed higher plant height in the null and soil competition partitioning treatment. In contrast, the competition was more intense in light + soil and light competition partitioning treatment (Figure 7A). Dry biomass average of the biotypes are shown in Figure 7B. Regardless of the competitor, the maximum dry biomass value was obtained from light + soil treatment. The null compartitioning showed the competition less dry biomass for the *F. miliacea* biotypes. Root competition from *Dactylis glomerata* had significant negative effects on *Fraxinus excelsior* seedling growth, in terms of height (Bloor et al., 2008). Additionally, the authors observed that *Fraxinus excelsior* seedlings biomass that below-ground competition was more

important than above-ground competition, and that root and shoot competition did not interact to influence plant growth.

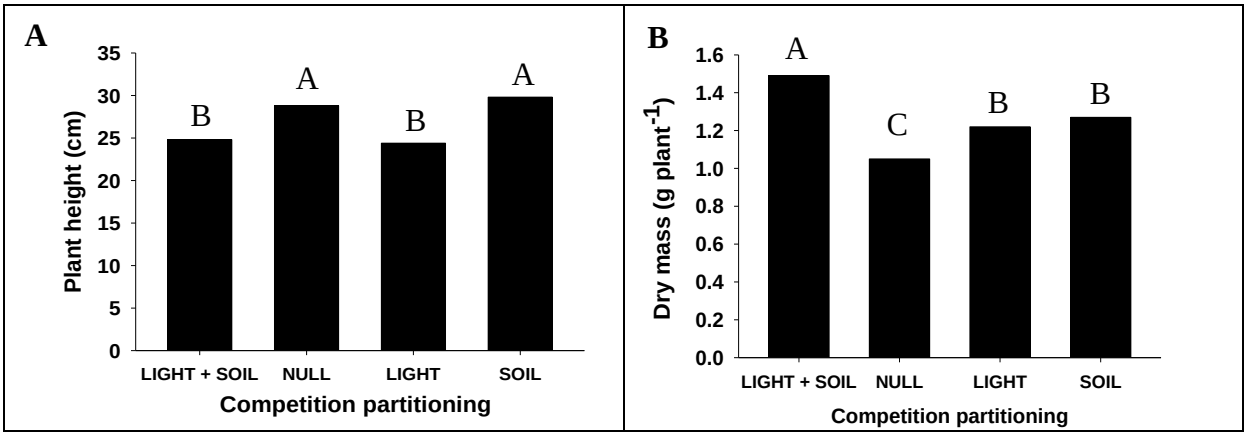

Figure 7. Plant height (A) and shoot dry biomass (B) of *F. miliacea* biotypes in four competition conditions. University of Arkansas, Fayetteville / AR, 2010. Bars with the same capital letters are not significantly different at p≤0.05 (Fischer`s test).

Plant height of FIMMI 13 presented higher value when in competition with the rice crop (Figure 8). On the other hand, the FIMMI 10 biotype had lower values in competition with the rice crop and FIMMI 13; however, there was no difference between biotypes. The rapid growth in plant height is a characteristic usually desired in the suppressive process of weed-crops, as gives them advantage in competition for light characteristics, allowing shade smaller competitor species (Ogg Jr.; Seefeldt, 1999).

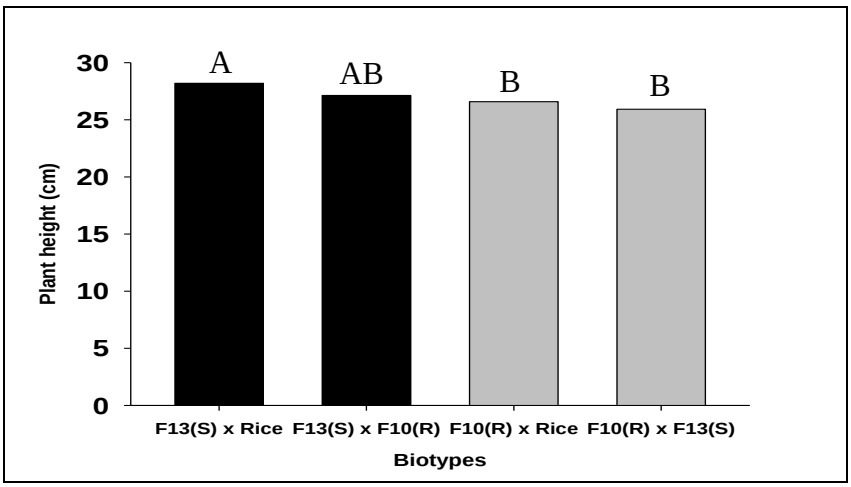

Figure 8. Height of *F. miliacea* biotypes FIMMI 13 and FIMMI 10 when competing with rice or each other. University of Arkansas, Fayetteville / AR, 2010. Bars with the same capital letters are not significantly different at p≤0.05 (Fischer`s test).

There was no interaction effect of *F. miliacea* biotype combination and competition partitioning at 36 DAT. However, there were significant differences in number of tillers, plant height, shoot dry biomass, root dry biomass and leaf area for all treatments (Figure 9). Rice plants produced lower number of tillers in competition null (Figure 9A). However, a greater value on plant height, leaf area and dry biomass was observed in treatment when the competition for resources was below ground (Figure 9B, 9C and 9E). For root dry biomass variable, the rice crop had the highest value when competing for resources light + soil. Root competition may indirectly structure diversity through light + soil competition interactions, this indirect effect of root competition occur because light + soil competition interactions alter the overall size-asymmetry of competition (Lamb et al., 2009).

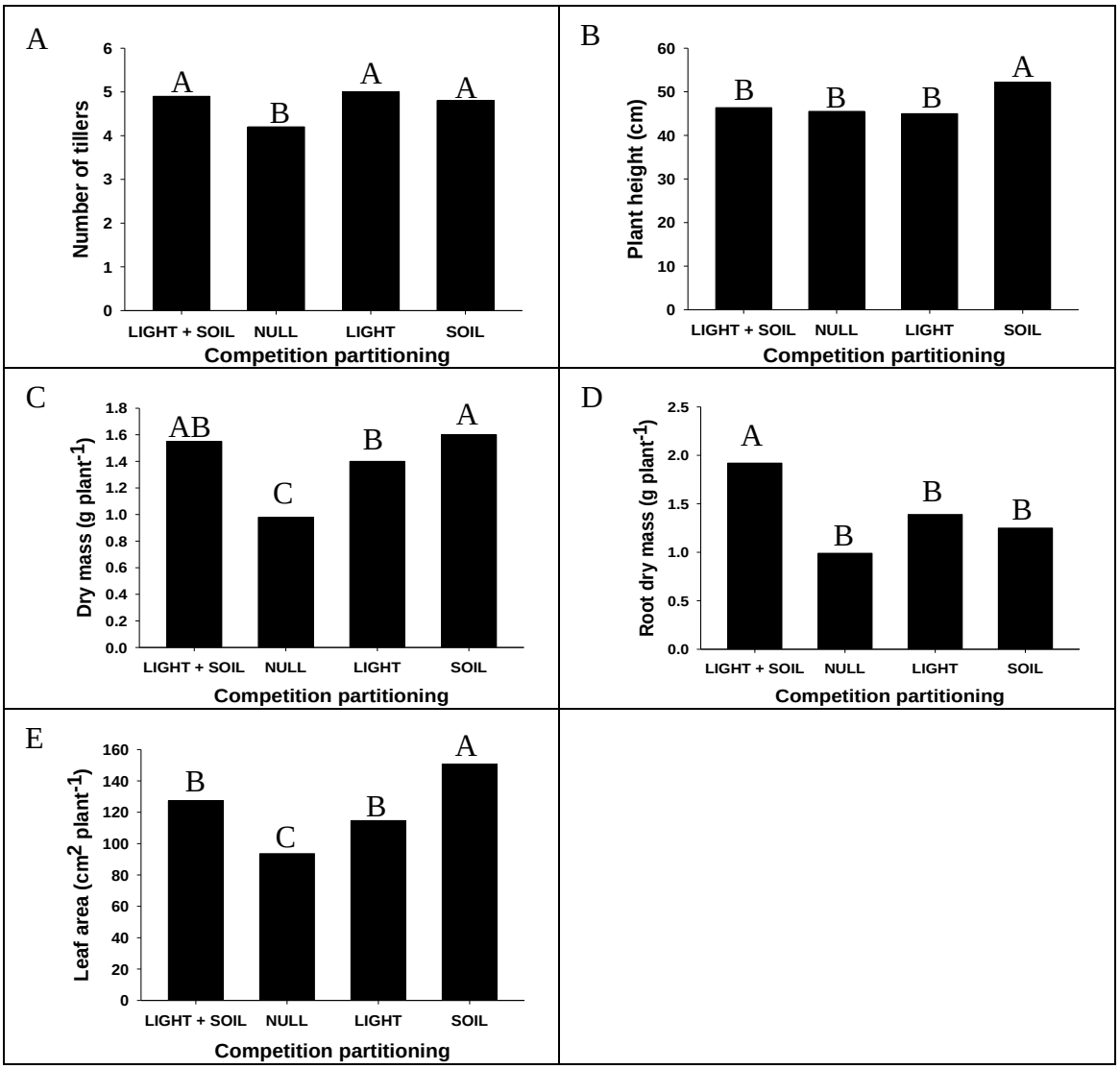

Figure 9. Number of tillers (A), plant height (B), shoot dry biomass (C), root dry biomass (D) and leaf area (E) of rice in four competition conditions. University of Arkansas, Fayetteville / AR, 2010. Bars with the same capital letters are not significantly different at $p \leq 0.05$ (Fischer`s test).

Generally, the lowest values in these variables were in treatment in which the rice crop was not in competition with the biotypes *F. miliacea*. Morphologically similar species, the advantage may not be favorable to crop, as observed for red rice competing as rice crop (Pantone and Baker, 1991), probably due to competition for the same niche, which may result competitive advantage for the weed. In the present study, root competition was essentially for soil nutrients since all treatments were watered daily. Generally, weeds respond efficiently to high nitrogen rates. Thus, competition between rice and red rice was observed higher dry biomass under high nitrogen levels to red rice (Burgos et al., 2006). Furthermore, in research evaluating the effect of nitrogen fertilization and growth of *F. miliacea*, the researchers observed that nitrogen treatment stimulated the formation of tillers, increased number of inflorescences and increased dry matter production, all of which contributed to production of higher number of seeds (Begum et al., 2008).

One disadvantage for the null competition treatment may affect the microclimate by reducing heat and water fluxes in the canopy, increasing both air temperature and humidity (Figure 6), even that pots were rotated to new positions every two days to minimize the environmental variability. In competition study with wheat crop and clover, the higher temperature and shading by the partitions may have led to increased senescence of wheat and clover leaves, and the mineralization of these may senesced leaves have contributed nitrogen to the wheat crop causing the slightly higher nitrogen concentration in wheat when there was above-ground division (Thorsted et al., 2006). Similarly, the acrylic above-ground partitions increased crop canopy temperature about 7°C (Brede and Duich, 1986).

It was found differences in plant height and shoot dry biomass values for rice in competition with *F. miliacea* biotypes (Figure 10). *F. miliacea* susceptible biotype was taller than the resistant one when both were competiting with rice; however, no difference was observed between the *F. miliacea* biotypes when competing with each other.

Rice plants had higher values in these variables in competition with resistant *F. miliacea* biotype to ALS-inhibiting herbicides (Figure 10A and B). Thus, the susceptible *F. miliacea* biotype can be more competitive than the resistant biotype in some variables when in competition with the rice.

The results suggest that rice has competitive superiority in relation to *F. miliacea* biotypes resistant and susceptible to ALS inhibitors herbicide and that these do not differ with each other. Rice in equal proportions, regardless of the biotype, showed, in general, increases in the variables studied. Interspecific competition for rice and intra-specific for *F. miliacea* biotypes were the most advantageous.

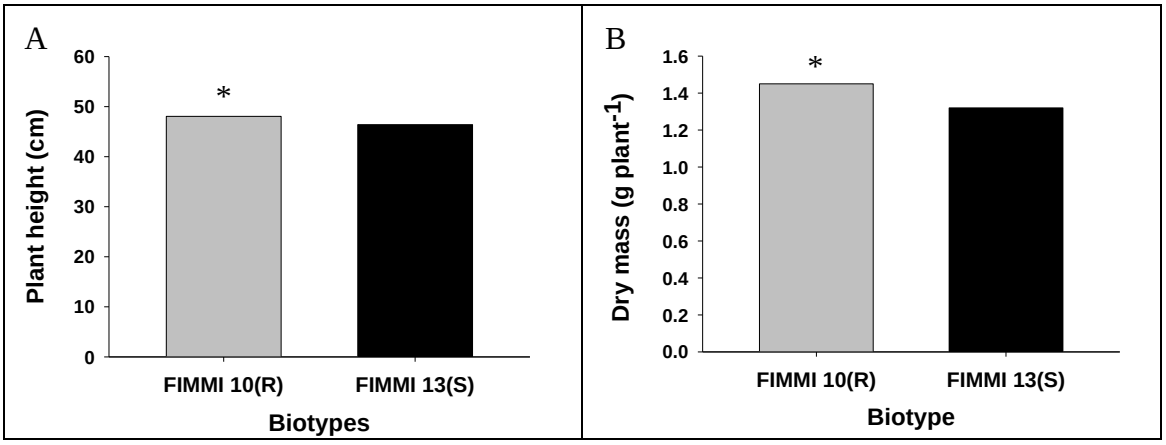

Figure 10. Rice height (A) and dry biomass (B) when competing with R or S biotypes of *F. miliacea*. Bars with asterisks are significantly different ($p \leq 0.05$) according to "Fischer`s test". University of Arkansas, Fayetteville / AR, 2010. Bars with the same capital letters are not significantly different at $p \leq 0.05$ (Fischer`s test).

The ALS-resistant *F. miliacea* biotype was less competitive with rice than the susceptible biotype. Intraspecific competition among rice plants is stronger than interspecific competition with *F. miliacea* biotypes for below ground competition has the most effect on these competing species.

The ALS-resistant *F. miliacea* biotype was less competitive with rice than the susceptible biotype. Because these populations were not near isolines, we cannot conclude that the difference in competitive ability was a physiological penalty for the resistance trait. Further experimentation is needed to address that question. In farmers' fields, though, which are dominated by resistant populations, rice yields are significantly reduced for the lack of weed control. Integrating chemical alternatives with agronomic tools such as the use of competitive varieties should be an effective strategy for this less competitive ALS-resistant *F. miliacea* biotype.

ACKNOWLEDGEMENTS

We are grateful to the CNPq for the scholarship to Carlos Eduardo Schaedler. We are grateful to the Departament of Plant Protection ("Fitossanidade") (Federal University at Pelotas) and Crop Soil and Environmental Science Department (University of Arkansas) for the structure availability and viability of the research conducted.

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Artigo 04 - Revista Planta Daninha

CROSS RESISTANCE TO *ALS*-INHIBITOR HERBICIDES AND SEQUENCING OF THE *ALS* GENE IN *Fimbristylis miliacea* (L.) VAHL

CROSS RESISTANCE TO *ALS*-INHIBITOR HERBICIDES AND SEQUENCING OF THE *ALS* GENE IN *Fimbristylis miliacea* (L.) VAHL[4]

ABSTRACT – Weeds resistant to acetolactate synthase (ALS)-inhibitors herbicides are the most widespread in number of species with resistance, compared to other herbicide modes of action. *Fimbristylis miliacea* is one of the most troublesome weeds in flooded rice fields in Santa Catarina, southern Brazil. ALS-inhibiting herbicides are widely used to control weeds in this crop. The continuous use of ALS-inhibitor herbicides has led to the evolution of herbicide-resistant *F. miliacea* populations. The objective of this research is to determine the level of resistance of *F. miliacea* to several ALS-inhibiting herbicides, and to compare the *ALS* gene nucleotide sequences between resistant and susceptible *F. miliacea* biotypes. To confirm the resistance of FIMMI to ALS inhibitors, whole-plant bioassays were conducted in 2008 and 2009. In the bioassay experiments, two ALS-resistant (FIMMI 10 and FIMMI 12) and one ALS-susceptible (FIMMI 13) biotypes were used. The results confirmed that FIMMI 10 is cross-resistant to three ALS chemical families while the FIMMI 12 is cross-resistant to two chemical families of ALS-inhibiting herbicides. To determine if target site mutation is the mechanism of resistance in *F. miliacea*, the ALS gene was sequenced and compared between the susceptible and resistant biotypes. Analysis of the nucleotide and amino acid sequences indicated that a single point mutation, Thymine-Adenine, in FIMMI 10 resulted in an amino acid substitution, $Asp_{376}Glu$, in the F region between the C, A, D and B, E domains.

Key words: Acetolactate synthase, ALS gene, herbicide resistance, globe fringerush.

RESUMO – Plantas daninhas resistentes a herbicidas inibidores da acetolactato sintase (ALS) são largamente difundida entre as espécies com resistência a herbicidas com outros modos de ação. *F. miliacea* é uma das plantas daninhas mais problemáticas em lavouras de arroz em Santa Catarina, no sul do Brasil. Herbicidas inibidores da (ALS) são amplamente utilizados para controlar as plantas daninhas nesta cultura. O uso contínuo desses herbicidas tem levado populações de *F. miliacea* à evolução de resistência aos herbicidas. O objetivo desta pesquisa foi determinar o nível de resistência de *F. miliacea* a alguns herbicidas inibidores da ALS, e comparar as seqüências de nucleotídeos dos genes da ALS entre biótipos resistentes e suscetível de *F. miliacea*. Para confirmar a resistência de *F. miliacea* aos inibidores da ALS,

[4] Recebido para publicação em __/__/__e na forma revisada em __/__/

bioensaios foram realizados em 2008 e 2009. Foram utilizados dois biótipos resistentes (FIMMI 10 e FIMMI 12) e um suscetível (FIMMI 13) aos herbicidas inibidores da ALS. Os resultados confirmam que o biótipo FIMMI 10 apresenta resistência cruzada para três grupos químicos da ALS enquanto FIMMI 12 apresentou resistência cruzada a dois grupos químicos inibidores da ALS. Para determinar se a mutação no local de ação era o mecanismo de resistência em *F. miliacea*, o gene ALS foi seqüenciado e comparado entre os biótipos resistentes e suscetível. A análise das seqüências nucleotídicas e aminoácidos entre os diferentes biótipos indicou um único ponto de mutação, timina-adenina, no biótipo FIMMI 10 que resultou na substituição de aminoácidos, Asp376Glu, na região F, entre os domínios C, A, D e B, E.

Palavras-chave: Acetolactato sintase, gene ALS, resistência a herbicidas, cuminho.

INTRODUCTION

Chemical weed control is the most commonly adopted weed management practice in rice fields in Santa Catarina, southern Brazil. However, the use of herbicides has limitations because weedy species that evolued resistance to herbicidal compound. Currently, there are over 360 biotypes resistant to herbicides worldwide, involving approximately 197 weed species (Heap, 2011). In Brazil, there are 18 weeds species recorded as resistant to herbicides, and about 60% of the cases involve resistance to ALS-inhibitor herbicides, mainly in soybean, maize and rice (Heap, 2011).

Acetolactate synthase (also referred Acetohydroxyacid synthase – AHAS, EC 2.2.1.6) is a plastidic enzyme that is found in bacteria, fungi and plants (Duggleby and Pang, 2000). AHAS catalyses the decarboxylation of pyruvate, and its condensation with another pyruvate, producing acetolactate, the precursor of valine and leucine; pyruvate reaction with an alfa-ketobutyrate produces acetohydroxy butyrate leading to isoleucine synthesis (Chipman et al., 1998). ALS is the target site of many classes of commercial herbicides including the sulfonylureas (SUs) (Chaleff and Mauvais, 1984), imidazolinones (IMIs) (Shaner et al., 1984), pyrimidinyloxybenzoates (PTBs) (Stidham, 1991); triazolopyrimidines (TPs) (Gerwick et al., 1990) and sulfonylaminocarbonyltriazolinones (SCTs) (Santel et al., 1999).

Generally, a point mutation in one of the conserved domains in the ALS gene is responsible for conferring resistance to ALS inhibitors (Boutsalis et al., 1999). So far,

substitution at position 376 [numbering standardized to *Arabidopsis thaliana* (L.) Heynh. ALS sequence] has been documented and associated with resistance to ALS-inhibitors in three biotypes: smooth pigweed (*Amaranthus hybridus* L.) Kochia (*Kochia scoparia* (L.) Roth) powell amaranth (*Amaranthus powellii* S. Wats.) (Whaley et al., 2007; Warwick et al., 2008; Ashigh et al., 2009). Recently, Massa et al. (2011) reported a new amino acid substitution of the ALS gene Arg_{377}His in loose silky-bent [*Apera spica-venti* (L.) Beauv.]; this mutation so far is associated with resistance to three chemical families of ALS inhibitors (sulfonylureas, triazolopyrimidines and sulfonylaminocarbonyltriazolinone). Therefore, eight target site mutations has been found to confer resistance to ALS inhibitors in weed species by rendering the enzyme insensitive to the herbicide: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653} and Gly_{654} [numbering standardized to *Arabidopsis thaliana* (L.) Heynh. ALS sequence] (Tranel et al., 2011). Nucleic acid point mutations can lead to amino acid substitutions that reduce the efficacy of the herbicide. For example, Ala_{122} and Ala_{205} mainly result in resistance to IMI herbicides; Asp_{376} and Trp_{574} are responsible for resistance to all classes of ALS inhibitors and Gly_{654} confer resistance to IMI, SU and SCTs.

Fimbristylis miliacea belongs to the Cyperacea family and is a troublesome weed in rice fields. This weed is a summer-germinating annual sedge often present at high densities and has envolved resistance to pyrazosulfuron-ethyl, an ALS-inhibitor herbicide (Noldin et al., 2002). *F. miliacea* is problematic weed in rice fields because of its prolific seed production, which allows it to become widespread after entry into any rice production area. In addition, *F. miliacea* has a continuous emergence pattern throughout the growing season (Watanabe, et al., 1997). *F. miliacea* can reduce grain yield of rice by about 42% due to season-long competition (Begum et al., 2008). Usually, *F. miliacea* is controlled by herbicides in flooded rice fields. In comparison with other herbicide alternatives, the ALS-inhibiting herbicides are most widely used in rice production due to their efficacy at low rates, flexibility of use and mainly because of their favorable environmental profile and low mammalian toxicity (Shaner, 1999). However, the use of herbicides as the main method of weed control without diversifying the mechanism of action which has resulted in the evolution of herbicide-resistant weeds (Gressel and Segel, 1990). Cross-resistance to different ALS inhibitors is commonly observed (Burgos et al., 2001; Lamego et al., 2009; Merotto et al., 2009; Jin et al., 2011).

Resistance in *F. miliacea* is an example of rapid adaptive evolution to ALS inhibitors. In 2001, after at least 10 consecutive years of pyrazosulfuron-ethyl use a resistant biotype was found in a rice area in Santa Catarina. The impact of resistance to pyrazosulfuron-ethyl in *F.*

miliacea and to other ALS-inhibitor herbicides is not known. Combining mutations with observed resistance would allow predicting what specific ALS-inhibiting herbicides may or may not be successfully used and, also helps to elucidate the evolution and spread of resistance to ALS-inhibitors in *F. miliacea* in rice fields in Southern Brazil.

Most cases of resistance to ALS-inhibiting herbicides involve a modified ALS enzyme with reduced herbicide binding properties (Tranel et al., 2011). The mechanism of resistance to ALS herbicides at the biochemical and genetic levels has not been reported for *Fimbristylis miliacea*. The objectives of this research were to 1) determine the level of resistance to several ALS-inhibiting herbicides, and 2) compare the ALS sequences between resistant and susceptible *F. miliacea* biotypes.

MATERIALS AND METHODS

Plant materials

Seeds of globe fringerush surviving applications of the registered field dose of pyrazosulfuron-ethyl (20 g a.i. ha^{-1}) were collected in February 2006 from a rice field in Forquilhinha county, Santa Catarina, southern Brazil (FIMMI 10: 28º47'912''S, 49º26'528''W, FIMMI 12: 28º47'881''S, 49º33'971''W). The field had been treated with ALS inhibitor herbicides for at least 10 consecutive years. Those biotypes (FIMMI 10 and FIMMI 12) are hereafter designated as ALS resistant (R). Susceptible (S) biotype (FIMMI 13: 27º16'978''S, 49º44'776''W) was collected from a fallow area where herbicides had not been sprayed in previous growing seasons.

Determination of resistance level

Dose response experiments were performed in 2008 and 2009, using greenhouse-grown seedlings. Seeds of susceptible (FIMMI 13) and two resistant biotypes (FIMMI 10 and FIMMI 12) were planted in 500mL pots filled with soil obtained from a rice field and placed in a glasshouse at the Federal University at Pelotas-RS. The growth conditions were 35/25°C day/night temperature (± 5°C), with a photoperiod of 14 h. The plants were treated with pyrazosulfuron-ethyl (Sirius 250 SC, Iharabras), penoxulam (Ricer 240 SC, Dow AgroScience), bispyribac-sodium (Nominee 400 SC, 400 g a.i. L^{-1}; Iharabras), (imazethapyr+imazapic) (Only®, BASF) and bentazon (Basagran 600, BASF). The dose range for the S biotype was 0, 1/32, 1/16 1/8, 1/4, 1/2, 1X and that of the R biotype was 0, 1, 2, 4, 8,

16, 32X of the labeled dose. The 1X doses were: 17.5, 30, 50, (75+25), and 960 g a.i. ha^{-1} for pyrazosulfuron-ethyl, penoxsulam, bispyribac-sodium, (imazethapyr+imazapic), and bentazon, respectively (Table 2). Herbicides were applied at the six-leaf stage of *F. miliacea* with a non-ionic surfactant (0.5% v/v), using a backpack sprayer delivering 150 L ha^{-1} at 200 kPa.

The experiment was a randomized complete block design consisting of three factors (biotype, herbicide and dose) in a 3 x 4 x 7 factorial hierarchical arrangement. The experimental unit consisted of one plant per pot replicated four times.

F. miliacea control was visually evaluated at 28 days after treatment (DAT), using a percentage scale of 0-100 where 100% = total mortality. At 28 DAT, above-ground plant material was cut and oven-dried for 72 h at 60°C and the dry weight recorded. GR_{50} values (the rate necessary to provide 50% reduction of control and dry matter) for resistant and susceptible biotypes were estimated from adjusted mathematical models.

Statistical analyses

Data were tested for homogeneity of variance and analyzed using ANOVA ($p \leq 0.05$) to determine biotype x herbicide x dose interactions, using Statistical Analysis System (SAS) software. When significant difference was detected, the data were fitted with a sigmoidal log-logistic model:

Equation 1:
$$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

Where: Y corresponds to control (%) or shoot biomass; X is the herbicide application rate in g a.i. ha^{-1}, a is the maximum asymptote. X_0 is the herbicide dose required to killing 50% of individuals or to reduce the shoot biomass or yield loss and b is the slope of the curve around X_0. Weed control and shoot biomass data were fitted with the sigmoidal log-logistic model (Seefeldt et al., 1995) using the herbicide rate as independent variable. Sigma Plot 10.0 (Sigma Plot, 2004) was used for the regression analysis and curve fitting.

The amount of herbicide that would control 50% of the individuals (LD_{50}), reduce above-ground dry weight 50% (GR_{50}) were calculated from the regression equations. The resistance index (RI) were calculated by dividing LD_{50} or GR_{50} values of each R biotype by the corresponding value of the S biotype.

Genomic DNA Extraction.

Leaves of ten individual plants per biotype were harvested at five-leaf stage and stored at -80°C. DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle, 1987). Leaf tissues were ground in liquid nitrogen and transferred to chilled Eppendorf tubes. After adding 500 µL of extraction buffer (Tris HCl pH 8.0; 20 mM EDTA pH 8.0, 2M NaCl, 2% CTAB, 2% PPV-40, 1mM phenanthroline, and 0.3% β-mercaptoethanol) to the tubes, they were vortexed vigorously for 1 min. The tubes were incubated in a water bath at 55°C for 45 min. Following incubation, 500 µL of chloroform : phenol : isoamylalcohol (25:24:1 by volume) were added to the tubes, mixed, and centrifuged at 12,000 rpm (15,000 X g) for 10 min at 4°C. The supernatant was transferred to a fresh tube and mixed with 500 µL of isopropyl alcohol and incubated at -80°C overnight. The next day, tubes were centrifuged for 10 min at 12,000 rpm (15,000 b) at 4°C, and the supernatant was discarded. The DNA pellet was washed in 100% ethanol, vacuum-dried and resuspended in 30 µL of sterile distilled deionized water. Genomic DNA was quantified by fluorometry and the DNA was diluted to a final concentration of 100 ng μL^{-1}.

Primer Design for ALS, DNA amplification and Gene Sequencing

Primer pairs were designed to amplify the ALS gene in four segments (Table 1). The primers used for D, F, B and E domains were the same as those for smallflower umbrella sedge (*Cyperus difformis* L.) (Merotto et al., 2009).

Table 1. Primer sequences used for the amplification of acetolactate synthase gene in *F. miliacea*.

Region	Primers	Sequence (5'-3')	Amplicon size obtained (bp)
III	M2cf	GTYGGRCARCAYCARATGTGGG	535
	M2cr	TCCKGCCATCWCCWTCCRKK	
III	W1f5	NATGYTNGGNATGCAYGG	591
	W1r1	CATCAGGAAGGAACCATCACCGTC	
II	W2f4	ACNGAYGCNTTYCARGARAC	492
	W2r4	TGCTTTGCTAACTGCATAAT	
I	RACE_R1	CCTGGAAGGCGCATTGGTGGATTCCA	913
	UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
		CTAATACGACTCACTATAGGGC	
I	ALS_RaceF2	TAGCGACAGCACAAGGAGAA	848
	ALS_RaceR2	CTTTGATTCGGAAACAAGACG	

Taq 2x Master Mix DNA polymerase (New England Biolabs) was used to amplify ALS gene fragments from genomic DNA in two separate polymerase chain reactions (PCR) for region III (Figure 1). The PCR cocktail consisted of 0.9 µl of genomic DNA (100 ng μl^{-1}), 1.5 µl of each primer (10 pmol), 15 µl of Taq 2X Master Mix PCR buffer [MgCl2 (1.5 mM), dNTPs (200 µM)], and 11.1µL water. Amplification was conducted in a MJ Research PTC-200 thermocycler using the following cycles: DNA denaturation for 3 min at 94 °C, and 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1.5 min elongation at 72 °C. The samples were then subjected to 10 min elongation at 72 °C. PCR products were analyzed in 1% agarose gels stained with ethidium bromide using a 1 kb DNA ladder (Fermentas) for reference. The degenerate oligonucleotide primers M2cf and M2cr (Table 1) produced a clear 535bp amplification product corresponding to the expected size. For region II, PCR cocktail was conducted as previously described; however, the amplification was conducted in a Techne TC 5000 thermocycler using the following cycles: DNA denaturation for 3 min at 94 °C, and 45 cycles of 1 min denaturation at 94 °C, 1min annealing at 49 °C, and 1.5min elongation at 72 °C. The samples were then subjected to a 10 min elongation at 72 °C. The amplified fragment was purified using Wizard® SV Gel and PCR Clean-Up System following the manufacture's instructions and sequenced. Purified fragments were then sequenced directly after purification at the DNA Resource Center of the University of Arkansas, Fayetteville, AR, using an ABI 3100 Genetic Analyzer. 13. When the sequencing of region II was completed, new primer pairs were designed in an attempt to obtain sequences for the remaining domains, C and A (region I). However, we were not able to obtain the ALS gene sequence from this last region.

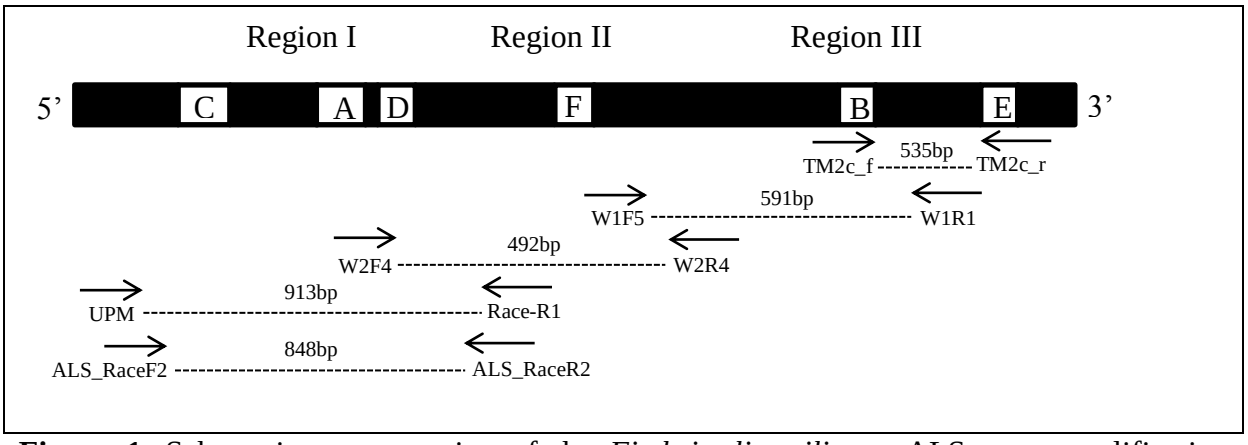


Figure 1. Schematic representation of the *Fimbristylis miliacea* ALS gene amplification procedure. The sequences of the primers are reported in Table 1. The highly conserved domains where mutations endowing herbicide resistance have previously been found are represented in white boxes. UPM = universal primer mix.

After sequencing the amplified fragments, RNA was extracted from fresh leaf tissue from six-leaf stage plants of the susceptible biotype (FIMMI 13), using PureLink™ RNA Mini Kit (Invitrogen). Complementary DNA was synthesized from the RNA using a cDNA synthesis kit (SMARTer RACE cDNA Amplification Kit) to obtain remaining sequences for C and A domains toward the 5' end of the ALS gene. RNA quality was evaluated in a formaldehyde gel through electrophoresis using 1.2% agarose gel stainded with ethidium bromide. The gene-specific regular reverse primer Race_R1 (Table 1) for the RACE amplification was based on the sequence obtained earlier (Figure 1). The RACE PCR cycling program was used following the manufacturer's instructions (Clontech). The RACE PCR fragment was excised from the gel, purified as described earlier, cloned into a plasmid vector and transformed into *Escherichia coli* competent cells using the Topo-TA Cloning (Topo-TA Cloning) procedure. Ten colonies were chosen and suspended individually in 20 μL of water. PCR was conducted using 1μL of this suspension as template and the universal oligonucleotide M13 reverse and M13 forward (-20) primers. PCR products were checked for the expected size and the amplicon excised and purified as described earlier. DNA sequencing was conducted in both directions using the M13 reverse and M13 forward (-20) primers

The ALS sequences were analyzed using Sequencher v. 4.8 and compared with sequences in the GenBank using BLASTN.

RESULTS AND DISCUSSION

Level of Resistance to ALS Inhibitors.

The whole-plant experiments showed that FIMMI 10 and FIMMI 12 biotypes are resistant to three and two chemical groups of ALS inhibitors, respectively (Table 2). FIMMI 10 biotype showed 46-, 192-, and 271-fold resistance to pyrazosulfuron-ethyl, bispyribac-sodium and penoxsulam, respectively, relative to FIMMI 13, the susceptible biotype. FIMMI 12 showed 566- and 114-fold resistance to pyrazosulfuron-ethyl and penoxsulam, respectively, relative to the S biotype. FIMMI 10 and FIMMI 12 biotypes collected in a rice field exhibited a high level of resistance (Table 2). It shoud be pointed out that the resistance level to pyrazosulfuron-ethyl was very different comparing the R biotypes: resistance index of 46 for FIMMI 10 and 566 for FIMMI 12. Herbicide sulfonylaminocarbonyltriazolinones (SCTs) have not selected for resistance in this population because is not registered for use in Brazil.

Table 2. Whole-plant bioassays for *Fimbristylis miliacea* Federal University at Pelotas, Capão do Leão / RS, 2008/09.

Herbicide	Biotype	GR_{50}[a]	RI[b]
pyrazosulfuron-ethyl	FIMMI 10	56.4 ± 2.93	46.2
	FIMMI 12	690.1 ± 186.9	565.6
	FIMMI 13	1.22 ± 0.11	-
bispyribac-sodium	FIMMI 10	161.4 ± 7.6	192.1
	FIMMI 12	20.1 ± 1.01	23.9
	FIMMI 13	0.84 ± 0.06	-
penoxsulam	FIMMI 10	66.1 ± 3.4	275.4
	FIMMI 12	28.6 ± 2.6	119.2
	FIMMI 13	0.24 ± 0.07	-
(imazetapyr+imazapic) [c]	FIMMI 10	32.1 ± 1.83	9.90
	FIMMI 12	63.3 ± 4.25	19.5
	FIMMI 13	3.24 ± 0.25	-

[a]Herbicide rate (± standard error) responsible for reducing growth rate of *F. miliacea*.
[b]Resistance Index = GR_{50} of R biotype/GR_{50} of S biotype.
[c] The somation of imazethapyr + imazapic.

This result is consistent with the hypothesis that ALS-inhibitor resistance in FIMMI 10 and FIMMI 12 are due to an altered enzyme. Thus, it can infered that an altered enzyme is responsible for ALS resistance in *F. miliacea*. The ALS nucleotide sequences of R and S biotypes were compared to identify possible point mutations associated with resistance.

ALS Sequencing and Identification of ALS Mutation

After purification of PCR products from *F. miliacea* biotypes the DNA sequence information was obtained for 1713 base pairs for FIMMI 13 biotype and 1379 base pairs for FIMMI 10 and FIMMI 12 biotypes. The sequences correspond to nucleotide positions 605 and 939 to 2317 of the standard Arabidopsis ALS gene sequences X51514 for FIMMI 13S and resistant biotypes, respectively. This covered about 67% ALS gene for FIMMI 10 and FIMMI 12 and 83% for FIMMI 13 in comparison with the ALS gene in Arabidopsis at start codon (ATG) (*Arabidopsis thaliana* L. Acc. no. X51514). The degenerate oligonucleotide primers W1f5 and W1r1 produced a clear 591bp fragment. These oligonucleotide primers amplified region I of the ALS gene corresponding to B and E domains (Figure 1). The degenerated oligonucleotide primers W2f4 and W2r4 (Table 1) produced a clear 492bp fragment and these oligonucleotide primers amplified region II of the ALS gene corresponding to D and F domains (Figure 1).

A single amplicon of about 913bp was obtained and queried with the C, A, D, F, B and E domains of *Cyperus difformis* and D, F, B and E domains of *Fimbristylis miliacea* ALS fragments. The ALS sequence from the two species was found to have high similarity. The nucleotide sequence from FIMMI 13 was 87% identical to the ALS from smallflower umbrella sedge (*Cyperus difformis* L.; Genbank acc. no. EF061294.2) and was 100% identical to FIMMI 12. However, the ALS sequences from FIMMI 10 had a mutation that was absent in the other biotypes with a substitution of thymine$_{1128}$ to adenine. This mutation coded for a substitution of Asp to Glu at position 376 (standardized to *Arabidopsis*) of the deduced protein sequence. In this case, Asp$_{376}$ (positions 1126, 1127 and 1128), nucleotide substitution (T to A) were observed at position 1128, i.e., susceptible FIMMI 13 biotype: GAT = asrpartic acid (Asp); and resistant FIMMI 10 biotype GAA = glutamic acid (Glu).

The RI values of FIMMI 12 for bispyribac-sodium and the premix formulation of (imazethapyr+imazapic) were 24 and 23, respectively. For FIMMI 10, the RI value was equivalent to 12 for (imazethapyr+imazapic) herbicide. These values were obtained from GR_{50} of R biotype divided by GR_{50} of S biotype are high due to the GR_{50} values for the susceptible biotype arevery low, even all biotypes were controlled. The reason for this is that GR_{50} was obtained from a mathematical model (logistic curve), thus it is an estimation, not a real value (Lamego et al., 2009).

The Asp$_{376}$Glu substitution has been associated with ALS-inhibitor resistance in smooth pigweed (*Amaranthus hybridus* L.). kochia (*Kochia scoparia* (L.) Roth), powell amaranth (*Amaranthus powellii* S. Wats.) (Whaley et al., 2007; Warwick et al., 2008; Ashigh et al., 2009). It is generally agreed that this substitution confers relatively high resistance, for example, in experiments conducted with smooth pigweed to evaluate a biotype that survived applications from ALS-inhibitors herbicides (Whaley et al., 2007). That paper reported 60- to >3,200-fold resistance to all four ALS inhibiting herbicide families compared to the S biotype (Whaley et al. 2007). This substitution confered resistance to SU, IMI, PTB, TP and SCT (Whaley et al., 2007), but in this study, it was found out a biotype (FIMMI 10) cross-resistant to three different ALS-inhibiting groups: SU, PTB and TP.

ALS-inhibitor herbicides have been widely used worldwide because these compounds present favorable environmental properties and high agronomic efficacy. On the other hand, the consecutive use associated with the high efficacy has exerted extensive selection pressure for resistant biotypes (Saari et al., 1994; Tranel and Wright, 2002). The resistant biotypes used in this study are from a rice field that had been sprayed with pyrazosulfuron-ethyl, a sulfonylurea herbicide, for at least 10 consecutive years. However, there is no reporting about

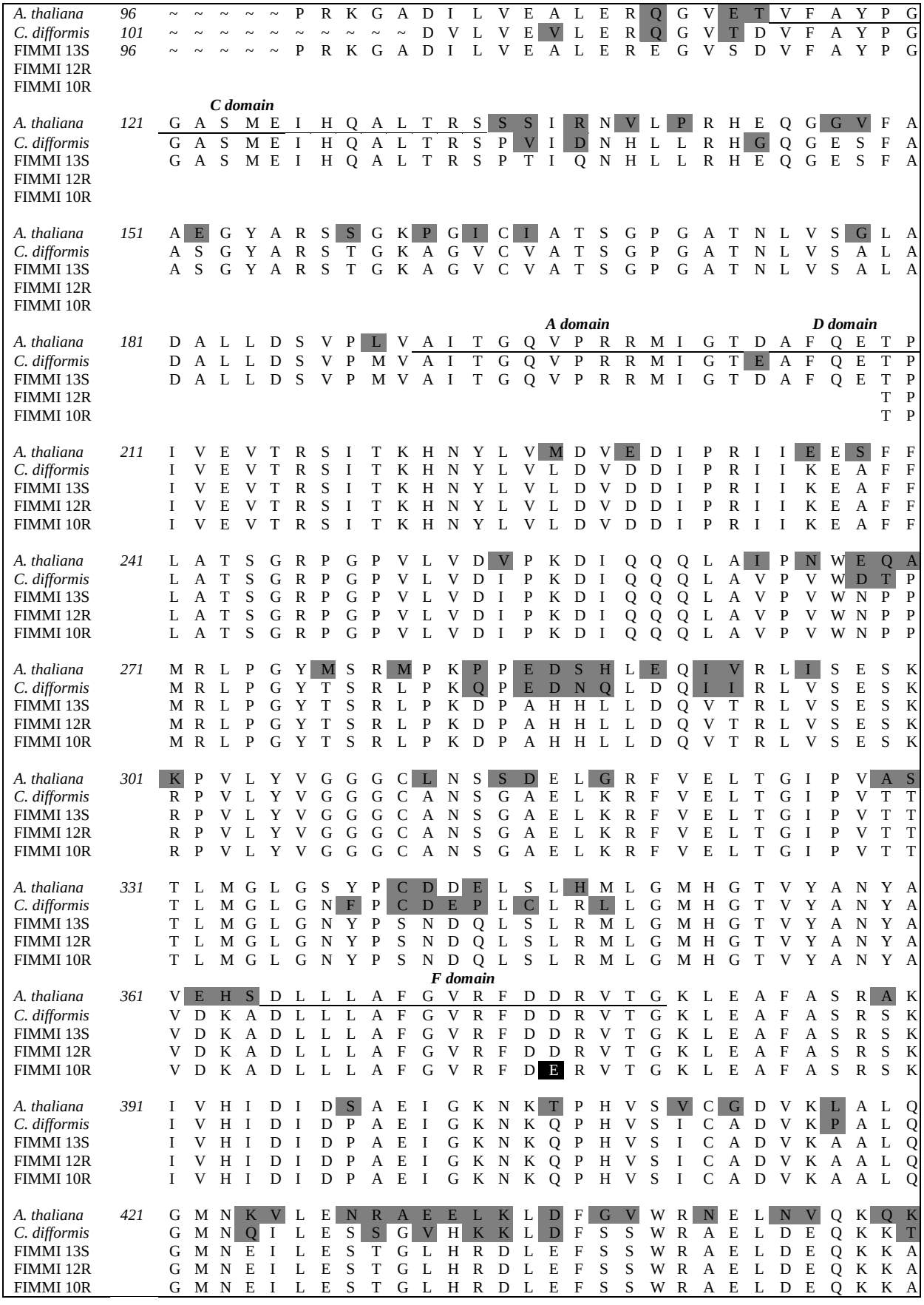
spraying ALS-inhibitor herbicide from the IMI group because the farmers use only conventional cultivars, not Clearfield rice. Moreover, the IMI herbicide used in this research was a premix formulation with imazethapyr+imazapic), two different molecules of the same group, and this may delay the selection pressure of these biotypes to the imidazolinone herbicides.

In this research, it was not detected any variation in nucleotide and amino acid sequences between FIMMI 12 and FIMMI 13 in the F, B and E regions analyzed. Thus, we suspect that there is a mutation in A or D domains of biotype FIMMI 10 and specially in FIMMI 12 due to the high level of resistance showed to sulfonylurea herbicide (Table 2) (Warwick et al., 2010; Massa et al., 2011; Jin et al., 2011). Usually, mutations at Pro_{197} can result in resistance to SU herbicides and to variable levels of resistance and even susceptibility to others groups (Tranel et al., 2011).

In this study, FIMMI 12 showed resistance to pyrazosulfuron-ethyl and penoxsulam but was sensitive to bispyribac-sodium. Kuk et al. (2004) reported that a single amino acid substitution in the channel of access to the active site of the ALS can result in insensitivity to an herbicide that normally binds to the amino acid residue. According to these authors, the same mutation may not affect as much the binding of other herbicides from the same chemical group that do not interact with this amino acid residue.

In summary, these results confirmed that the biotype FIMMI 10 is cross-resistant to three ALS chemical families while FIMMI 12 is cross resistant to two chemical families of ALS-inhibiting herbicides. These results suggested that certain ALS-inhibiting herbicides can still be used to control some resistant populations like FIMMI 12. For instance, it was observed susceptibility to the premix formulation of (imazethapyr+imazapic) for all biotypes, but this herbicide can only be applied in Clearfield rice. The FIMMI 12 biotype was susceptible to bispyribac-sodium, commonly used in rice fields and can still is used for weed control in rice fields infested with this biotype. However, because of the potential to select for resistance to any of these herbicides, ALS-inhibiting herbicides should only be used as part of an integrated weed management program.

This research indicated that ALS-inhibiting resistance in *F. miliacea* biotype is based on an altered target site conferred by a single-point mutation for FIMMI 10 biotype. However, the C and A region have not yet been sequenced in biotypes FIMMI 10 and FIMMI 12 indicating that further investigation is still needed.


A. thaliana 96 ~ ~ ~ ~ ~ P R K G A D I L V E A L E R Q G V E T V F A Y P G
C. difformis 101 ~ ~ ~ ~ ~ ~ ~ ~ ~ D V L V E V L E R Q G V T D V F A Y P G
FIMMI 13S 96 ~ ~ ~ ~ ~ P R K G A D I L V E A L E R E G V S D V F A Y P G
FIMMI 12R
FIMMI 10R
C domain
A. thaliana 121 G A S M E I H Q A L T R S S S I R N V L P R H E Q G G V F A
C. difformis G A S M E I H Q A L T R S P V I D N H L L R H G Q G E S F A
FIMMI 13S G A S M E I H Q A L T R S P T I Q N H L L R H E Q G E S F A
FIMMI 12R
FIMMI 10R
A. thaliana 151 A E G Y A R S S G K P G I C I A T S G P G A T N L V S G L A
C. difformis A S G Y A R S T G K A G V C V A T S G P G A T N L V S A L A
FIMMI 13S A S G Y A R S T G K A G V C V A T S G P G A T N L V S A L A
FIMMI 12R
FIMMI 10R
A domain
D domain
A. thaliana 181 D A L L D S V P L V A I T G Q V P R R M I G T D A F Q E T P
C. difformis D A L L D S V P M V A I T G Q V P R R M I G T E A F Q E T P
FIMMI 13S D A L L D S V P M V A I T G Q V P R R M I G T D A F Q E T P
FIMMI 12R T P
FIMMI 10R T P
A. thaliana 211 I V E V T R S I T K H N Y L V M D V E D I P R I I E E S F F
C. difformis I V E V T R S I T K H N Y L V L D V D D I P R I I K E A F F
FIMMI 13S I V E V T R S I T K H N Y L V L D V D D I P R I I K E A F F
FIMMI 12R I V E V T R S I T K H N Y L V L D V D D I P R I I K E A F F
FIMMI 10R I V E V T R S I T K H N Y L V L D V D D I P R I I K E A F F
A. thaliana 241 L A T S G R P G P V L V D V P K D I Q Q Q L A I P N W E Q A
C. difformis L A T S G R P G P V L V D I P K D I Q Q Q L A V P V W D T P
FIMMI 13S L A T S G R P G P V L V D I P K D I Q Q Q L A V P V W N P P
FIMMI 12R L A T S G R P G P V L V D I P K D I Q Q Q L A V P V W N P P
FIMMI 10R L A T S G R P G P V L V D I P K D I Q Q Q L A V P V W N P P
A. thaliana 271 M R L P G Y M S R M P K P P E D S H L E Q I V R L I S E S K
C. difformis M R L P G Y T S R L P K Q P E D N Q L D Q I I R L V S E S K
FIMMI 13S M R L P G Y T S R L P K D P A H H L L D Q V T R L V S E S K
FIMMI 12R M R L P G Y T S R L P K D P A H H L L D Q V T R L V S E S K
FIMMI 10R M R L P G Y T S R L P K D P A H H L L D Q V T R L V S E S K
A. thaliana 301 K P V L Y V G G G C L N S S D E L G R F V E L T G I P V A S
C. difformis R P V L Y V G G G C A N S G A E L K R F V E L T G I P V T T
FIMMI 13S R P V L Y V G G G C A N S G A E L K R F V E L T G I P V T T
FIMMI 12R R P V L Y V G G G C A N S G A E L K R F V E L T G I P V T T
FIMMI 10R R P V L Y V G G G C A N S G A E L K R F V E L T G I P V T T
A. thaliana 331 T L M G L G S Y P C D D E L S L H M L G M H G T V Y A N Y A
C. difformis T L M G L G N F P C D E P L C L R L L G M H G T V Y A N Y A
FIMMI 13S T L M G L G N Y P S N D Q L S L R M L G M H G T V Y A N Y A
FIMMI 12R T L M G L G N Y P S N D Q L S L R M L G M H G T V Y A N Y A
FIMMI 10R T L M G L G N Y P S N D Q L S L R M L G M H G T V Y A N Y A
F domain
A. thaliana 361 V E H S D L L L A F G V R F D D R V T G K L E A F A S R A K
C. difformis V D K A D L L L A F G V R F D D R V T G K L E A F A S R S K
FIMMI 13S V D K A D L L L A F G V R F D D R V T G K L E A F A S R S K
FIMMI 12R V D K A D L L L A F G V R F D D R V T G K L E A F A S R S K
FIMMI 10R V D K A D L L L A F G V R F D E R V T G K L E A F A S R S K
A. thaliana 391 I V H I D I D S A E I G K N K T P H V S V C G D V K L A L Q
C. difformis I V H I D I D P A E I G K N K Q P H V S I C A D V K P A L Q
FIMMI 13S I V H I D I D P A E I G K N K Q P H V S I C A D V K A A L Q
FIMMI 12R I V H I D I D P A E I G K N K Q P H V S I C A D V K A A L Q
FIMMI 10R I V H I D I D P A E I G K N K Q P H V S I C A D V K A A L Q
A. thaliana 421 G M N K V L E N R A E E L K L D F G V W R N E L N V Q K Q K
C. difformis G M N Q I L E S S G V H K K L D F S S W R A E L D E Q K K T
FIMMI 13S G M N E I L E S T G L H R D L E F S S W R A E L D E Q K K A
FIMMI 12R G M N E I L E S T G L H R D L E F S S W R A E L D E Q K K A
FIMMI 10R G M N E I L E S T G L H R D L E F S S W R A E L D E Q K K A

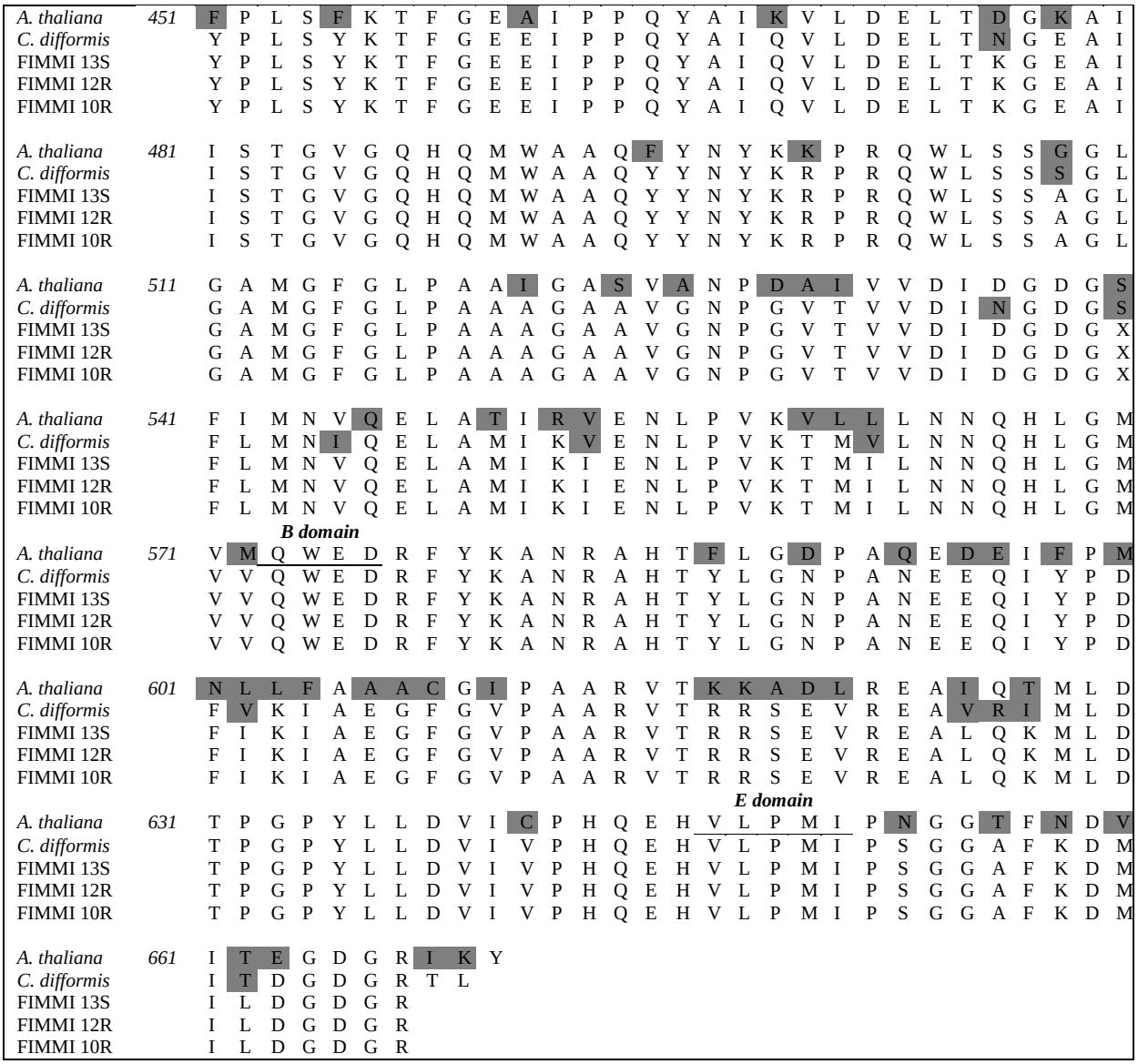


Figure 2. Sequence alignment of a fragment of the acetolactate synthase (ALS) protein from *F. miliacea* (FIMMI 13, FIMMI 12 and FIMMI 10). Aside from *F. miliacea* biotypes, proteins shown are wild type sequences from *Arabidopsis thaliana* (GenBank accession X51514) and *C. differmis* (EF061294). Shaded residues highlight differences from the consensus. Numbers refer to the first amino acid position on each line. The black box indicates the amino acid change described in this article FIMMI 10 and is at position 376 as numbered for the *Arabidopsis* protein. Position conserved amino acid domains are underline.

ACKNOWLEDGEMENTS

We are grateful to the CNPq for the scholarship to Carlos Eduardo Schaedler. We are grateful to Departamento de Fitossanidade (Federal University at Pelotas) and Crop Soil and Environmental Science Department (University of Arkansas) for the structure availability and viability of the research conducted.

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Conclusões gerais

Em condições de campo a população de *Fimbristylis miliacea* mostrou resistência cruzada aos herbicidas inibidores da enzima ALS para pyrazosulfuorn-ethyl, penoxsulam, bispyribac-sodium e ethoxysulfuron em lavoura de arroz irrigado.

No ensaio de curvas-dose respostas, o biótipo FIMMI 10 apresentou resistência cruzada para três grupos de herbicidas inibidores da ALS (pyrimidinylthiobenzoates, sulfonylureas and triazolopyrimidines). Já, o biótipo FIMMI 12 apresentou resistência cruzada para dois grupos de herbicidas inibidores da ALS (sulfonylureas and triazolopyrimidines).

Nenhum dos biótipos avaliados mostrou resistência cruzada a imidazolinonas, como por exemplo, a formulação de (imazethapyr+imazapic).

Em termos de habilidade competitiva, os biótipos de *F. miliacea* resistente e suscetível aos herbicidas inibidores da ALS não diferiram entre si e apresentam inferioridade competitiva em relação a cultura de arroz.

A competição intraespecífica para biótipos de *F. miliacea* e inter-específica para o arroz, foram as mais vantajosas.

O biótipo de *F. miliacea* resistente apresentou menor competitividade com arroz do que o biótipo suscetível.

A competição por recursos do solo tem maior efeito para os biotipos *F. miliacea* resistentes aos herbicidas inibidores da ALS e para a cultura do arroz.

A análise das seqüências nucleotídicas e aminoácidos entre os diferentes biótipos indicou um único ponto de mutação, timina-adenina, no biótipo FIMMI 10 que resultou na substituição de aminoácidos, Asp376Glu, na região F, entre os domínios C, A, D e B, E.

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Vita

Carlos Eduardo Schaedler é filho de Ismar Schaedler e Beatriz Margarida Schaedler, nasceu em 9 de Novermber de 1981, no município de Santa Rosa, Rio Grande do Sul. No ano 2000 ingressou no curso de Agronomia da Universidade Federal do Rio Grande do Sul (UFRGS), onde se graduou como Engenheiro Agrônomo em 2006. No período de abril de 2001 a fevereiro de 2006 desenvolveu atividades como estagiário e Bolsista de Iniciação Científica (CNPq), atuando no Departamento de Plantas de Lavoura, na área de Herbologia. Em 2006, iniciou o curso de mestrado, junto ao Programa de Pós-Graduação em Fitossanidade da Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, em Pelotas/RS. Em março de 2008, ingressou no curso de Doutorado em Fitossanidade da UFPel. No período de março de 2010 a fevereiro de 2011 realizou doutorado sanduíche na University of Arkansas, em Fayetteville – AR, EUA, com apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).