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ALTERAÇÕES FISIOLÓGICAS E MORFOLÓGICAS DE DUAS CULTIVARES DE ARROZ IRRIGADO APÓS APLICAÇÃO DO HERBICIDA IMAZAMOX NA FASE REPRODUTIVA

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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Bibiana Silveira Moraes

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Orientador: Prof. Dr. Fernando Teixeira Nicoloso

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elaborado por
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Ao meu esposo Guilherme

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"Foi o melhor dos tempos e o pior dos tempos,
a idade da sabedoria e da insensatez,
a era da fé e da incredulidade,
a primavera da esperança e o inverno do desespero.
Tínhamos tudo e nada tínhamos."

Charles Dickens

RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Agronomia

Universidade Federal de Santa Maria

ALTERAÇÕES FISIOLÓGICAS E MORFOLÓGICAS DE DUAS CULTIVARES DE ARROZ IRRIGADO APÓS APLICAÇÃO DO HERBICIDA IMAZAMOX NA FASE REPRODUTIVA

AUTOR: BIBIANA SILVEIRA MORAES
ORIENTADOR: FERNANDO TEIXEIRA NICOLOSO
Santa Maria, RS, 01 de março de 2013

O controle de plantas daninhas é uma das práticas agrícolas indispensáveis para garantir rentabilidade e sucesso no cultivo. No cultivo de arroz irrigado, o arroz vermelho é a planta daninha de maior importância, devido sua dificuldade de controle. Um método de controle bastante difundido é o uso de cultivares resistentes aos herbicidas inibidores da ALS, pois permite um controle químico seletivo. Estudos demonstram que o controle tardio com o herbicida imazamox promove controle eficiente de escapes de arroz vermelho. Por isso, o objetivo deste trabalho foi verificar os efeitos da aplicação do imazamox na fase reprodutiva de duas cultivares de arroz irrigado (IRGA 422 CL e PUITÁ INTA CL) que diferem quanto ao nível de resistência as imidazolinonas. Em vista do exposto, foram conduzidos dois estudos na área experimental da Universidade Federal de Santa Maria (2009/10 e 2010/11). O imazamox foi aplicado em diferentes estádios de desenvolvimento e doses, sendo que a dose final foi de 80 g i.a ha^{-1}. Com os resultados obtidos conclui-se que independente da data de aplicação do imazamox na fase reprodutiva da cultura ocorreu redução da produtividade de grãos e aumentou a esterilidade de espiguetas da cultivar IRGA 422 CL. De maneira geral, os parâmetros: peso de mil grãos, comprimento da folha bandeira, comprimento de panícula, peso fresco e seco de panículas, e número de panículas por metro quadrado mostraram redução em praticamente todos os tratamentos na cultivar IRGA 422 CL. Alterações nos parâmetros bioquímicos (clorofila, carotenoides, substâncias reativas ao ácido tiobarbitúrico, superóxido dismutase, catalase e ascorbato peroxidase) foram observadas em folhas e panículas do colmo principal em alguns tratamentos, demonstrando que o estresse oxidativo provocado pela aplicação do imazamox pode ter contribuído para a redução da produtividade de grãos e o elevado percentual de espiguetas estéreis da cultivar IRGA 422 CL. A cultivar PUITÁ INTA CL não sofreu alterações em todos os parâmetros avaliados neste estudo. As alterações morfológicas e anatômicas demonstraram que a aplicação de 80 g i.a ha^{-1} imazamox na diferenciação da panícula promoveu alterações semelhantes às alterações homeóticas observadas em arroz mutante. Além disso, nas plantas que receberam a dose de 80 g i.a ha^{-1} após 14 dias da diferenciação do primórdio floral (DPF) e as plantas que receberam a dose de 80 g i.a ha^{-1} em aplicação fracionada (metade da dose 7 dias após DPF e metade da dose aos 14 dias após DPF) mostraram alterações morfológicas e anatômicas do grão de pólen. Dado o exposto, os resultados obtidos sugerem que as alterações morfológicas e anatômicas foram responsáveis pela redução da produtividade de grãos e alto percentual de espiguetas estéreis da cultivar IRGA 422 CL.

Palavras-chave: Arroz. Herbicida inibidor da ALS. Redução da produtividade de grãos. Esterilidade de espiguetas.

ABSTRACT

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PHYSIOLOGICAL AND MORPHOLOGICAL CHANGES OF TWO RICE CULTIVARS AFTER IMAZAMOX HERBICIDE APPLICATION IN REPRODUCTIVE PHASE

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Santa Maria, RS, January 01th, 2013

Weed control is one of the main agricultural practices indispensable to ensure profitability and crop success. In paddy rice field, red rice is the most important weed due to its difficult control. A widespread control method is the use of rice cultivars resistant to herbicides which are inhibitors of ALS, since it is possible to have a selective chemical control. Studies showed that the late control with imazamox promotes efficient control of red rice escapes. Thus, the objective of this research was to check the effects of imazamox application in the reproductive phase of two rice cultivars that differ in the level of resistance to imidazolinones. Two studies were carried out at the Federal University of Santa Maria in the years of 2009/10 and 2010/11. Imazamox was applied in different stadium of development and doses. At the end of the application the final dose was 80 g a.i ha^{-1} for all treatments. Results showed that independent of the date of the imazamox application in the reproductive phase of rice, the grain yield reduced and spikelet sterility of IRGA 422 CL increased. In general, the parameters 1000-grain weight, flag leaf length, panicule length, fresh and dry weight of panicles, and panicles per m^2 showed a reduction in practically all imazamox treatments in the IRGA 422 CL cultivar. Changes in the biochemical parameters (chlorophyll, carotenoids, thiobarbituric acid reactive substances, superoxide dismutase, catalase and ascorbate peroxidase) were observed in leaves and panicles from main culm in some treatments, demonstrating that the oxidative stress promoted by imazamox may have contributed to grain yield reduction and the high percentage of sterile spikelet from IRGA 422 CL cultivar. Morphologic and anatomical changes showed that imazamox application in the panicle differentiation promoted similar changes to homeotic changes observed in rice mutant. Moreover, in the other treatments different morphologic and anatomical changes were observed. Therefore, morphologic and anatomical changes were likely to be responsible for grain yield reduction and high percentage of spikelet sterile from IRGA 422 CL.

Key words: Rice. ALS herbicide inhibitor. Grain yield reduction. Spikelet sterility.

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INTRODUÇÃO

O arroz (*Oryza sativa* L.) é um dos cereais mais produzidos no mundo, sendo responsável por 2 % na produção mundial de grãos (FAO, 2009). O Rio Grande do Sul é o maior produtor de arroz no país, alcançando uma produção de 7.441 kg ha^{-1} na safra 2011/12 (IRGA, 2012). Apesar do crescimento gradual da produção de grãos nos últimos anos, esta produtividade está aquém do alcançado pelas lavouras que adotam as práticas agronômicas de forma adequada (manejo da lavoura, época semeadura, utilização correta de produtos químicos, etc). Um dos motivos que contribuem para que não sejam atingidas produtividades maiores é a elevada infestação por plantas daninhas e consequentemente a dificuldade de controle. O controle de plantas daninhas é uma das etapas importantes durante o cultivo agrícola, pois devido à competição por água, luz e nutrientes, as plantas daninhas limitam a produção de grãos da cultura. Na cultura do arroz, o arroz vermelho (*Oryza sp*) é a principal planta daninha, pois causa redução da qualidade do produto comercial e da qualidade das sementes (MARCHEZAN, 1994). Ambas pertencem botanicamente a mesma espécie, ou seja, são morfologicamente e fisiologicamente similares, dificultando o controle químico por meio de herbicidas seletivos. Devido a estas similaridades, o uso de apenas um método de controle se torna ineficaz, por isso é necessário que ele seja baseado em um manejo integrado.

Dentre as práticas que podem ser usadas para o controle do arroz vermelho em arroz irrigado, merece destaque o sistema de produção Clearfield®. Esse sistema é uma ferramenta baseada no uso de herbicidas do grupo das imidazolinonas em cultivares de arroz portadoras dos genes que conferem resistência a esses herbicidas (SOSBAI, 2010). O uso desses herbicidas em cultivos com cultivares CL proporciona um controle seletivo eficiente de arroz vermelho (OTTIS et al., 2003). Porém, o controle não chega a 100% ocorrendo o escape de algumas plantas. O controle desses escapes de arroz vermelho faz-se necessário para que se obtenha sustentabilidade do sistema de produção. Além disso, para um controle eficiente de plantas de arroz vermelho, é preciso que se utilizem sementes de qualidade, uso do herbicida recomendado e adoção de um programa de monitoramento de arroz vermelho. Para garantir o controle eficiente de escapes de arroz vermelho é importante o conhecimento da melhor época e dose de aplicação

do herbicida imazamox. Em estudos realizados nos EUA foi demonstrado que os herbicidas imazethapyr e imazamox podem ser usados em aplicação tardia para o controle dos escapes de arroz vermelho, com resultados bastante promissores (MEINS et al., 2004; SCOTT et al., 2007). Além de reduzir a produção de sementes do arroz vermelho, a aplicação na fase reprodutiva do arroz pode diminuir as chances de florescimento simultâneo entre o arroz vermelho e o arroz tolerante, diminuindo assim as chances de cruzamento entre as duas espécies (MEINS et al., 2004). Esse cruzamento pode ocorrer devido às condições ambientais, de acordo com a época de florescimento, da distância da fonte doadora e receptora de pólen e do nível de infestação da área. Devido a isso, pode ocorrer o fluxo gênico que é a transferência do gene resistente ao herbicida para o arroz vermelho, sendo este fluxo menor que 1% (GEALY et al., 2003). No Brasil, Villa et al. (2006) encontraram taxas de cruzamento entre arroz vermelho e arroz tolerante de 0,065%. Roso et al. (2010) sugeriram que o fluxo gênico estava ocorrendo nas lavouras arrozeiras do Sul do país entre cultivares e arroz vermelho, devido a mutação observada do gene da ALS ser na mesma posição (S653D) que o da cultivar resistente ao herbicida.

Os herbicidas imidazolinonas estão entre os cinco grupos químicos que inibem a enzima Acetolactato sintase (ALS) ou Acetohidroxiácido sintase (AHAS) (TAN et al., 2005). A ALS atua como precursora da biossíntese dos aminoácidos de cadeia ramificada (valina, leucina e isoleucina), sendo a primeira enzima da rota de síntese da valina e da leucina e a segunda enzima na rota da isoleucina(SHANER; O`CONNOR, 1991; COBB; READE, 2010). Os herbicidas inibidores da ALS bloqueiam o ciclo celular na fase G2 para mitose e na fase G1 para a síntese de DNA (VIDAL; MEROTTO, 2001). Embora sem nenhum efeito direto sobre o aparato mitótico, é possível que haja inibição muito rápida da divisão celular (COBB; READE, 2010). As imidazolinonas são absorvidas através das folhas, sendo também absorvido via radicular em menor grau (SENSEMAN, 2007). A translocação se dá via xilema e floema acumulando-se nas zonas de crescimento, levando as plantas à paralisação do crescimento e à morte num período de quatro a seis semanas. Entretanto, o processo fitotóxico ainda permanece obscuro (SHANER; O`CONNOR, 1991; COBB; READE, 2010). Os sintomas de fitotoxicidade observados são a inibição do crescimento em poucas horas após a aplicação, clorose das regiões meristemáticas, seguida por uma leve clorose geral e necrose (SHANER;

O`CONNOR, 1991). O efeito observado nas regiões meristemáticas pode ser atribuído à inibição da biossíntese dos aminoácidos de cadeia ramificada nesta região.

No Brasil, os herbicidas recomendados para utilização no sistema Clearfield são o Only® (imazethapyr + imazapic) e o Kifix® (imazapyr + imazapic). Nos EUA o herbicida imazamox é registrado para uso no sistema Clearfield® de trigo, canola e girassol e no controle de escapes de arroz vermelho em cultivares e híbridos resistentes aos herbicidas imidazolinonas (MEINS et al., 2004; RAINBOLT et al., 2005). O herbicida imazamox, 2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methoxymethylnicotinic acid (IUPAC) é registrado no Brasil para o controle seletivo de várias gramíneas anuais e de espécies de folhas larga em pós-emergência nas culturas de soja e feijão, sendo comercializado como Sweeper® pela BASF®. É um herbicida que possui características de ácido fraco, ou seja, o pH externo altera seu estado de associação-dissociação e apresenta meia vida de 20-30 dias no solo (SENSEMAN, 2007).

As primeiras cultivares tolerantes aos herbicidas imidazolinonas comercializadas nos EUA em 2001 foram CL 121 e a CL 141 que foram derivadas da linhagem resistente 93AS-3510 (GEALY et al., 2003; WEBSTER; MASSON 2001). Posteriormente, a cultivar CL 161 originária da linhagem resistente PWC16, também foi lançada no mercado norte americano em 2003 (GEALY et al., 2003; TAN et al., 2005). Meins et al. (2004) observaram que a cultivar CL161 mostrou uma alta tolerância ao imazamox sendo equivalente ao imazethapyr. No Brasil, a partir da safra 1998/1999, pesquisadores do Instituto Riograndense do Arroz (IRGA) iniciaram o processo de transferência da característica do gene mutante para os genótipos de arroz através de retrocruzamentos (LOPES et al., 2001). A cultivar IRGA 422 CL é um exemplo, sendo a primeira cultivar resistente lançada no ano de 2002. É oriunda do cruzamento entre a linhagem 93AS-3510 e a cultivar IRGA 417. A mutação do gene da ALS que proporciona resistência à cultivar IRGA 422 CL é $G_{654}E$ (Roso et al., 2010). A cultivar originária da Argentina PUITÁ INTA CL, foi obtida por mutagênese e seleção de linhagens resistentes as imidazolinonas, sendo a cultivar IRGA 417 utilizada no cruzamento. A mutação do gene da ALS que proporciona resistência à cultivar PUITÁ INTA CL é $A_{122}T$ (ROSO et al., 2010).

O efeito *"primário"* dos herbicidas imidazolinonas é a inibição da atividade da enzima ALS com consequente inibição da formação dos aminoácidos de cadeia ramificada e assim a redução do crescimento e até mesmo a morte da planta. Apesar de alguns autores demonstrarem que não ocorre prejuízo no desenvolvimento de algumas cultivares de arroz tolerantes aos herbicidas inibidores da ALS (MEINS et al., 2004; SCOTT et al., 2007), existem estudos que mostram efeitos secundários desses herbicidas sobre a cultura do milho e ervilha (SCARPONI et al., 2001; ZABALZA et al., 2004). Os efeitos secundários causados por herbicidas podem ocorrer tanto na cultura presente (não-alvo) quanto na própria planta daninha. Além disso, alterações morfológicas e bioquímicas podem acontecer como efeito secundário de herbicidas (GUO et al., 2007; AHSAN et al., 2008; HENSLEY, 2009).

O estresse oxidativo é um fenômeno bioquímico bastante complexo que inicia com a formação de espécies reativas ao oxigênio (EROs). As EROs são geradas como sub-produtos em processos celulares que geram energia, como a fotossíntese e respiração celular (VAN BREUSEGEM; DAT, 2006). As espécies de radicais livres centradas no oxigênio podem originar as chamadas EROs, as quais podem se tornar danosas à célula causando danos as proteínas, aos lipídios, carboidratos e DNA levando a mesma a morte (GILL; TUTEJA, 2010). O estresse induzido pelo acúmulo de EROs pode ser minimizado pelo sistema de defesa antioxidante presente na célula vegetal, que pode ser enzimático ou não-enzimático. Entretanto, se acontecer um excesso da produção de EROs e/ou falha na atividade do sistema antioxidante instala-se uma situação de estresse oxidativo celular. O equilíbrio entre a produção de EROs e o sistema antioxidante pode ser afetado por vários estressores bióticos ou abióticos, como por exemplo, salinidade, exposição a temperaturas extremas, seca, exposição a poluentes do ar, radiação ultravioleta, metais pesados, deficiência nutricional, ataque de patógenos e herbicidas de diferentes grupos químicos (AHSAN et al., 2005; GUO et al., 2007; TABALDI et al., 2007; ZABALZA et al., 2007, GONÇALVES et al., 2009; CALGAROTO et al., 2010; GILL; TUTEJA, 2010). A acumulação de oxigênio singleto já foi mencionada na literatura como possível efeito da aplicação de herbicidas inibidores da ALS, porém devido aos sintomas observados nas plantas serem diferentes dos efeitos causados por esse grupo, sugere-se que não seja a causa primaria da toxicidade na planta (COBB; READE,

2010). Zabalza et al. (2007) avaliaram o efeito de herbicidas inibidores da ALS em plantas de ervilha e concluíram que o leve aumento na peroxidação lipídica gerado após a aplicação não foi relacionado ao modo de ação de inibidores da ALS.

O desenvolvimento floral é coordenado por um determinado grupo de genes. Existem genes, por exemplo, responsáveis pela manutenção dos meristemas reprodutivos e pelo controle da identidade das espiguetas (YOSHIDA; NAGATO, 2011). Além disso, existe um modelo central chamado “modelo ABCDE” que explica o mecanismo genético do desenvolvimento da flor (DORNELAS et al., 2011; YOSHIDA; NAGATO, 2011). Diversos estudos demonstram que mutações homeóticas nesses genes causam perda de função do órgão, alteração de identidade e número dos órgãos florais (FORNARA et al., 2003; LI et al., 2007; ZHANG et al., 2007). Além disso, a aplicação de herbicidas pode promover redução da viabilidade polínica e /ou alterar a morfologia floral que pode ou não reduzir a eficiência da polinização. Hensley (2009) observou alterações morfológicas em panículas de arroz submetidas à deriva do herbicida imazamox.

Os objetivos da tese foram: a) investigar os efeitos do herbicida imazamox aplicado na fase reprodutiva de duas cultivares de arroz que diferem quanto ao nível de resistência aos herbicidas inibidores da enzima acetolactato sintase (ALS); b) caracterizar as alterações morfológicas e anatômicas causadas pela aplicação de imazamox na cultivar IRGA 422 CL.

ARTIGO 1

EFFECTS OF IMAZAMOX APPLIED IN THE REPRODUCTIVE STAGE ON THE OXIDATIVE STRESS OF IMIDAZOLINONE-RESISTANT RICE CULTIVARS

(Artigo submetido para "Pest Management Science")

Effects of imazamox applied in the reproductive stage on the oxidative stress of imidazolinone-resistant rice cultivars

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Abstract

Imazamox is an imidazolinone (IMI) herbicide that inhibits acetolactate synthase (ALS). This compound is used for controlling weeds in agricultural practice and the morphological adverse effects on rice are known. The aim of this study was to investigate the effects of imazamox applied in the reproductive phase of two Clearfield rice cultivars (IRGA 422 CL and PUITÁ INTA CL) that differ in the level of resistance to IMI herbicides. PUITÁ INTA CL did not show changes in the parameters evaluated in the each growing seasons evaluated. IRGA 422 CL was affected by imazamox applied at reproductive stage reducing the length of the flag leaf in the main culm, the 1000-grain weight and grain yield. Furthermore, panicle exertion was delayed resulting in greater level of spikelet sterility and reduction in panicle dry weight and panicle length. Imazamox caused changes in the oxidative stress parameters of leaves and panicles of IRGA 422 CL. The changes observed in this parameter demonstrated that the oxidative stress promoted by imazamox may have contributed to grain yield reduction and the high percentage of sterile spikelet from IRGA 422 CL cultivar. Moreover, the most common alteration of imazamox treatments was in the morphological structure of the reproductive organs of IRGA 422 CL.

Keywords: herbicide ALS inhibitor, antioxidant system, grain yield, oxidative stress, spikelet sterility

1. Introduction

Weed control is a relevant factor for maximizing rice yield. Red rice is the most troublesome weed in rice, because it can reduce grain yield, quality of commercial grain and seeds (Marchesan, 1994). Both cultivated rice and red rice are the same species (*Oryza* sp.) and due to their morphologically and physiologically similar, control with selective herbicides is difficult (Agostinetto et al., 2001; Roso et al., 2010). Imidazolinone-resistant rice cultivars (IMI rice) allowed selective control of red rice with imidazolinone herbicides (Webster and Massom, 2001). The IRGA 422 CL and PUITÁ INTA CL cultivars differ in the level of resistance to acetolactate synthase (ALS) inhibitors herbicides. These cultivars were obtained by the mutagenesis and crossing with IRGA 417 cultivar (sensitive to imidazolinone) (Tan et al., 2005; Livore et al., 2003). The ALS gene mutation that confers imidazolinone herbicide resistance in the IRGA 422 CL is $Gly_{654}Glu$ and in the PUITÁ INTA CL is $Ala_{122}Thr$ (Roso et al., 2010). Although the imidazolinone herbicides are very efficient for red rice control, the control rarely reaches 100%, allowing the occurrence of late-season red rice escapes. Studies have shown that IMI rice was tolerant to late-growing season applications of imazamox, which can result in an effective tool for preventing red rice seed formation in the Clearfield® system (Meins et al., 2004; Scott et al., 2007).

Imazamox herbicide belongs to the chemical group of imidazolinones. This group is characterized by inhibiting ALS (EC 4.1.3.18), also known as acetohydroxyacid synthase (AHAS), enzyme that catalyzes the synthesis of branched chain amino acids isoleucine, leucine and valine (Little and Shaner, 1991). Imazamox has a half-life of 20 to 30 days in soil and translocation occurs via the phloem and xylem after foliar absorption (Senseman, 2007).

There are studies that showed secondary effects of IMI herbicides in maize and pea crops (Scarponi et al., 2001; Zabalza et al., 2004). These secondary effects may occur either in target weeds or in the crop, which can be observed at morphological and biochemical levels, such as: deformation in rice panicle caused by imazamox application (Hensley, 2009), oxidative stress observed in rice shoots after paraquat and glyphosate herbicide application (Guo et al., 2007; Ahsan et al., 2008).

Herbicides from different chemical groups can disrupt normal biochemical and/or physiological metabolism in plants (Hensley, 2009; Jiang and Yang, 2009; Song et al., 2007). Superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are enzymes from antioxidant system that are responsible by protection against to cell damage. The disruption in the basal metabolism or ROS production excessive cause oxidative stress (Gill and Tuteja, 2010). Various biotic and abiotic stressors, such as salinity (Khan and Panda, 2008), drought (Slatev et al., 2006), exposure to herbicides (Guo et al., 2007; Ahsan et al., 2008), heavy metals (Calgaroto et al., 2010; Rossato et al., 2011; Tabaldi et al., 2009), nutritional deficiencies and attack of pathogens (Gill and Tuteja, 2010) can affect the balance between ROS production and antioxidant system. Recent studies have demonstrated that herbicides are able to directly or indirectly induce intracellular over-production of ROS and thus damage plant cells (Guo et al., 2007; Ahsan et al., 2008; Yin et al., 2008). The accumulation of ROS as a result of various environmental stresses can be the main cause of crop yield loss. Moreover, the ROS can initiate the expression of many genes involved in the xenobiotic detoxification (Gill and Tuteja, 2010).

Therefore, the hypothesis of the present study is that rice cultivars resistant to ALS inhibitors may show different levels of resistance to imazamox when applied at the onset of the reproductive phase and, in such case, the cause of the reduction in

grain yield is due to oxidative stress in leaves and panicle of the least resistant cultivar. To confirm these hypotheses, two cultivars that differ in resistance to the ALS inhibitors herbicides were assessed.

2. Material and methods

2.1. Rice growth condition

Field experiments were conducted in the paddy field at Universidade Federal de Santa Maria, RS, Brazil (29º41'24"S, 53º48'42"W, 95 m altitude) in 2009-2010 and 2010-2011. The experiment was a split-spot design arrangement with cultivar as the main plot and herbicide treatment as subplot, with four replications. The rice cultivars used were IRGA 422 CL and PUITÁ INTA CL. These cultivars were chosen because the PUITÁ INTA CL (enhanced resistant) is more resistant to ALS inhibitor herbicides than IRGA 422 CL cultivar (resistant). The treatments were an arrangement of imazamox total rate (80 g ai ha^{-1}) and timing of application (item 2.2). The cultivars seeds were drilled at 100 kg ha^{-1}, previously treated with the insecticide fipronil (250 g ai per 100 kg of seeds). Rice seeds were sown on October 21st 2009 and October 5th 2010, on 2009/10 and 2010/11 growing season, respectively. Nitrogen fertilizer in form of urea (350 kg ha^{-1}) was applied 5% on planting, 50% as topdressing fertilizer for promoting tillering (applied at V3-V4 stadiums) (Counce et al., 2000), 45% for promoting panicle initiation (applied at V9 or R0) (Counce et al., 2000; SOSBAI, 2010). All phosphorous and potassium, respectively fertilizer was used on planting. Except for the control treatment, all treatments received sequential applications of formulated mixture of imazethapyr and imazapic (75 + 25 g ai L^{-1}) at a dose of 0.75 L ha^{-1} pre-emergence followed by 0.75 L ha^{-1} post-emergence for weed control first. Permanent flood was established when plants were at V3-V4 stadium (Counce et al., 2000). Other

standard agronomic practices were implemented through the growing season, according to the recommendations (SOSBAI, 2010).

2.2 Herbicide treatments

All treatments received sequential applications of formulated mixture of the herbicides imazethapyr + imazapic at 56.25 + 18.75 g ai ha^{-1} pre-emergence followed by 56.25 + 18.75 g ai ha^{-1} post-emergence. The imazamox herbicide 2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methoxymethylnicotinic acid (IUPAC) is registered in Brazil for selective control of post-emergence weeds in soybean and bean crops, and is commercialized as Sweeper® by BASF®. The herbicide applications were performed using a CO_2-backpack sprayer using a volume of 180 L ha^{-1}. The treatments were: untreated check (0 g ai ha^{-1}) (T1) and for herbicides treatments the imazamox was applied in different stadiums and rates: at panicle differentiation (PD, R0 stadium) (80 g ai ha^{-1}) (T2); PD+7 days (R1- R2 stadiums; 80 g ai ha^{-1}) (T3); PD+14 days (R2 stadium; 80 g ai ha^{-1}) (T4); at PD followed by (fb) PD+7 (R0 and R1-R2 stadiums; 40 fb 40 g ai ha^{-1}) (T5); PD fb PD+14 (R1-R2 and R2 stadiums; 40 fb 40 g ai ha^{-1}) (T6); PD+7 fb PD+14 (R1-R2 and R2; 40 fb 40 g ai ha^{-1}) (T7) (Counce et al., 2000).

2.3 Growth and yield parameters

In 2009/10, growth of *O. sativa* crop plants was determined by measuring the aboveground rice fresh and dry weight and number of panicles per m^2. In 2010/11, the growth was evaluated by length of flag leaf in the main culm, aboveground rice fresh and dry weight and number of panicles per m^2. Aboveground rice fresh and dry weights were evaluated at R1 (63 DAE), R1-R2 (70 DAE), R2 (77 DAE), R3 (85 DAE)

and R6-R7 (92 DAE) in 2009, and at R1 (61 DAE), R1-R2 (68 DAE), R2 (75 DAE), R2-R3 (82 DAE) and R5 (89 DAE) in 2010. These dates (DAE) indicate the stadium of development that the plant was at the time of imazamox treatments application according to Counce et al. (2000). The plant was split into tillers and main culm, but no significant differences were observed between these portions. Plants were oven-dried at 65 °C to a constant weight for the determination of dry weight. The number of panicles per m^2 was evaluated by counting the number of panicles in one-meter row of each plot. The counting was conducted every five days in the period between heading (the date when more than 50% of all panicles had emerged from the flag leaf sheath) and harvesting. At R8 (102 DAE), R9 (109 DAE) stadiums and before harvest (117 and 123 DAE), the flag leaf length from the main culm (five for each plot) was measured.

In the 2010/11 growing season, the panicle fresh weight, dry weight and length were evaluated in all treatments. At crop establishment, each plot had one meter of row marked to collect panicles at R1-R2 (75 DAE), R2-R3 (82 DAE), R5 (89 DAE), R7-R8 (95 DAE), R8 (102 DAE), R9 (109 DAE) stadiums and before harvest (117 and 123 DAE). Subsequently, the panicles (five from each plot) were weighed and measured, and then the panicles were oven-dried for 10 days at 65 °C to determine the dry weight.

In 2009/10 and 2010/11 growing seasons, to estimate grain yield, rice was harvested in an area of 4.1 m^2 when the grain moisture averaged 22%. After weighing the grain, data were corrected to 13% moisture and converted into kg ha^{-1}. The 1000-grain weight (g) was determined from the average weight of four subsamples of 100 grains. After harvest, ten panicles of each plot were collected and the percentage of

spikelet sterility was determined by counting the number of unfilled and filled grains in the panicles.

2.4 Biochemical parameters

In the 2010/11 growing season, at 1, 8 and 16 DAT (days after treatment), ten rice plants were harvested and the main culm was separated. From the main culm, the flag leaf, penultimate leaf and panicle were removed and frozen immediately in liquid nitrogen and stored at -30 °C for biochemical analysis.

The chlorophyll and carotenoids concentrations in leaves of both cultivars were also evaluated using the method described by Hiscox and Israeslstam, (1979) and estimated using the Arnon's formula (1949).

2.4.1 Determination of hydrogen peroxide

The H_2O_2 concentration was determined using the method described by Loreto and Velikova (2001). Initially, 0.05 g of macerated tissue was homogenized in 1.5 mL of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12,000 *x* g for 15 min at 4 °C. Then, 0.5 mL of the supernatant was add to 0.5 mL of 10 mM potassium phosphate buffer, (pH 7.0), and 1 mL of 1M potassium iodide. The H_2O_2 concentration of the supernatant was estimated by comparing its absorbance at 390 nm with a standard calibration curve. The H_2O_2 concentration was expressed as µmol g^{-1} fresh weight.

2.4.2 Lipid peroxidation estimation

The level of lipid peroxidation products in leaves or panicles samples (0.1 g fresh weight) were estimated using the method described by El-Moshaty et al (1993)

measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA). The absorbance of the supernatant was measured at 532 nm and 600 nm. The MDA concentration was calculated using an extinction coefficient 155 L^{-1} mol^{-1} cm^{-1}, and lipid peroxides were expressed as nmol MDA (mg $protein^{-1}$).

2.4.3 Enzyme activities: superoxide dismutase, catalase and ascorbate peroxidase

Samples of macerated tissue (0.06 g) were homogenized in 1.5 mL of 50 mM K-phosphate buffer (pH 7.0), containing 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The homogenates were centrifuged at 12,000 *x* g for 20 min at 4 °C and then, the supernatants were used for the enzyme assays. An aliquot of the supernatant was used to determine soluble proteins as described by Bradford (1976).

Superoxide dismutase (SOD) was assayed through the method described by Misra and Fridovich (1972). The assay mixture consisted of a total volume of 1 ml containing glycine buffer (pH 10.5), 1mM epinephrine and tissue extract. Epinephrine was the last component to be added. Adrenochrome formation over the next 2 min was spectrophotometrically recorded at 480 nm. One unit of SOD activity was expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

Catalase activity was assayed through the method described by Aebi (1984) with some modifications. Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm from a reaction mixture containing 2 mL 15 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 30 µL of extract. Activity was expressed as nmol min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX) was measured throughout the method described by Zhu et al (2004). The reaction mixture, a total volume of 2 mL, contained 25 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µL enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (E = 2.8 mM^{-1} cm^{-1}) and activity was expressed as µM ascorbate oxidated min^{-1} mg $protein^{-1}$.

2.5 External morphology of panicles and spikelets

In the 2010/11 growing season, panicles from field-grown plants of the IRGA 422 CL cultivar were collected and fixed in a solution with glutaraldehyde 3% in sodium phosphate buffer at pH 7.2 and Tween20 2mL L^{-1} (adapted from Freudenstein, 2002) for 24 h, at room temperature. Then, the spikelets were removed from the panicle and images were taken under stereomicroscopes Olympus SZH10 and Leica EZ4 from images digitally collected.

2.6 Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests through SISVAR software. The results are the means of at least four replicates and the mean differences were compared using the Tukey Test ($P \leq 0.05$). Asterisk (*) indicates significant difference between the untreated check and herbicide treated plants ($P \leq 0.05$) at a given day of treatment in Fisher's test.

3. Results and discussion

3.1 Effect of imazamox on growth and yield parameters

At R8 stadium (102 DAE) the length of flag leaf in IRGA 422 CL was slightly reduced in treatments T4 and T5 whereas in the T2, T3, T6 and T7 it was reduced when compared to the untreated check of IRGA 422 CL (Fig. 1A). Only treatment T7 resulted in significant reduction in this parameter when compared to the untreated check at 109 DAE. At before harvest (117 and 123 DAE), treatments T3 and T7 showed a reduction in the length of flag leaf. The PUITÁ INTA CL cultivar did not show change in flag leaf length in all evaluations (data not shown). The top three leaves of rice plants, mainly the flag leaf, contribute significantly to the grain yield. The flag leaf has an important role in rice yield by increasing grain weight from 41 to 43% (Tari et al., 2009). This leaf remains green and maintains active photosynthesis until maturity. The dry weight of rice grain partially comes from the non-structural carbohydrates that are stored in the culm and leaves before heading and are transferred to panicles after heading, and partially from the photosynthetic products of leaves after heading (Wu et al., 2008).

In general, the fresh and dry weight of panicles of plants exposed to imazamox decreased significantly in the IRGA 422 CL, but not in PUITÁ INTA CL (Fig. 1C and D). At R2-R3 stadium (82 DAE), IRGA 422 CL cultivar showed a significant reduction in panicle fresh weight in the T3, T6 and T7 treatments. At R7-R8 stadium (95 DAE), only T3 and T7 treatments promoted a decrease in this parameter when compared to the untreated check. At R8 (102 DAE), R9 (109 DAE) and before harvest (117 and 123 DAE) all treatments resulted in significant reduction for this parameter in the IRGA 422 CL when compared to the untreated check. The panicle dry weight of IRGA 422 CL was significantly reduced in all treatments at R8 (102 DAE), R9 (109 DAE) and before harvest (117 and 123 DAE). This corroborated with data showed in Fig. 3A and B, which demonstrate that imazamox treatments caused a delay in the exertion of

panicles, providing asynchrony of flowering between treated plants and untreated check. In 2010/11, main culm panicle length was reduced in response to imazamox application (Fig. 1B). The T3 treatment resulted in reduction of panicle length in all periods evaluated, with an exception at R9 (109 DAE). Similarly, the T7 treatment promoted reduction in all periods with an exception at R1-R2 (75 DAE). These results suggest that the effect of imazamox application was much more pronounced on reproductive organs than on vegetative structure, because the fresh and dry weights of plant were not significantly changed (Fig. 2).

Aboveground rice fresh and dry weights of IRGA 422 CL and PUITÁ INTA CL were not significantly affected by imazamox treatments in both growing seasons. However, for these parameters a significant difference was observed between cultivars at R2 (77 DAE) in 2009/10 growing season (Figs. 2A and C). Fresh and dry weight did not appear to be sensitive to imazamox applications after PD. This result make sense, because the mass production peak is reached around PD and the possible changes can has been diluted in the vegetative biomass The reproductive phase (number of days from PD to harvest) increased in the IRGA 422 CL plants treated with imazamox. In 2010/11 growing season, harvesting was delayed 21 days in treatments T3, T4, T5, T6 and T7. Only the treatment receiving a single application of imazamox at PD (T2) did not delayed harvesting. The reproductive phase of PUITÁ INTA CL cultivar was not altered with used of imazamox in both growing seasons. Delay in harvesting can be explained mainly by new panicles arising from the secondary nodes of the main culm. However, the effect of imazamox on the process of cell division could slowing the growth rate and development of the panicle. Although no direct effect on the mitotic apparatus has been reported for ALS herbicides

inhibitors, it is possible that these herbicides affect the regulatory mechanisms of the cell cycle (Vidal and Merotto, 2001).

Grain yield of IRGA 422 CL was significantly reduced with applications of imazamox during the reproductive phase (Table 1). For this cultivar, in 2009/10, a drastic reduction in the T4 treatment was observed, whereas in the other treatments this reduction was less when compared to the untreated check. Grain yield for IRGA 422 CL was slightly reduced in treatments T2 and T6, in 2009/10 and 2010/11 growing seasons, respectively. As far as known there is no scientific information available regarding the resistance of first development cultivars, such as IRGA 422 CL, to imazamox. Studies demonstrated that lines of first developed cultivars were significant less tolerant to imazethapyr than the second developed ones (Avila et al., 2005). O 'Barr et al *(*2005) reported a reduction in grain yield of red rice after imazamox application at booting stadium (R2). Similarly, it was observed that wheat yield decreased due to a simulated drift of imazamox application at the elongation of 1st node or at the flowering (anthers visible) (Deeds et al., 2006).

For PUITÁ INTA CL no reduction in grain yield was observed in response to imazamox application. Similarly, in study conducted by Meins et al (2004) in the United States it was showed that imazamox application at PI (panicle initiation) did not alter the grain yield of CL 161 cultivar. This result may be related to the level of cultivar resistance to imazamox. Study conducted by Bond and Walker (2011) showed that grain yield of CL161 cultivar was not affected by the imazamox treatments (44 and 88 g ai) applied in different stadiums of reproductive phase of rice, but the grain yield of hybrid CL was reduced when compared to the CL161. According to these authors, the reasons for the grain yield differences between genotypes are not well understood. Rice cultivar and/or growth stage at application may influence rice tolerance to

herbicides (Zhang et al., 2005; Zhang and Webster, 2002). Interestingly, the ALS enzyme of CL161 cultivar was 420-fold more resistant to imazethapyr when compared to conventional rice cultivar (Cypress) (Avila et al., 2005). Results observed in the present study allow us to infer that the late-growing season application of imazamox, independently of the reproductive stage, caused the same negative effect on grain yield in IRGA 422 CL cultivar.

In addition, results showed a significant decrease in 1000-grain weight of IRGA 422 CL cultivar (Table 1). In 2009/10 growing season, the T4 treatment promoted a significant reduction in this parameter, whereas the T7 treatment promoted a slight reduction when compared to the untreated check. In 2010/11, only the T3 treatment promoted significant reduction, whereas the T6 and T7 treatments promoted a slight reduction when compared to the untreated check. As grain weight is a component of grain yield these results might be related to the yield observed in the same treatments.

The percentage of spikelet sterility of the main panicle was evaluated in both cultivars in both growing seasons. In 2009/10, at before harvest (112 DAE), the IRGA 422 CL cultivar demonstrated a significant higher percentage of spikelet sterility in the T4 and T7 treatments (Table 1). In 2010/11 growing season, at before harvest (125 DAE) all treatments containing imazamox resulted in higher percentage of spikelet sterility (34.56 to 86.97% for IRGA 422 CL). These results can explain the reduction observed in grain yield in both growing seasons, especially in the 2010/11. The PUITÁ INTA CL cultivar did not have alteration in this parameter in either growing season.

3.2 Biochemical parameters

Biochemical parameters results only were showed of some imazamox treatments due to results similarity between the treatments evaluated in the IRGA 422 CL cultivar.

3.2.1 Chlorophyll and carotenoid determination

In general, the effect of imazamox treatments on the total chlorophyll and carotenoids concentrations in penultimate and flag leaves was similar (Table 2). However, the response of the flag leaf was more consistent than in the penultimate leaf. Moreover, chlorophyll and carotenoids concentrations in the leaves did not show a clear response pattern to the imazamox treatments at 8 and 16 DAT for IRGA 422 CL (Table 2). These results indicate that these pigments may not be a suitable parameter for evaluation of the effects of this herbicide on rice plants. ALS inhibitor herbicides are not known to cause direct change in photosynthesis, because the primary effect is on biosynthesis of branched-chain amino acids in plants (Senseman, 2007).

3.2.2 Hydrogen peroxide concentration and lipid peroxidation

In the flag leaf of IRGA 422 CL, H_2O_2 concentration was significantly decreased in the T3 and T4 treatment at 8 DAT, when compared to the untreated check (Table 2). Interestingly, at 16 DAA the H_2O_2 concentration was increased in T4 of this same leaf. In the penultimate leaf no change was observed for this parameter in both times evaluated (Table 2).

In the present study, MDA (malondialdehyde) content did not show changes in both leaves and panicle of IRGA 422 CL cultivar in different periods evaluated (Table 2). Lipid peroxidation (LPO) is caused by free radicals on biological membranes and it

is considered one of the most damaging processes that occur as a consequence of ROS (Gill and Tuteja, 2010). Lipid peroxidation can be evaluated according to levels of primary products or the end products of peroxidation, such as the malondialdehyde (MDA) which is assayed with thiobarbituric acid and expressed as thiobarbituric acid reactive substances (TBARS) (Garg and Manchanda, 2009). Zabalza et al., (2007) reported a slight increase of lipid peroxidation in pea leaves exposed to the imazethapyr herbicide, but the authors of this study suggest that the effects observed were not related to the mode of action of ALS inhibitors.

3.2.3 Enzyme activities of antioxidants system

SOD activity varied with the imazamox treatment and leaf evaluated (Table 2). SOD activity in both leaves either decreased or increased. Superoxide dismutase (SOD) is one of the most important enzymes of the antioxidant system as a first defense mechanism against ROS (Gill and Tuteja, 2010). SOD is responsible for catalyzing the dismutation of superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2), preventing the formation of hydroxyl radical ($OH^{\bullet-}$) which is more reactive than other ROS (Gill and Tuteja, 2010). Panicle SOD activity decreased in T3 (8 DAT) and T4 (1 DAT) in IRGA 422 CL, when compared to the untreated check (Fig. 4C and D). The reduction observed in SOD activity in panicles can be involved partially in the reduction of grain yield showed in the same treatments (Table 1).

In general, CAT activity in leaves did not vary with the imazamox treatment (Table 2). However, in T4 treatment, the effect of imazamox on CAT activity was increased in penultimate leaf at 8 DAT. The CAT activity in panicles did not show changes in both imazamox treatments evaluated (Fig. 4E and F). These results are not in accordance with Jiang and Yang (2009) that reported inhibition of CAT activity in

leaves of wheat exposed to prometryne, a selective herbicide of the s-triazine chemical family. Leaves and roots of rice plants showed that the bound residues of sulfonylurea, a group of herbicide that inhibit ALS enzyme activity, may affect metabolism of this plants due to inhibition observed in SOD and CAT activities (Li et al., 2007).

APX activity was significantly increased in both leaves of IRGA 422 CL plants treated with T3 and T4 treatments at 8 DAT, when compared to the untreated check (Table 2). Results are not corroborating with others researches that reported that CAT and APX activity decreased in leaves of both rice cultivars after the exposure to paraquat herbicide that acts as interceptor of electrons from the electron transport chain at PSI and results in the production of $O_2^{\bullet-}$ (Guo et al., 2007; Iannelli et al., 1999). Panicle APX activity was significantly increased in T4 treatment (1 DAT), when compared to the untreated check (Fig. 4H). The CAT is responsible for breaking down H_2O_2 in water (H_2O) and oxygen (O_2), which is essential for detoxification of ROS. This enzyme is able to convert about 6 million molecules of H_2O_2 to H_2O (Gill and Tuteja, 2010). APX enzyme converts H_2O_2 to H_2O by using ascorbate as electron donor and could be responsible for the fine modulation of ROS for signaling (Mittler, 2002). According to Gill and Tuteja (2010), APX has a higher affinity for H_2O_2 (μM range) than CAT (mM range) and it may have a more crucial role in the management of ROS during stress. Therefore, this can explain the results observed in the present study, which showed more pronounced changes in APX than CAT activities in panicles of rice plants.

Catalase (CAT) and ascorbate peroxidase (APX) are enzymes responsible for the protecting the plant against oxidative damage caused by H_2O_2. CAT activity inhibition has been reported in plants under water stress, salt, herbicides and

exposure to heavy metals (Calgaroto et al., 2010; Rossato et al., 2011; Tabaldi et al., 2009; Li et al., 2007; Pan et al., 2006; Sharma and Dubey, 2005). Therefore, the alterations in the biochemical parameters and theirs effects on the growth parameters had little contribution to explain the significant difference in grain yield between the two cultivars studied.

3.3 External morphology of panicles and spikelets

The untreated rice spikelet showed a pair of rudimentary glumes, two empty glumes, lemma and palea, a pair of lodicules, six stamens and one pistil in the center (Fig. 5A and D). The panicles that received imazamox application evaluated at 109 DAE showed changes in the morphological structure, when compared to the untreated check (Fig. 5A and B). A curvature in the rachis, primary branches and secondary branches of panicles was observed (Fig. 5B) when imazamox was applied at 60 DAE. Each internode possessed a slightly curved structure and/or it grew in a slightly different direction in relation to both inferior and superior internode, differing, therefore, from the usual linear structure of the reproductive branch. In our experiment it was observed the budding of new culms from secondary nodes of main culm. Moreover, some spikelets showed the tip of the lemma excessively curved toward the palea (Fig. 5E and F), causing an appearance very similar to the physiological disorder, called "parrots beak". Similar data were observed by Hensley (2009).

It was observed changes in the number and identity of the whorls, mainly structures that are alike carpels but occupying stamens positions and numbers. These spikelets showed a reduction in the number of stamens or no differentiation of such organs (Fig. 5C, G and H). These results may justify the high percentage of spikelet sterility and hence the low grain yield of IRGA 422 CL. Results from these studies

suggest that imazamox had its effects much more pronounced on the reproductive organ than on the vegetative organs of rice plant (IRGA 422 CL). In rice, the genes, *SPW1*, *MADS2* and *MADS4*, are required for the specification of the stamen (Yoshida and Nagato, 2011). Our results, specifically in this subject, (Fig. 5H), bring resemblance with those reported by Zhang et al. (2007) for *ah* rice mutant that presented stamens homeoticaly transformed into carpeloid structures. The authors also observed that the florets containing zero, three, or five stamens. According to Zhang et al. (2007) the mutation in *ah* resulted in homeotic alterations of the second and the third whorls.

In the present study, the imazamox application was made when the source-sink relationship in the plants was changing, i.e., plants were at the initiation of floral primordium development. Consequently, the transport of imazamox through the phloem might have increased its concentration in the panicle that was in formation. Therefore, the reduction of the branched chain amino acids content upon addition of imazamox could have caused changes in the synthesis of proteins that are important to the process of coordinated expression of homeotic genes and other genes of the cell cycle, consequently increasing the number of spikelet sterile.

Some studies have shown a rapid increase of carbohydrate content in leaves of plants treated with ALS inhibitors herbicides (Bestman et al., 1990; Zabalza et al., 2004). This accumulation was suggested to be related to a decreased photoassimilate translocation to sink tissues (Bestman et al., 1990). In the present study, if there was a decrease in the photoassimilate translocation it might be due to an alteration in the relationship between the source (leaves) and sink (panicles in development) caused by a negative effect of imazamox on the percentage of spikelet sterility which lowered the sink strength of the panicles.

4. Conclusion

The cause of the reduction in grain yield observed in IRGA 422 CL cultivar is not due to only one factor such as high oxidative stress in leaves and panicles, but it configures itself as part of a more complex process in combinations of factors. The oxidative stress promoted by imazamox (80 g ai ha^{-1}) may have contributed to grain yield reduction and the high percentage of sterile spikelet from IRGA 422 CL cultivar. However, the biochemical parameters and theirs effects on the growth parameters have little contribution to explain the significant difference in grain yield between the two cultivars. However, morphological and anatomical alterations seem to be the main cause of the differences in grain yield of imazamox herbicide-resistant rice cultivars. Therefore, it is necessary to further characterize the morphological alterations in rice.

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Table 1. Grain yield, 1000-grain weight and percentage of spikelet sterility of two rice cultivars in response to imazamox application in reproductive phase.

	2009/10		2010/11	
	IRGA 422 CL	PUITÄ INTA CL	IRGA 422 CL	PUITÄ INTA CL
Treatments	Grain yield (kg ha^{-1})			
T1	8507.5 A a[1]	8751.7 A a	10627.2 A a	11058.8 A a
T2	6795.2 B ab	8532.6 A a	6493.5 B b	10540.0 A a
T3	5878.9 B b	8740.1 A a	5773.4 B b	11073.8 A a
T4	3143.3 B c	8531.3 A a	6316.0 B b	10796.6 A a
T5	6231.0 B b	8658.2 A a	5913.6 B b	10933.4 A a
T6	6395.9 B b	8568.2 A a	7060.6 B ab	11673.9 A a
T7	5310.7 B b	8930.0 A a	6112.9 B b	11427.3 A a
CV_1(%)[2]	16.01		16.09	
CV_2(%)[3]	11.12		8.91	
	1000-grain weight (g)			
T1	29.5 A a[1]	24.2 B a	29.2 A a	24.7 B a
T2	28.6 A a	26.3 A a	28.2 A a	24.9 B a
T3	29.0 A a	24.8 B a	24.7 A b	25.4 A a
T4	24.4 A b	24.2 A a	26.4 A a	25.5 A a
T5	28.7 A a	24.0 B a	25.5 A a	25.9 A a
T6	29.3 A a	24.2 B a	26.8 A ab	25.3 A a
T7	26.9 A ab	25.5 A a	25.6 A ab	25.7 A a
CV_1(%)[2]	7.57		7.55	
CV_2(%)[3]	6.00		6.12	
	Spikelet sterility (%)			
T1	8.29 A b[1]	5.13 A a	4.32 A c	3.64 A a
T2	24.52 A ab	4.18 B a	34.56 A b	3.10 B a
T3	28.30 A ab	4.66 B a	74.28 A a	3.16 B a
T4	45.20 A a	6.51 B a	71.02 A a	3.70 B a
T5	24.56 A ab	6.54 B a	83.35 A a	3.81 B a
T6	17.10 A b	4.90 B a	74.50 A a	3.15 B a
T7	25.45 A ab	4.97 A a	86.97 A a	2.92 B a
CV_1(%)[2]	52.05		31.83	
CV_2(%)[3]	44.06		23.89	

[1]Means values followed by the same small letters in the column, and capital letters in the line did not differ significantly by Tukey test of $P \leq 0.05$. [2]Coefficient of variation of main plot. [3]Coefficient of variation of subplot. T1: untreated check; T2: 80 g ai in PD (panicle differentiation); T3: 80 g ai in PD+7 days; T4: 80 g ai in PD+14 days; T5: 40 g ai in PD and 40 g ai in PD+14 days; T6: 40 g ai in PD and 40 g ai in PD+7 days; T7: 40 g ai in PD+7 days and 40 g ai in PD+14 days.

Table 2. Chlorophyll and carotenoids concentration (CHLO and CAR; mg g^{-1} FW), lipid peroxidation (TBARS; nmol MDA mg^{-1} protein), hydrogen peroxide levels (H_2O_2; µmol g^{-1} FW), superoxide dismutase (SOD; U mg^{-1} protein), catalase (CAT; nmol mg^{-1} protein) and ascorbate peroxidase (APX; µmol oxided ascorbate min^{-1} mg^{-1} protein) enzyme activities, in the penultimate leaf and flag leaf of IRGA 422 CL cultivar in response to imazamox application in reproductive phase. T1: untreated check; T3: PD+7 days (80 g ai ha^{-1}); T4: PD+14 days (80 g ai ha^{-1}).

8 DAT[1]							
	CHLO	CAR	TBARS	H_2O_2	SOD	CAT	APX
Penultimate leaf							
T1	1.23 + 0.001	0.32 + 0.002	0.040 + 0.002	3.34 + 0.56	0.37 + 0.01	6.19 + 2.40	0.52 + 0.01
T3	1.43 + 0.002*	0.37 + 0.001*	0.042 + 0.006	3.89 + 0.19	0.48 + 0.01*	4.81 + 0.39	0.65 + 0.04*
T1	1.76 + 0.030	0.44 + 0.007	0.030 + 0.001	5.06 + 0.33	0.37 + 0.01	6.30 + 0.57	0.42 + 0.03
T4	1.27 + 0.002*	0.35 + 0.003*	0.040 + 0.007	6.06 + 0.92	1.05 + 0.06*	8.17 + 0.99*	0.86 + 0.07*
Flag leaf							
T1	0.92 0.008	0.26 + 0.003	0.021 + 0.002	4.18 + 0.17	1.21 + 0.05	9.42 + 2.09	0.54 + 0.05
T3	0.99 + 0.06	0.21 + 0.026	0.045 + 0.009	3.90 + 0.19*	0.75 + 0.10*	10.98 + 2.49	0.71 + 0,03*
T1	1.43 + 0.068	0.39 + 0.014	0.022 + 0.004	6.25 + 0.42	0.83 + 0.06	10.98 + 2.49	0.59 + 0.02
T4	1.38 + 0.007	0.37 + 0.001	0.032 + 0.006	4.49+ 0.042*	1.37 + 0.09*	6.70 + 0.01	0.44 + 0.05
16 DAT[1]							
Penultimate leaf							
T1	1.76 + 0.03	0.44 + 0.007	0.030 + 0.001	5.06 + 0.33	1.19 + 0.08	6.30 + 0.57	0.42 + 0.03
T3	1.17 + 0.011*	0.33 + 0.001*	0.043 + 0.002	6.00 + 0.65	0.75 + 0.16*	4.84 + 2.13	0.61 + 0.02*
T1	1.34 + 0.005	0.36 + 0.002	0.080 + 0.007	6.96 + 0.20	1.19 + 0.08	3.92 + 2.31	0.74 + 0.13
T4	1.41 + 0.005*	0.37 + 0.007*	0.080 + 0.001	7.68 + 0.57	0.71 + 0.10	4.77 + 2.43	0.57 + 0.08
Flag leaf							
T1	1.43 + 0.068	0.39 + 0.014	0.022 + 0.004	6.25 + 0.42	0.83 + 0.06	10.98 + 2.50	0.59 + 0.02
T3	1.20 + 0.003	0.34 + 0.005*	0.039 + 0.007	5.52 + 0.56	0.29 + 0.03*	9.57 + 1.49	0.53 + 0.03
T1	1.50 + 0.016	0.40 + 0.007	0.060 + 0.01	8.35 + 0.33	0.52 + 0.16	4.49 + 1.13	0.57 + 0.09
T4	1.64 + 0.035*	0.43 + 0.002	0.088 + 0.006	9.96 + 0.14*	0.68 + 0.11	6.88 + 2.64	0.52 + 0,03

[1]Days after treatment. Asterisks (*) indicate that mean values are significantly different between the herbicide treatments and the untreated check ($P \leq 0.05$).

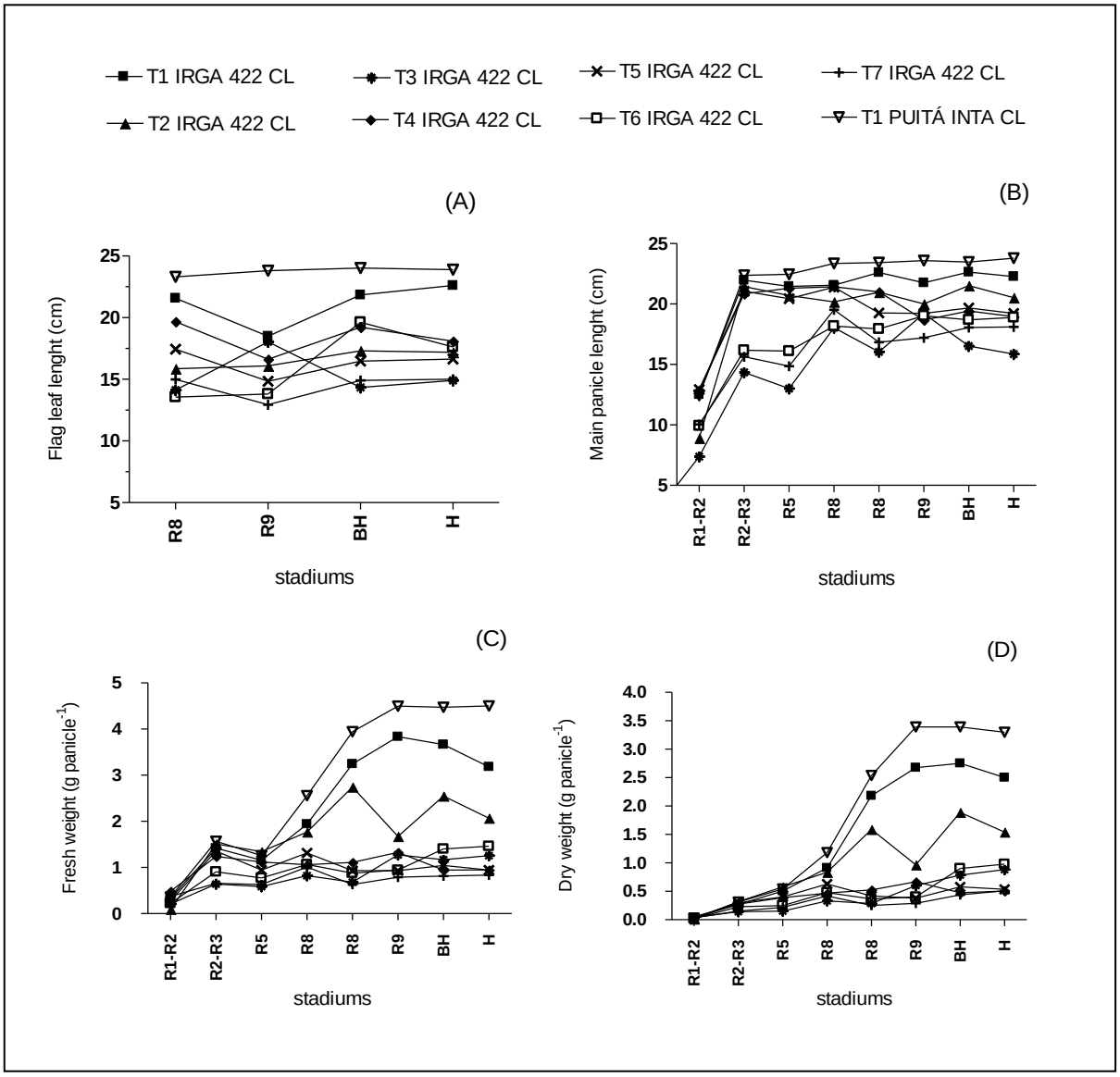

Fig. 1. Flag leaf length (A), length of panicles (B), fresh (C) and dry weight (D) of panicles from main culm of of two rice cultivars in response to imazamox application in 2010/11 growing season. T1: untreated check of IRGA 422 CL and PUITÁ INTA CL cultivars; T2: imazamox application at panicle differentiation (PD) (80 g ai ha^{-1}); T3: PD+7 days (80 g ai ha^{-1}); T4: PD+14 days (80 g ai ha^{-1}); T5: application at PD followed by (fb) PD+7 (40 fb 40 g ai ha^{-1}); T6: PD fb PD+14 (40 fb 40 g ai ha^{-1}); T7: PD+7 fb PD+14 (40 fb 40 g ai ha^{-1}). BH: before harvest; H: at harvest.

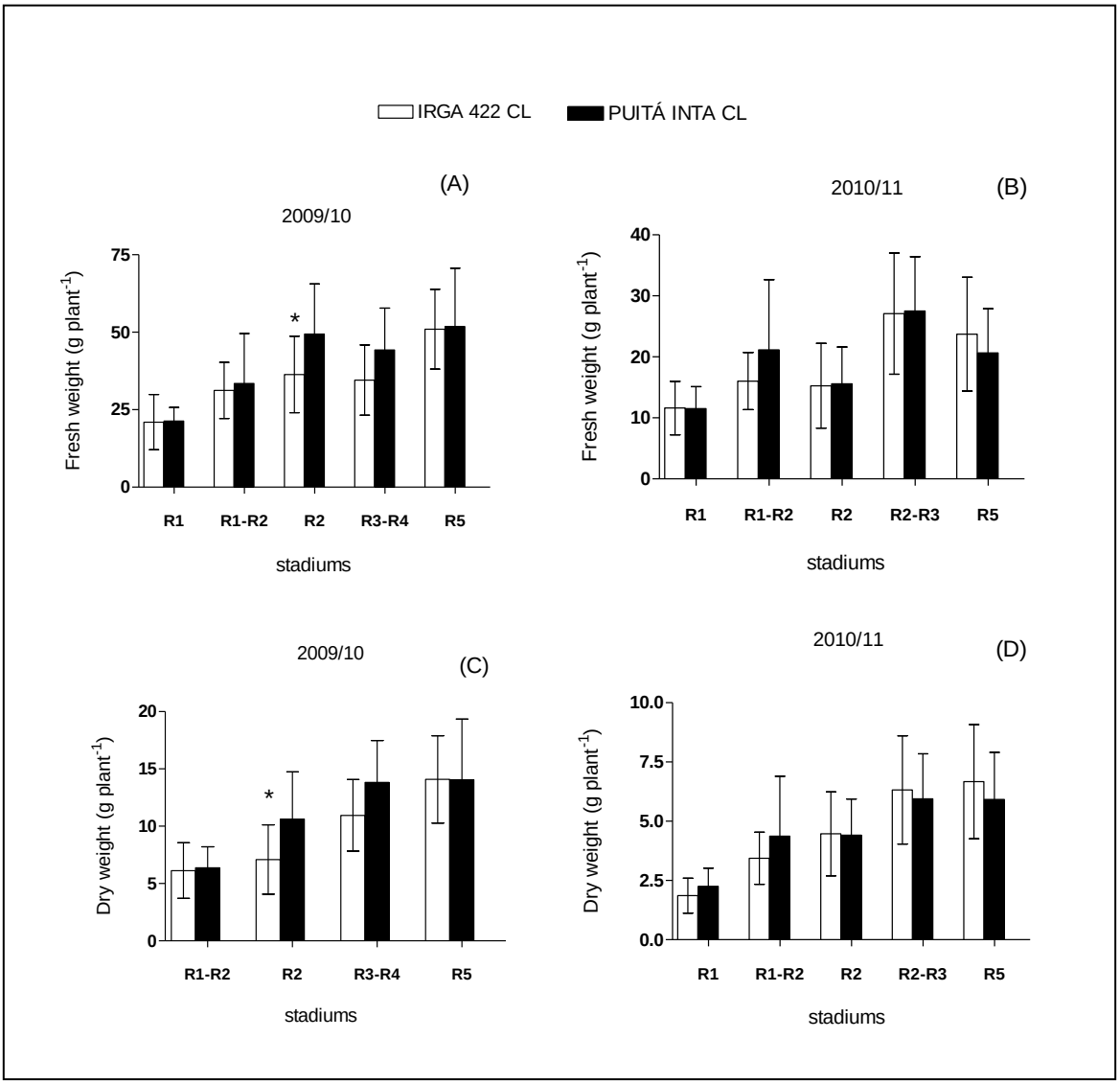

Fig. 2. Aboveground rice plants fresh (A and B) and dry weight (C and D) in response to imazamox application in reproductive phase in 2009/10 and 2010/11, respectively. The data represent the average of treatments. * Indicates difference between the cultivars.

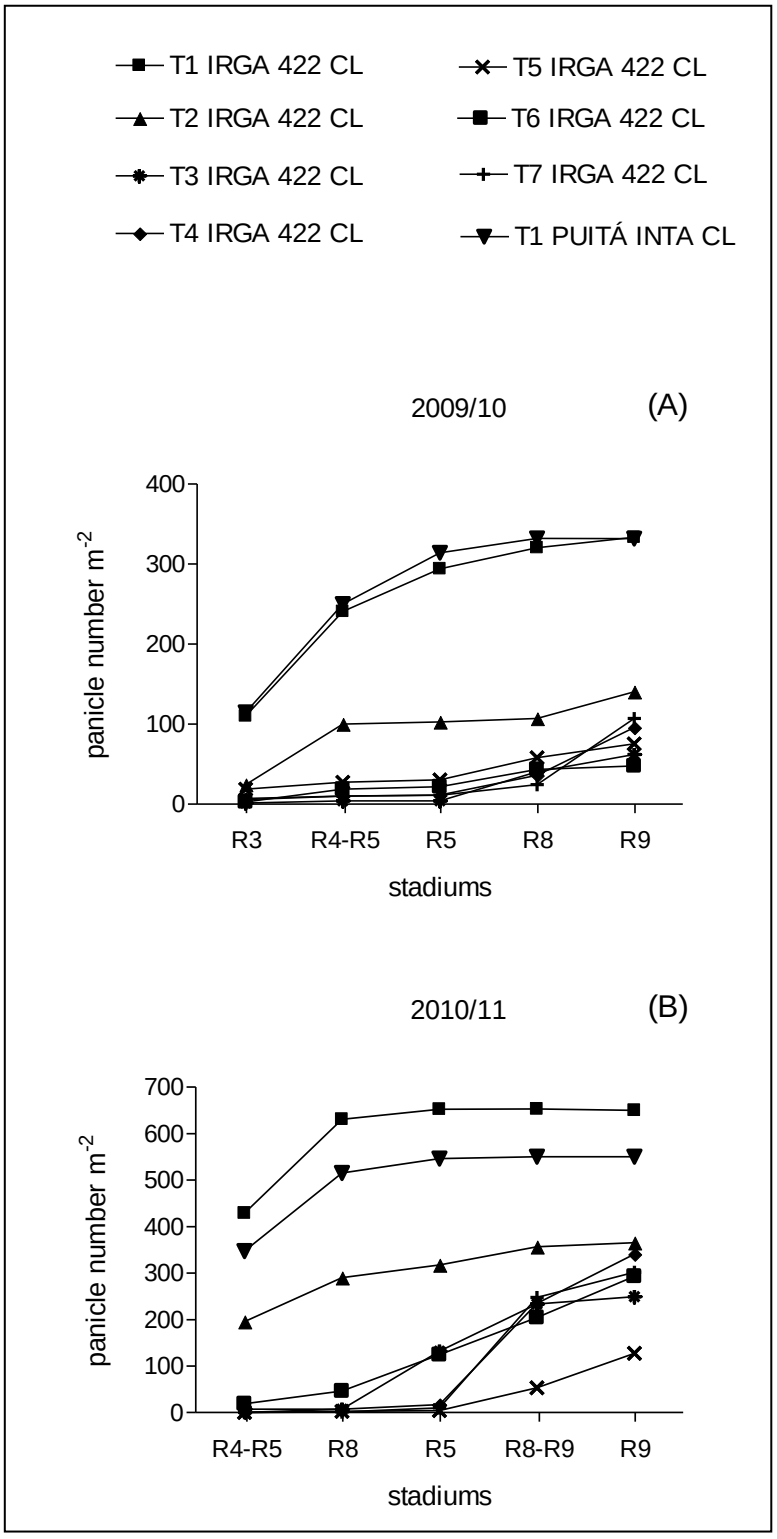

Fig. 3. Effect of imazamox application on number of panicles per m^2 of IRGA 422 CL and PUITÁ INTA CL cultivars in 2009/10 (A) and 2010/11 (B). T1: untreated check; T2: imazamox application at panicle differentiation (PD) (80 g ai ha^{-1}); T3: PD+7 days (80 g ai ha^{-1}); T4: PD+14 days (80 g ai ha^{-1}); T5: application at PD followed by (fb) PD+7 (40 fb 40 g ai ha^{-1}); T6: PD fb PD+14 (40 fb 40 g ai ha^{-1}); T7: PD+7 fb PD+14 (40 fb 40 g ai ha^{-1}).

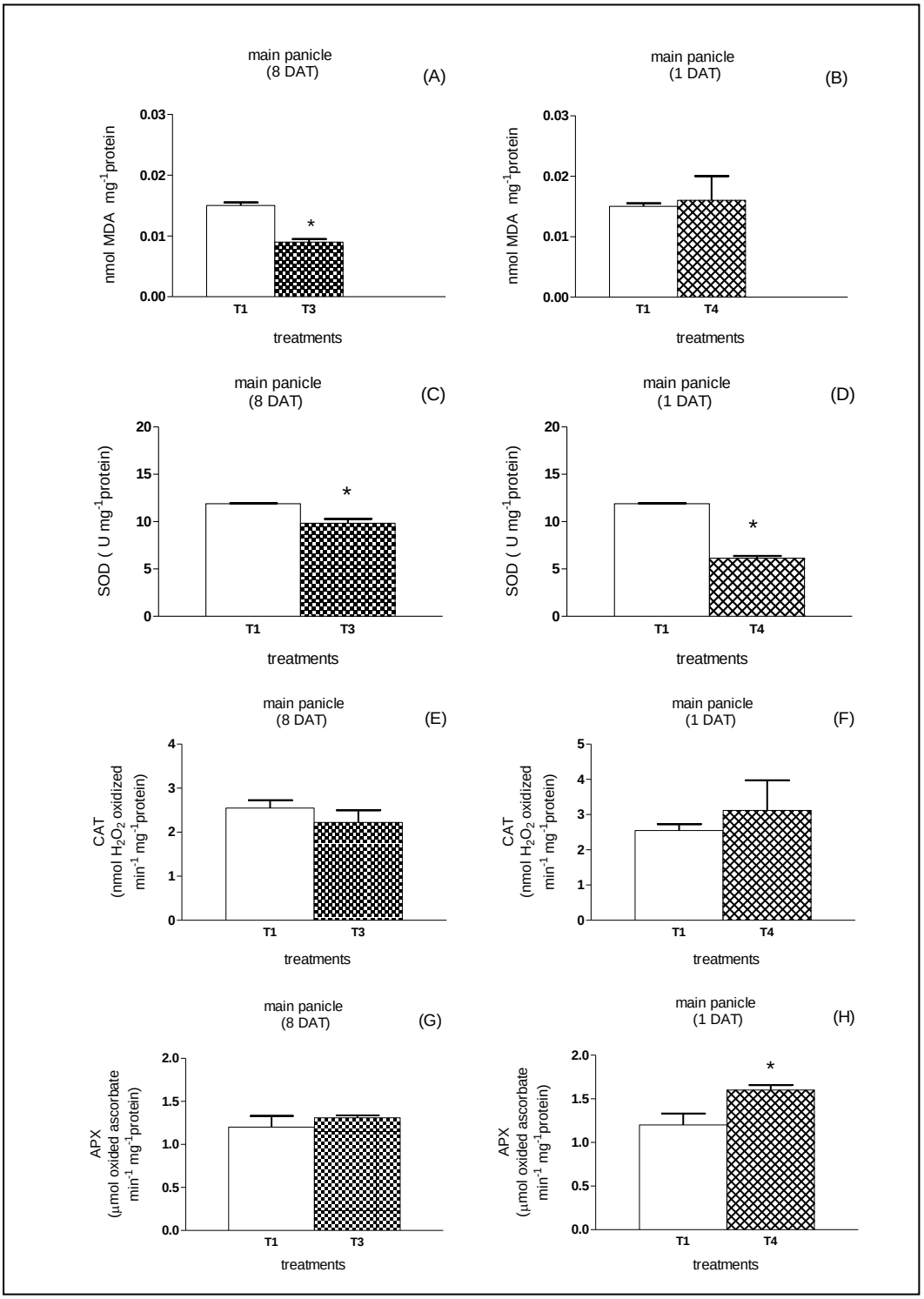

Fig. 4. Lipid peroxidation (A and B), superoxide dismutase (SOD) (C and D), catalase (CAT) (E and F) and ascorbate peroxidase (APX) (G and H) activity in panicles from main culm of IRGA 422 CL in response to imazamox application in reproductive phase at 8 and 1 DAT, respectively in 2010/11 growing season. Asterisks (*) indicate that mean values are significantly different between the herbicide treatments and the untreated check ($P < 0.05$). T1: untreated check; T3: PD+7 days (80 g ai ha^{-1}); T4: PD+14 days (80 g ai ha^{-1}).

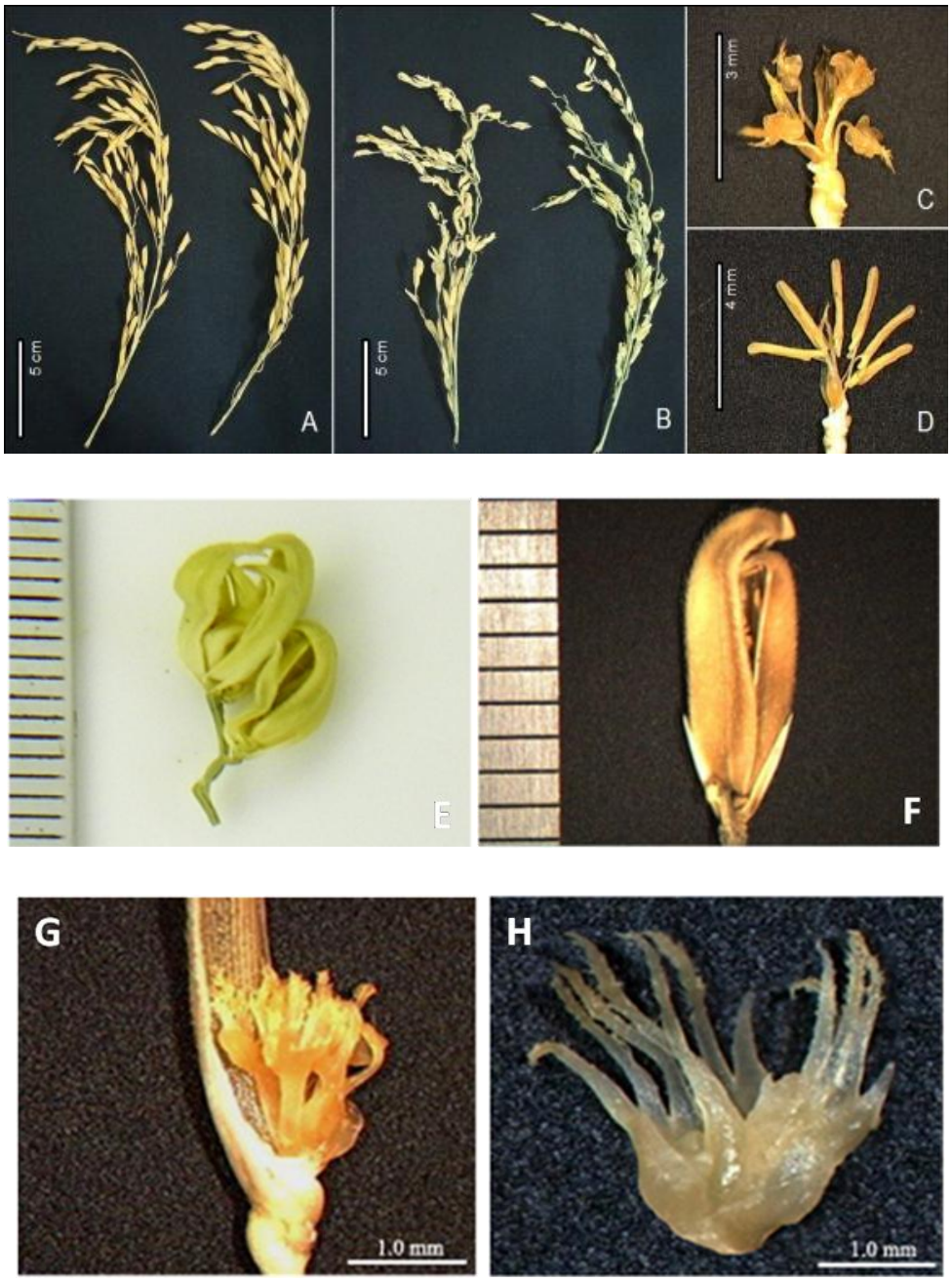

Fig. 5. Morphology of panicles and spikelets from IRGA 422 CL cultivar. Panicles from the untreated check (A). Panicles treated with imazamox (B). Spikelets showed a reduction in the number of stamens or no differentiation of stamens (C). Reproductive organs from the untreated check (D). Spikelets with the tip of the lemma excessively curved toward the palea (E and F). Increase in the carpels number (G and H).

ARTIGO 2

MORPHOLOGICAL AND ANATOMICAL CHARACTERIZATION OF REPRODUCTIVE ORGANS OF IMIDAZOLINONE-RESISTANT RICE AFTER IMAZAMOX APPLICATION IN REPRODUCTIVE PHASE

(Artigo foi redigido de acordo com as normas para autores do periódico "Planta")

Morphological and anatomical characterization of reproductive organs of imidazolinone-resistant rice after imazamox application in reproductive phase

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Abstract

Red rice is considered the main troublesome weed in rice paddy fields because it decreases the rice grain yield through the competition by water, light and nutrients. Herbicide-resistant Clearfield® rice technology allows the use of ALS inhibitors to control weedy rice. However, morphological changes are known as effect of ALS-inhibiting herbicides in non-resistant rice. The aim of this study was to characterize the floral alterations caused by imazamox herbicide applied in the reproductive phase of IRGA 422 CL cultivar. This cultivar is resistant to herbicides ALS inhibitors Two field experiments were carried out where imazamox was applied in different stadiums of rice development and rates (40 + 40 or 80 g ai ha^{-1}). In 2010/11 growing season, IRGA 422 CL showed a significant increase of spikelet sterility and reduction of pollen grain viability in all treatments when compared to the untreated check. Remarkably, transformations observed in florets from T2 treatment (80 g a.i ha^{-1} applied at panicle differentiation (PD) were similar to changes promoted by homeotic genes in rice mutants. However, T4 (80 g a.i ha^{-1} applied at PD+14 days) and T7 (40 g a.i ha^{-1} at PD+7 days and 40 g a.i ha^{-1} at PD+14 days) treatments promoted other morphological and anatomical alterations in the reproductive organs. The collapse of pollen grains was one of the main alterations observed in these treatments. Results showed that the increase of spikelet sterility of IRGA 422 CL was a consequence of morphological and anatomical alterations in florets that received imazamox application in the reproductive phase.

Keywords: *Oryza sativa*, floret, ALS herbicide, pollen grain, gynoecium and androecium.

1. Introduction

Rice (*Oryza sativa* L.) is a very important agricultural crop and the grain yield can be decreased by weedy red rice due to its competition by water, light and nutrients. Cultivated rice and red rice are morphologically and physiologically similar, making the control with selective herbicides difficult (Agostinetto et al. 2001; Roso et al. 2010). In the U.S., the introduction of herbicide-resistant Clearfield® rice occurred in 2001 with the imazethapyr and imazamox, in which the imazamox is applied during mid-season to control red rice and other weeds (Webster and Masson 2001; Shivrain et al. 2010). In Brazil, the introduction was in 2004, but only the imazethapyr and imazapic herbicides were registered for red rice controlling. The development of imidazolinone-resistant rice cultivars provides the selective control of red rice. The gene flow from imidazolinone-resistant rice is the main reason in the progression of herbicide-resistance in red rice of Southern Brazil (Goulart et al. 2012). The imazamox herbicide belongs to the chemical group of imidazolinone, which inhibits acetolactate synthase enzyme (ALS); also known as acetohydroxyacid synthase (AHAS) (Cobb and Reade 2010). Different mutations in the ALS gene can affect the cultivar response to imazamox application in the reproductive phase (Bond and Walker 2011). The ALS gene mutation that confers imidazolinone herbicide resistance in the IRGA 422 CL is $Gly_{654}Glu$ and in the PUITÁ INTA CL is $Ala_{122}Thr$ (Roso et al. 2010). Several researches have indicated diverse results when evaluating rice cultivar tolerance to ALS herbicides. The main explanation for varied tolerance between cultivars are the differences among the parent lines used in the cultivar development (Zhang and Webster 2002; Zhang et al. 2005).

Plant behavior, after herbicide exposure, could be sensed at different structural levels being considered, for such subject, the alterations in external morphology and/or histological constitution. Considering the complexity of the reproductive process through a developmental program, a sequence of cell and tissue transformation will not achieve success after drastic alterations in former sequences. Plants exposed to herbicides could present reductions in pollen viability and alterations in floral morphology that brings consequences to crop efficiency, which may reduce or not the production efficiency (Pline et al. 2003; Thomas et al. 2004).

Appropriate floral development in higher plants is the result of a sequence of events and need of a coordinated activity of a number of genes that control number, type and form of organs (Coen 1991). A central model simply explaining the genetic mechanism in flower development is the so-called 'ABC' model. The basic concept is that these flowers are composed of four layers of ring-shaped regions called 'whorls', and combinations of A-, B- and C-class gene functions exerted in each whorl specify the floral organ identities. Studies on MADS-box genes revealed another class of floral genes, known as the E function genes, which were necessary for specifying the organ identity of petals, stamens, and carpels (Yoshida and Nagato 2011)

There are few studies regarding the biology and effects of ALS inhibitor herbicides in the resistant rice cultivars. Some studies have showed that imidazolinone herbicides caused secondary effects on different plant species (Scarponi et al. 2001; Zabalza et al. 2004). Hensley (2009) reported that some spikelets of rice in response to imazamox drift showed the tip of the lemma excessively curved toward the palea, causing an appearance very similar to the physiological disorder, called "parrot's beak". The imazamox applications in the reproductive phase of IRGA 422 CL cultivar promoted a reduction in grain yield and morphologic alterations (Moraes et al. submitted).

The hypothesis of this study is that imazamox applied in different times of the reproductive phase of IRGA 422 CL causes reduction in grain yield through morphologic transformations in florets. Thus, the aim of the present study was to characterize the changes caused by imazamox herbicide applied in the reproductive phase of IRGA 422 CL cultivar.

2. Material and Methods

2.1 Rice growth condition

Plants were grown at Universidade Federal de Santa Maria in an experimental paddy fields in RS, Brazil (29º41'24"S, 53º48'42"W, 95 m altitude) in 2009/10 and 2010/11 growing seasons. The experiment was in randomized block design in a factorial scheme (2x7) in split-plot arrangement with cultivar as the main plot and treatments as subplot, with four replications. The rice cultivars used were IRGA 422 CL and PUITÁ INTA CL. The herbicide treatments were an arrangement of

imazamox rate (80 g ai ha^{-1}) and timing of application. The cultivars were drill seeded at 100 kg ha^{-1}, previously treated with the insecticide fipronil (250 g ai per 100 kg of seeds). Rice seeds were sown on October 21st, 2009 and October 5th, 2010. Nitrogen fertilizer at 350 kg ha^{-1} as urea was applied 5% as basal fertilizer, 50% as topdressing fertilizer for promoting tillering (applied at V3-V4 stadiums), 45% for promoting panicle initiation (applied at V9 or R0 stadiums, according to Counce et al. 2000). Phosphorous and potassium fertilizer was used as basal fertilizer. Permanent flood was established when plants were at the three to four-leaf stadium. Other standard agronomic and pest management practices were implemented through the growing season, according to the recommendations of the research (SOSBAI, 2010).

2.2 Herbicide treatments

The Imazamox herbicide 2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methoxymethylnicotinic acid (IUPAC) is registered in Brazil for selective control of post-emergence weeds in the soybean and dry bean cultures, and is commercialized as Sweeper® by BASF®. The herbicide applications were performed using a CO_2-backpack sprayer in an application volume of 180 L ha^{-1}. The imazamox treatments were: T1: untreated check; T2: 80 g ai at PD (panicle differentiation); T3: 80 g ai at PD+7 days; T4: 80 g ai at PD+14 days; T5: 40 g ai at PD and 40 g ai at PD+14 days; T6: 40 g ai at PD and 40 g ai at PD+7 days; T7: 40 g ai at PD+7 days and 40 g ai at PD+14 days.

2.3 Spikelet sterility

In the 2010/11 growing season, the main culm panicle from the IRGA 422 CL cultivar at before harvest and harvest (117 and 123 DAE) was split in three parts, apical, medium and basal portions and counted the number of filled and unfilled grains.

2.4 Pollen viability

In both growing seasons, when rice plants were in R2 stadium (Counce et al. 2000), the main culm panicle from field-grown plants of IRGA 422 CL cultivar was collected and fixed in Carnoy's (3:1 v/v of ethanol: acetic acid) solution. The fixing

solution in each sample was replaced after 24 h with a 70% ethanol and stored in a refrigerator. Anthers from three spikelets were randomly selected and the analysis of viability of pollen grains was estimated by their staining capacity by colorimetric test using Alexander's stain. Using Alexander's stain, which contains malachite green and acid fuchsin, unviable pollen grains stain green while viable pollen grains stain purple. After uniformly dispersing the pollen and stains grains were covered with a coverslip. In 2010/11, for better characterizing the imazamox effect, the panicle was split into three parts, apical, medium and basal portions. Afterwards, the slide was observed in Leica 400X light microscope. Three hundred pollen grains were counted for replication of each treatment.

2.5 External morphology of panicles and spikelets

In the 2010/11 growing season, panicles from field-grown plants of the IRGA 422 CL cultivar were collected and fixed in a solution with glutaraldehyde 3% in sodium phosphate buffer at pH 7.2 and Tween20 2mL L^{-1} (adapted from Freudenstein 2002) for 24 h, at room temperature. Then, the spikelets were removed from the panicle and the images were taken under stereomicroscopes Olympus SZH10 and Leica EZ4 from images digitally collected.

2.6 Anatomy of spikelets

Spikelets were fixed in glutaraldehyde 3% in sodium phosphate buffer at pH 7.2 and Tween20 2mL L^{-1} (adapted from Freudenstein 2002), at room temperature and then dehydrated in a graded ethanol series. Histological sections, with 6 µm thick, were made using a Leica RM2245 microtome. Sections were stained with Toluidine blue O 0.05% in sodium benzoate buffer at pH 4.4 (O'Brien and McCully 1981). The observations and photomicrographs were made with Leica DM2000 light microscope from images digitally collected.

2.7 Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. Results are the means of at least four replicates and the mean differences were compared using the Tukey test ($P < 0.05$).

3. Results and Discussion

3.1 Spikelet sterility and Pollen viability

At before harvest (117 and 123 DAE), IRGA 422 CL showed a significant increase of spikelet sterility in all treatments when compared to the untreated check (Figure 1A and B). However, no changes in the spikelet sterility between the apical, medium and basal parts in any period tested. The high sterility observed for this cultivar is correlated with the grain yield results, which were reduced in all treatments in the 2010/11 growing season (Manuscript 1).

In the 2009/10 and 2010/11 growing seasons, at R2 stadium (77 and 80 DAE), respectively, four rice plants were harvested and the main culm was separated. From the main culm, the panicle was removed for pollen viability analysis. In the 2009/10 growing season, a high rate of pollen viability (> 83%) was observed regardless of the staining method used (Table 1). In 2010/2011 growing season, T4 and T7 treatments promoted a reduction in the pollen viability from medium portion, whereas T2 show decrease of pollen viability only from the basal portion (Figure 2A and B). Results of T3 and T6 treatments are not shown because the pollen grains of these spikelets were not mature.

Several staining techniques have been employed to estimate pollen grain viability. In the present study, data suggest that no dye efficiently characterized the pollen viability of IRGA 422 CL cultivar. The pollen grains that were partly stained demonstrated that the method can overestimate the results of viability percentage. Previously, pollen grains without dye were analyzed and showed that pollen grains were in different phases of gametogenesis. The level of starch accumulation and size of the vacuole observed in pollen grains of the present study served as indicative of the different stages of microsporogenesis after the uninucleate stage (Chang and Neuffer 1989). Thomas et al (2004) found a reduction in pollen viability of corn plants treated with glyphosate herbicide. Glyphosate herbicide belongs to the chemical group that inhibits the biosynthesis of aromatic amino acids by blocking the activity of EPSPS (5-enolpyruvylshikimate 3-phosphate synthase), an enzyme in the shikimate pathway (Senseman 2007). Orcaray et al (2010) showed that the accumulation of quinate compound was a common effect of the two different classes of herbicide

(inhibitor of aromatic and branched-chain amino acid biosynthesis). Although the primary mechanism of action of these herbicides is widely known, it is not fully understood how plants in fact die after the inhibition of EPSPS or ALS. Our results suggest that the dyes used were not efficient to estimate the pollen viability, but the reduction of pollen grain viability could be involved in the reduction of grain yield of IRGA 422 CL cultivar (Manuscript 1).

3.2 External morphology of spikelets

The rice flower structure of untreated check consisted of one pair of rudimentary glumes, two empty glumes, lemma and palea, a pair of lodicules, six stamens and one pistil in the center as already described in the literature (Takeoka et al. 1993; Fornara et al. 2003; Itoh et al. 2005; Yoshida and Nagato 2011).

The external morphology analysis showed remarkable differences when comparing anther sporangia from the untreated check with that under imazamox treatment. Anther sporangia from the untreated check showed a dense yellow color caused by the great amount of pollen grains inside each sporangium (Figure 3A-C). The spikelets under imazamox treatment showed empty and translucent anther sporangia (Figures 3D, H and L). Rice flower from panicles in T2 treatment showed structures with multiple stigmas and expanded tissue between the filament and the feather-like stigma, reduction in the number of stamens, or no differentiation of such organs in all panicle portions analyzed (Figure 3D, E and F). Results observed in the present study were also observed by Li et al (2007) in the *fon(t)* rice mutants. Flowers in T4 treatment showed a delay of reproductive structure development (Figure 3G, H and I). T7 treatment promoted reduction or absence in the number of stamens from flowers evaluated at R3 stadium (85 DAE) (Figure 3J and K, respectively). Noteworthy, in T7 treatment the reproductive structures showing delayed development were more pronounced in the basal portion of the inflorescence (Figure 3L).

3.3 Anatomy of spikelets

3.3.1 Androecium of untreated check at R3 stadium (85 DAE)

Mature florets showed stamens with typical fillet and anther. Fillet under cross section presented a cylindrical contour where a centrally located collateral vascular bundle occurred being covered by a parenchimatous tissue that in turn appeared covered by an epidermis. The anther was ditecate and tetrasporongiate.

In the present study we observed that the anthers were mature at R3 stadium (85 DAE) and no difference was observed between anthers from the apical and medium panicle portions. In the anthers, the sporangia showed epidermis and endothecium (Figure 4A). The epidermis is composed of longitudinally elongated cells and some were vacuolated and exhibit compact nucleus.

Stomata were observed in the epidermis. The endothecium was predominantly composed of one cell layer, although in the distal apex sporangia two layers were developed. The shape of the endothecial cells ranged from isodiametric to radially elongated and tangentially elongate, and a great variation in cell volume was observed. Cells with tangential stretching were common in the protruding sporangia. Besides the cellular morphological variation, the endothecium typically presented a ring or ‘U’ shape secondary thickening. Thickening in 'U' shape or reticulate thickening, along the inner periclinal walls, was described for Oryza (Pullaiah and Febulaus 2000). Apparently, the types of parietal thickening occur in specific regions according to the contours of sporangia. According to Manning and Linder (1990), the Poaceae endothecium exhibits two types of thickenings. In general, the endothecium presents discontinuity between cells, allowing direct contact between the locular fluid and epidermis. Apparently, lignification observed in the epidermis allows the maintenance of locular fluid. At this stage of maturation, cells from the middle layer and tapetum were no longer observable. In Poaceae, both layers were described as ephemeral (Pullaiah and Febulaus 2000).

In general, pollen grains in different developmental stages were found in all panicle portions evaluated. Vacuolated grains, grains with phenolic compounds accumulated and grain with nucleolus were observed. In some grains plasmolysis was also observed (Figure 4A and B). Pollen grains were spherical, although in the histological analysis they appear with facets due to the contact between grains. Mature pollen grains showed vegetative cell accumulating large quantities of starch grains (Figure 4C). The differentiation of a vacuole was commonly observed in the

cytoplasm of the vegetative cell associated with aperture region, usually containing sugars in this vacuole. Male gametes were long and thin, like filaments with difficult visualization due to the large accumulation of starch grains above mentioned. It was difficult to distinguish cytoplasm and nucleus in male gametes. The sporoderm was composed of exine and a thin intine (Figure 4C). The grains had a single aperture, which was circular and resulted from combined differentiation of exine and intine. Thus, in the exine region we observed a great thickness of sporopollenin which delimits the opening, besides the differentiation of an operculum. The intine presented a greater thickness only in the region of the opening. The intine showed a pectic constitution.

Among the mature pollen grains, immature grains were found, but they were apparently functional (vacuolated and uninucleate or vacuolated and binucleate) (Figure 4B). These grains showed a delay in their development. Pollen grains with delayed development usually occupy the central locular position and the mature grains occupy the peripheral locular position.

3.3.2 Gynoecium of untreated check at R3 stadium (85 DAE)

The gynoecium was represented by a single carpel. The regions of the ovary, style and stigma were morphologically distinct. The ovary was unilocular with a single ovule inserted laterally in the ventral region of the carpel.

The ovule has two integument with the micropyle formed by the inner integument only and similar to what is described as common to Poaceae (Pullaia and Febulaus 2000; Itoh et al. 2005) (Figure 5A). In Poaceae, different morphologies have been described, including *Oryza sativa*. In this species, ovules hemianatropous or campilotropous are commonly observed (Itoh et al. 2005). In this study, a traditional ovule morphology classification was not presented mainly because the funiculus was not developed. The nucellus is pseudocrassinucelar. The mature gametophyte presents an oosferic apparatus in the micropylar region, antipodes in the lateral position, and a bulky central cell with two polar nuclei that were not fused. The synergids developed a fibrillar apparatus along the micropylar region, which configures a specialization of their cell walls, attracting the pollen tube (Maze and Lin 1975). Antipodes in this study showed a higher citoplasmatic density after the

staining procedure in relation to other cells of the female gametophyte. Additionally to particular position, cells presented a proliferative behavior and bi or trinucleated cells. Nuclei in process of fusion and probably polyploidy nuclei were also observed (Figure 5B). In Pooideae the antipodal lateral positioning is a common pattern (Figure 5C). Generally, antipodal cells have a nutritional or secretory function, where such conclusions are mainly influenced by the dense cytoplasmic structure, by its persistence after fertilization, and by seed abortion after antipode death (Pullaiah and Febulaus 2000).

In the present study, the characteristics observed and described for *O. sativa* showed the differentiation of functional reproductive structures by structural context of organs, tissues and cells observed and compared with the embryological literature. Therefore, the descriptions were configured as a basis for comparison of samples subjected to imazamox treatment.

3.3.3 Androecium and gynoecium of treatment 2 at R3 stadium (85 DAE)

Abnormalities were mainly detected in the florets that received imazamox application in PD (treatment T2). Androecium showed total or partial transformation of stamens into carpels when compared with untreated check. In the group with partial transformation, morphogenesis was observed only in the sporangial region. Although in such situations it was observed a similar morphology to carpel, an irregular tissue proliferation from sporangia already formed was also verified. In such structures, some portions of sporangia were not affected by cellular proliferation.

In the anthers, the proliferation of irregular tissue was observed in two distinct ways. Firstly, a massive structure was formed by a compact tissue, which is originated from connective and proliferates through the sporangium via locular space, projecting externally (Figure 6). Secondly, the other mode of cell proliferation built up a structure similar to a carpel that encloses its ovule (Figure 6). Both proliferative patterns occur in the same androecium. It is noteworthy that the stigmoid structures are originated from each modified stamen, being that such stigmoids rose from distal connectival portion of the anther. Results observed in this treatment were very similar to homeotic transformations found in mutants. Recently, a number of regulatory genes belonging to the MADS-box transcription factor family have been cloned in rice

and some of their functions have been studied in details (Fornara et al. 2003; Li et al. 2007; Zhang et al. 2007). Jack et al (1994) reported that carpels of flowers from transgenic Arabidopsis plants were converted to stamens in the fourth whorl, which was a result of the combined expression of B-class and C-class genes in this whorl. In rice, the genes, *SPW1, MADS2* and MADS4 are required for stamen establishment (Yoshida and Nagato 2011). Yoshida and Nagato (2011) showed that *AP3* orthologue *SPW1* caused transformation from stamen to carpel-like organs in rice. Zhang et al (2007) reported that *ah* rice mutant presented stamens homeoticaly transformed into carpeloid structures. Moreover, it was observed that the florets contained zero, three, or five stamens. Complementarily, the mutation in *ah* resulted in homeotic alterations of the second and the third whorls (Zhang et al. 2007).

Anatomical analysis of the stamens showed that in some anthers two groups of pollen grains were observed. One group with grains completely collapsed and the other with spherical grains as observed in the untreated check (Figure 6). In general, the pollen grain showed modifications in the sporoderm, mainly in the structure of the exine, which was presented with curvy and irregular thickness.

The gynoecium showed a diversity of structural arrangements. Gynoecium with two connate carpels was present (Figure 7 A and B). Besides the formation of two fused carpels, alterations in the morphology and organization of the female gametophyte were observed. In the situation of the two connate carpels, antipodal cells occur at a terminal position, facing calazal region and not lateral as expected. Additionally, nucellar cells are developed among antipodal cells, showing similar cytological structure, except for their shape. Remarkably, the nucellar cell expanded part of its wall into the antipodal group of cells (Figure 7C) that apparently causes its particular shape like a glandular trichome which presents a dilated terminal portion. In the floret, with the whorl structure drastically changed, it was not possible to observe if these carpeloid structures were from changes in the androecium or gynoecium.

The presence of nucelar cells among antipodal cells configures an important morphological variation because the altered pattern does not affect the fertilization since we observed fruits that originate from two connate carpels. Li et al (2007) reported that *fon(t)* rice mutant presented mature flower with two seeds. Probably the

new positioning of nucelar cell induced its development toward an antipodal structure.

Antipodes in Poaceae are very important for embryological process and embryogenesis (for review see Pullaiah and Febulaus 2000) thus the differentiation of these cells may be indicative of a functional gametophyte. To the contrary, antipodal cells do not proliferate, the endosperm fails to develop, and the embryo ultimately aborts.

3.3.4 Androecium and gynoecium of treatment 4 at R3 stadium (85 DAE)

In general, the whorl and stamens did not show morphological changes in any panicle portions (apical, medium and basal) evaluated when compared to the untreated check. In the anther was shown pollen grains totally collapsed (Figure 8A-D).

Carpel and ovule of flowers of all panicle portions showed no morphological changes when compared to the untreated check. Gametophyte also remained morphologically unchanged. Moreover, it was observed that the fertilization occurred, indicating the functionality of the structure.

3.3.5 Androecium and gynoecium of treatment 7 at R3 stadium (85 DAE)

Empty anther sporangium was also observed in another group of florets (Figure 9A). In this treatment, we observed pollen grains almost completely collapsed, with the exine apparently differentiated, unlike intine which appears only in the region of the opening (Figure 9B). The sporangium showed epidermis and endothecium similar to that observed in the untreated check. In the parietal position we observed that tapetal cells were collapsed and sometimes showed two nuclei. The changed esporoderm structure and the presence of cell debris from tapetum could indicate the interruption of the development in a few florets. Gametogenesis was apparently normal (Figure 9C). The early stage of fertilization was also observed.

4. Conclusions

Morphologic and anatomical alterations observed in florets from IRGA 422 CL cultivar may explain the high spikelet sterility observed in all imazamox treatments. Transformations observed in florets from T2 treatment were similar to changes promoted by homeotic genes in rice mutants. T4 and T7 treatments promoted other morphological and anatomical alterations in the reproductive organs than those observed in T2 treatment. The collapse of pollen grains was one of the main alterations observed in these treatments, while the gynoecium showed usual morphology indicating the functionality of the structure. Additionally, the collapse observed in pollen grains may be confirmed by the high pollen grain inviability. Differences among T2 and the T3 and T7 treatments on the reproductive organs of rice can be justified by the period of imazamox application.

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Table 1. Viability of pollen grain using colorimetric test with Alexander's stain in response to imazamox application in reproductive phase of rice cultivar.

Treatments	IRGA 422 CL
T1	83.79 [1] ns
T2	85.87
T3	87.45
T4	87.25
T5	89.37
T6	88.16
T7	85.45
CV (%)[2]	4.50

[1]ns = not significant between treatments by Tukey test of $P < 0.05$. [2]Coefficient of variation of main plot. T1: untreated check; T2: imazamox application at panicle differentiation (PD) (80 g ai ha $^{-1}$); T3: PD+7 days (80 g ai ha $^{-1}$); T4: PD+14 days (80 g ai ha $^{-1}$); T5: application at PD followed by (fb) PD+7 (40 fb 40 g ai ha $^{-1}$); T6: PD fb PD+14 (40 fb 40 g ai ha $^{-1}$); T7: PD+7 fb PD+14 (40 fb 40 g ai ha $^{-1}$).

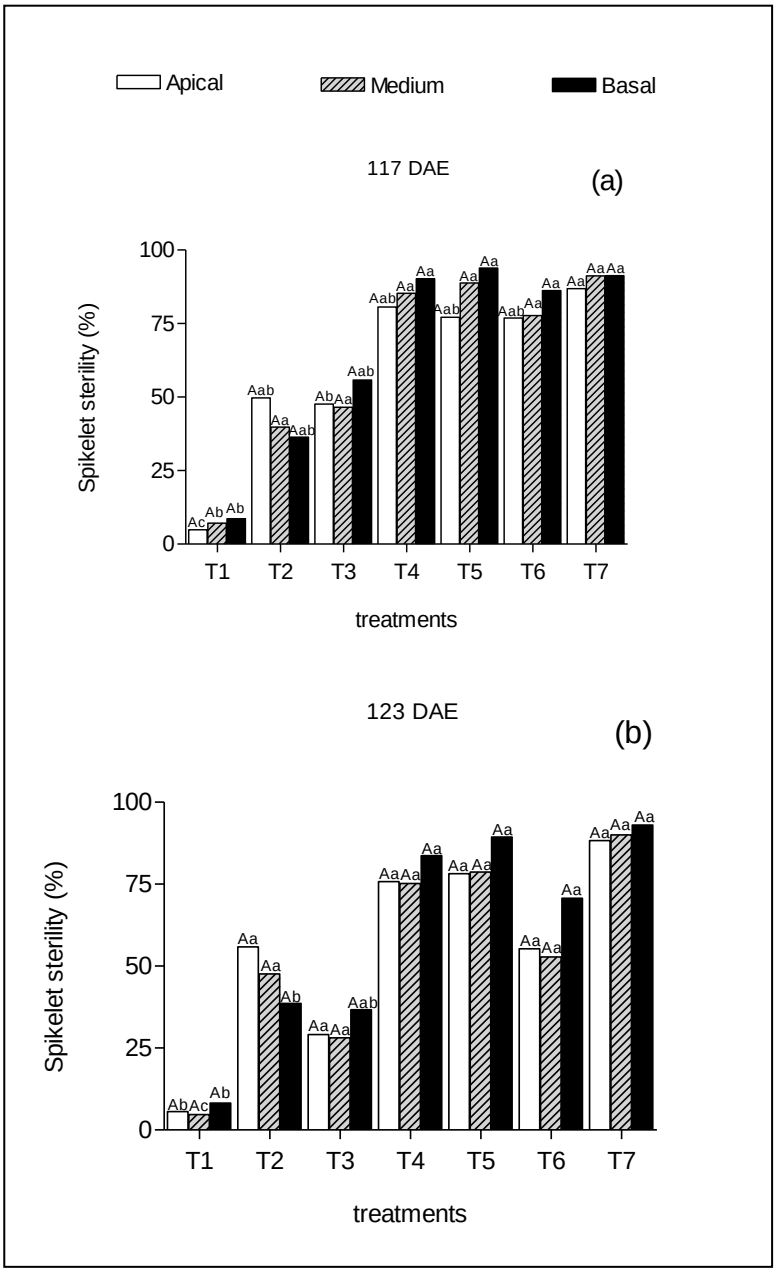

Figure 1: Percentage of spikelet sterility at before harvest and harvest (117 and 123 DAE) (a and b, respectively) from main panicles in response to imazamox application in reproductive phase of IRGA 422 CL cultivar in 2010/11 growing season. Means values followed by the same small letters and capital letters did not differ significantly between panicle portions and treatments, respectively, by Tukey test of $P \leq 0.05$. T1: untreated check; T2: 80 g ai in PD (panicle differentiation); T3: 80 g ai in PD+7 days; T4: 80 g ai in PD+14 days; T5: 40 g ai in PD and 40 g ai in PD+14 days; T6: 40 g ai in PD and 40 g ai in PD+7 days; T7: 40 g ai in PD+7 days and 40 g ai in PD+14 days.

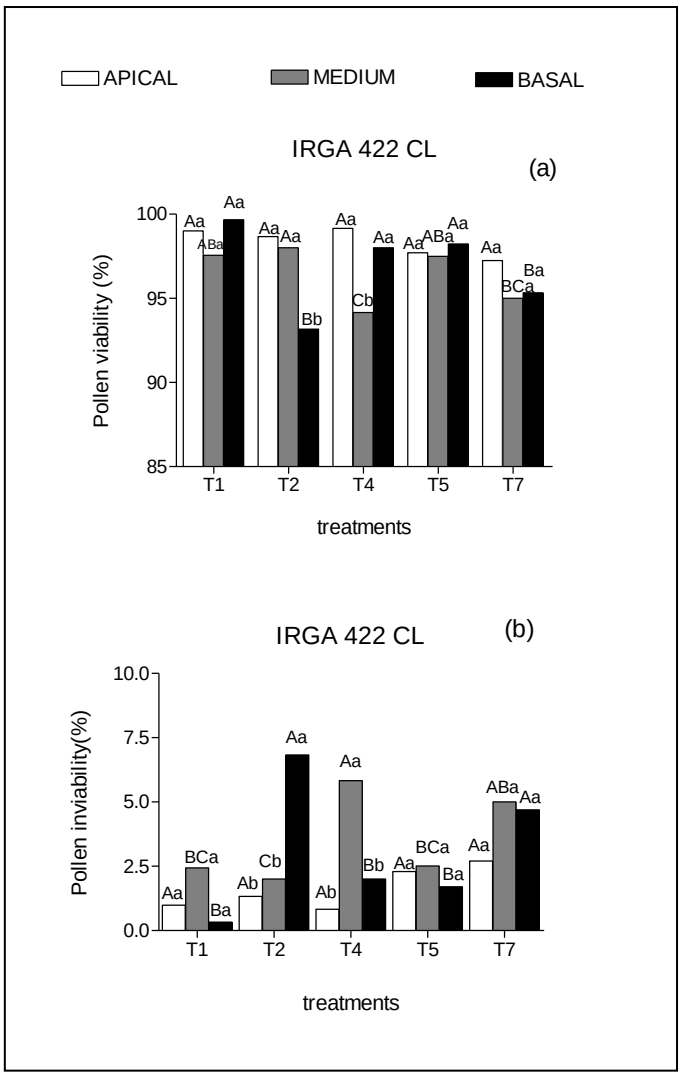

Figure 2: Pollen viability (a) and inviability (b) from main panicles in response to imazamox application in reproductive phase of IRGA 422 CL cultivar in 2010/11 growing season. Means values followed by the same small letters and capital letters did not differ significantly between panicle portions inside of each treatment and treatments in each panicle portion, respectively, by Tukey test of $P \leq 0.05$.T1: untreated check; T2: imazamox application at panicle differentiation (PD) (80 g ai ha $^{-1}$); T4: PD+14 days (80 g ai ha $^{-1}$); T5: application at PD followed by (fb) PD+7 (40 fb 40 g ai ha $^{-1}$); T7: PD+7 fb PD+14 (40 fb 40 g ai ha $^{-1}$).

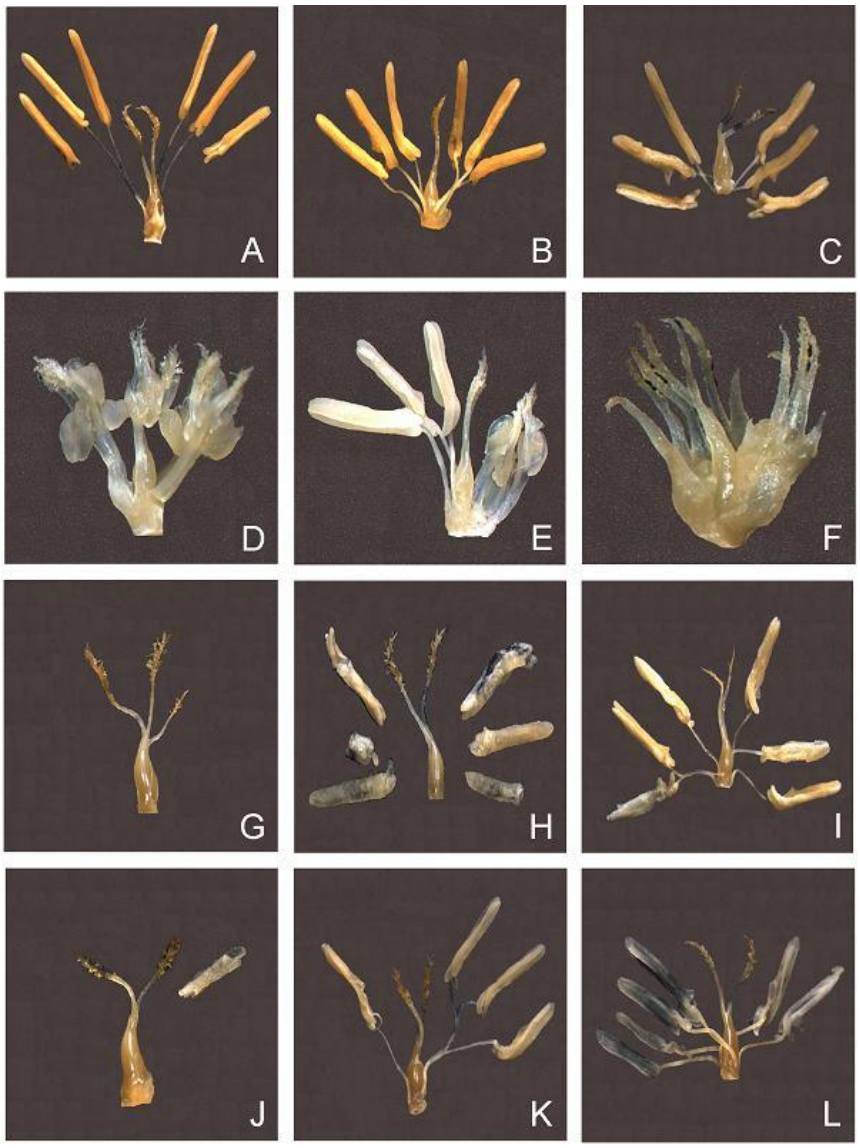

Figure 3: Morphology of rice spikelets. **A**: a mature flower of apical portion of the rice panicle of untreated check. **B**: a mature flower of medium portion of the rice panicle of untreated check. **C**: a mature flower of basal portion of the rice panicle of untreated check. **D**: flower from apical portion of panicle from T2 treatment with multiple stigmas and bulged tissue between the filament and the feather-like stigma.**E**: flower from medium portion of panicle from T2 treatment with three stamens and bulged tissue between the filament and the feather-like stigma. **F**: flower from basal portion of panicle from T2 treatment with multiple stigmas. **G**: flower from apical portion of panicle from T4 treatment with three stigmas and ausence of stamens. **H** and **I**: flower from medium and basal portions of panicle from T4 treatment with one pistil and six stamens. **J** and **K**: flower from apical and medium portions of panicle from T7 treatment with one pistil and number of reduced and translucent stamens. **L**: flower from basal portion of panicle from T7 treatment with one pistil and six translucent stamens.

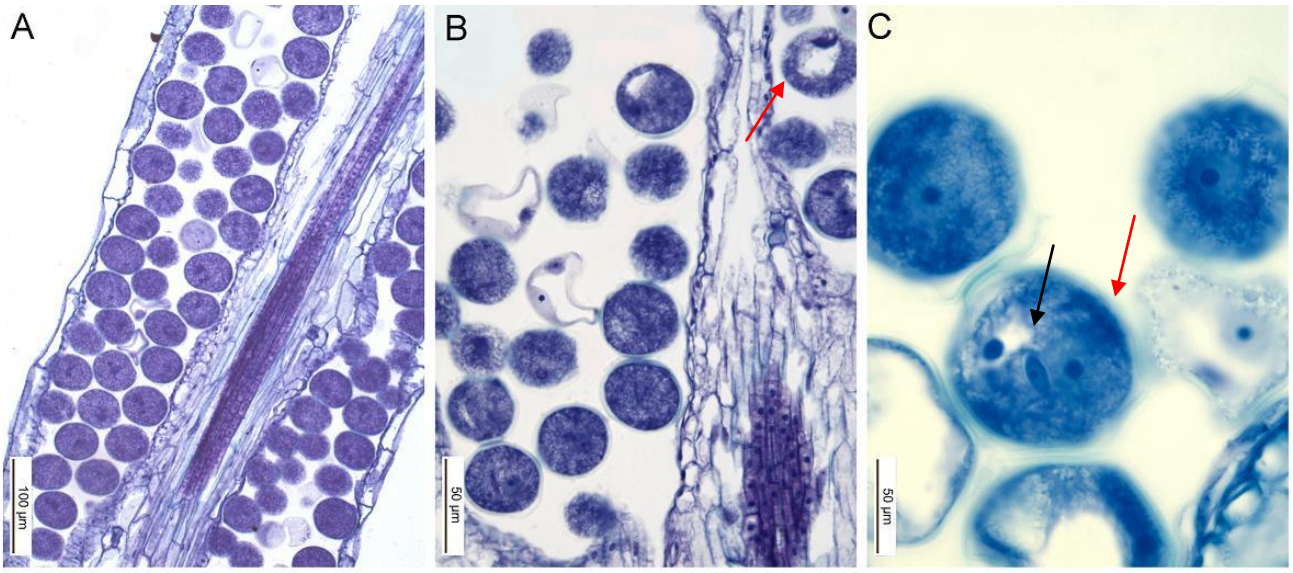

Figure 4: Photomicrographs of pollen grains from panicles of untreated check in transversal section. A: anther containing pollen grain of IRGA 422 CL cultivar, scale bar = 100 µm. B: detail of pollen grains from IRGA 422 CL cultivar, vacuolated grain (red arrow), scale bar = 50 µm. C: vegetative cells of pollen grain of IRGA 422 CL cultivar (black arrow) , sporoderm (red arrow), scale bar = 20 µm.

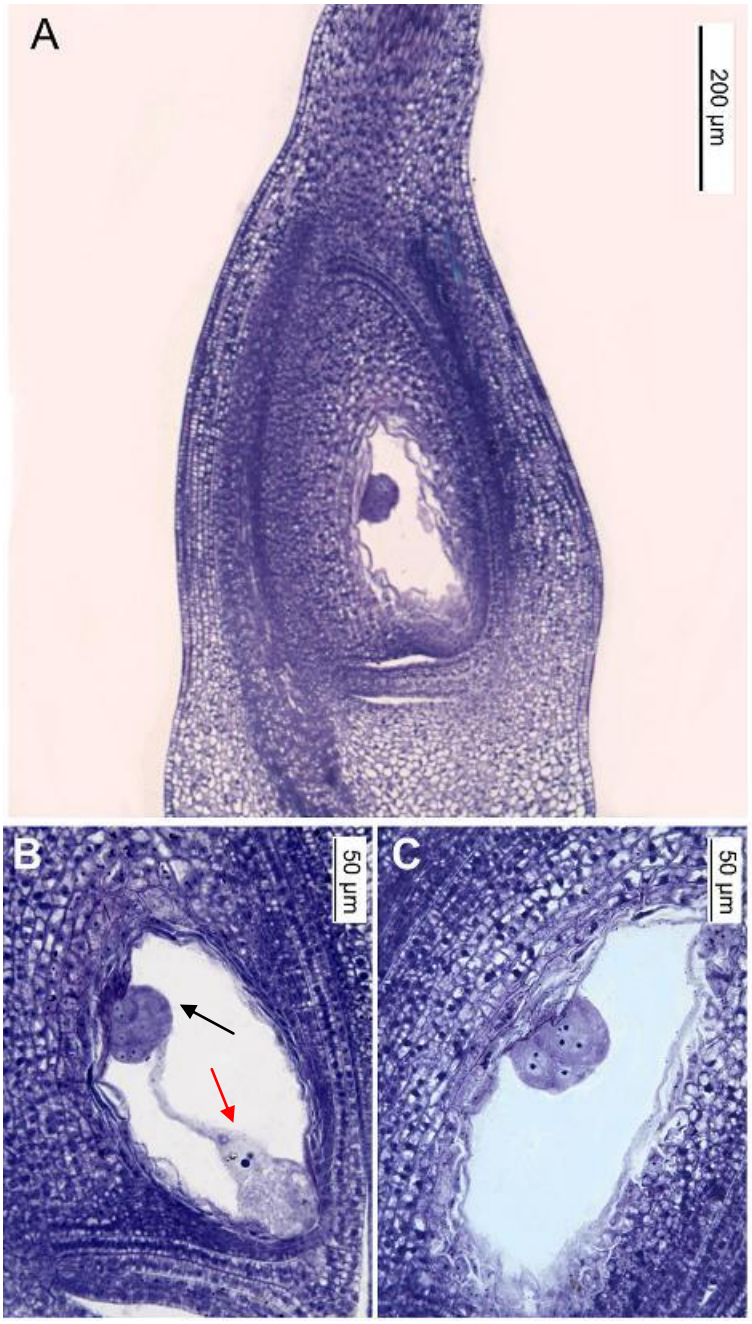

Figure 5: Photomicrographs of gynoecium and antipodes from floret of untreated check. A: general vision of gynoecium containing antipodes scale bar = 200 µm. B: antipodes and vegetative cell of IRGA 422 CL cultivar scale bar = 50 µm (black and red arrows, respectively). C: detail of antipodes of IRGA 422 CL cultivar, scale bar = 50 µm.

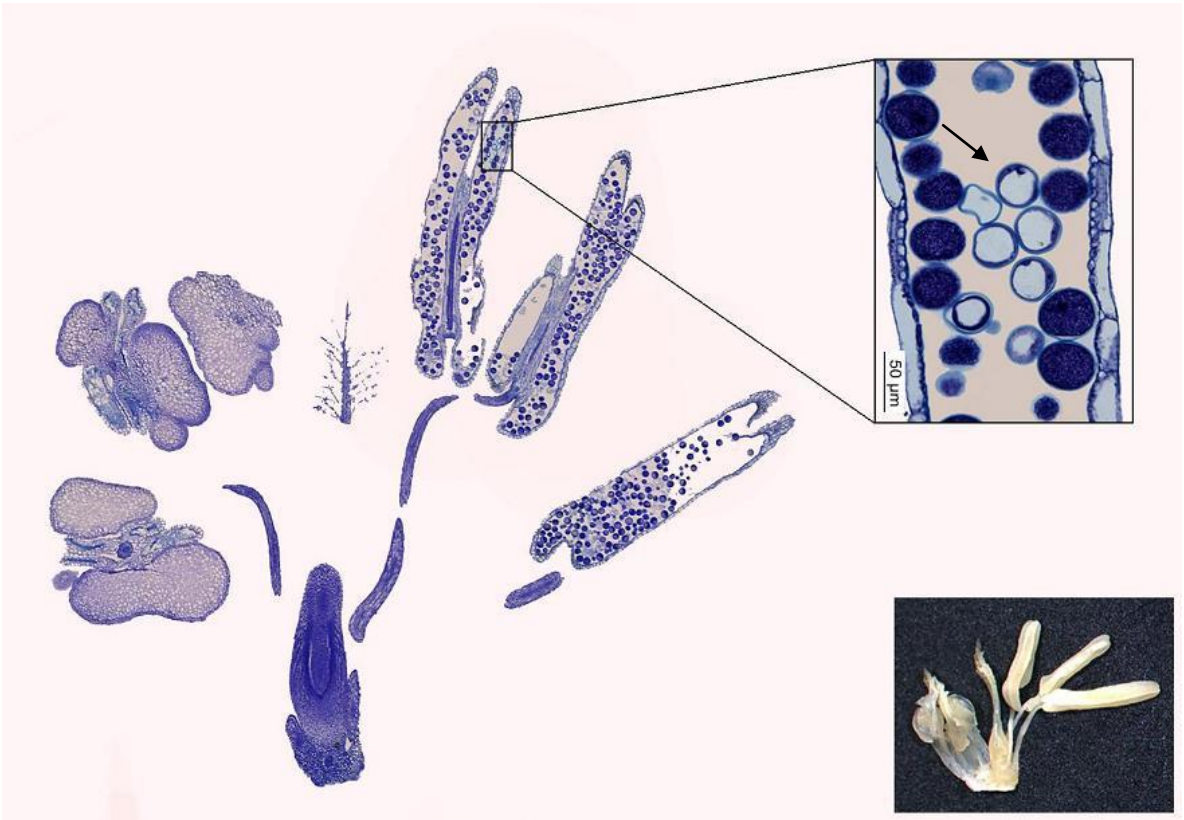

Figure 6: Photomicrographs of a floret from IRGA 422 CL submitted to imazamox applied in PD (T2 treatment), with a major detail of an anther containing pollen grain collapsed (black arrow), scale bar = 50 µm.

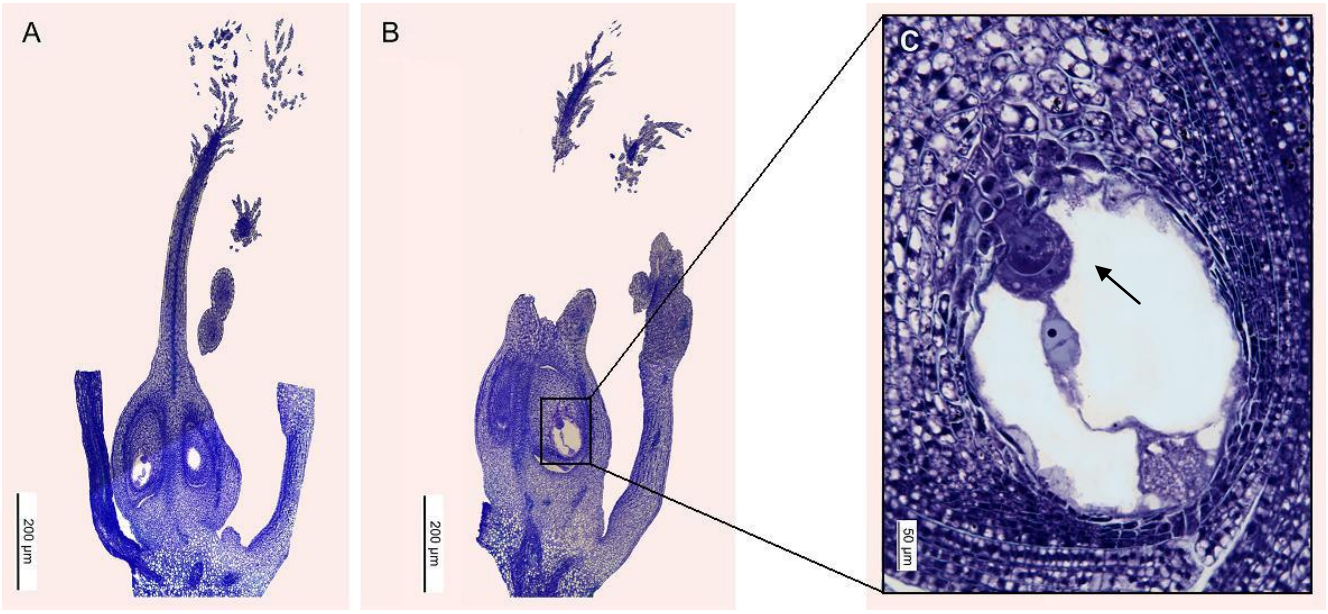

Figure 7: Photomicrographs of changes promoted by imazamox applied at PD (T2 treatment) in gynoecium from IRGA 422 CL. A and B: general vision of gynoecium connate, scale bar = 200 µm. C: detail of antipodes (black arrow) from IRGA 422 CL cultivar, scale bar = 50 µm.

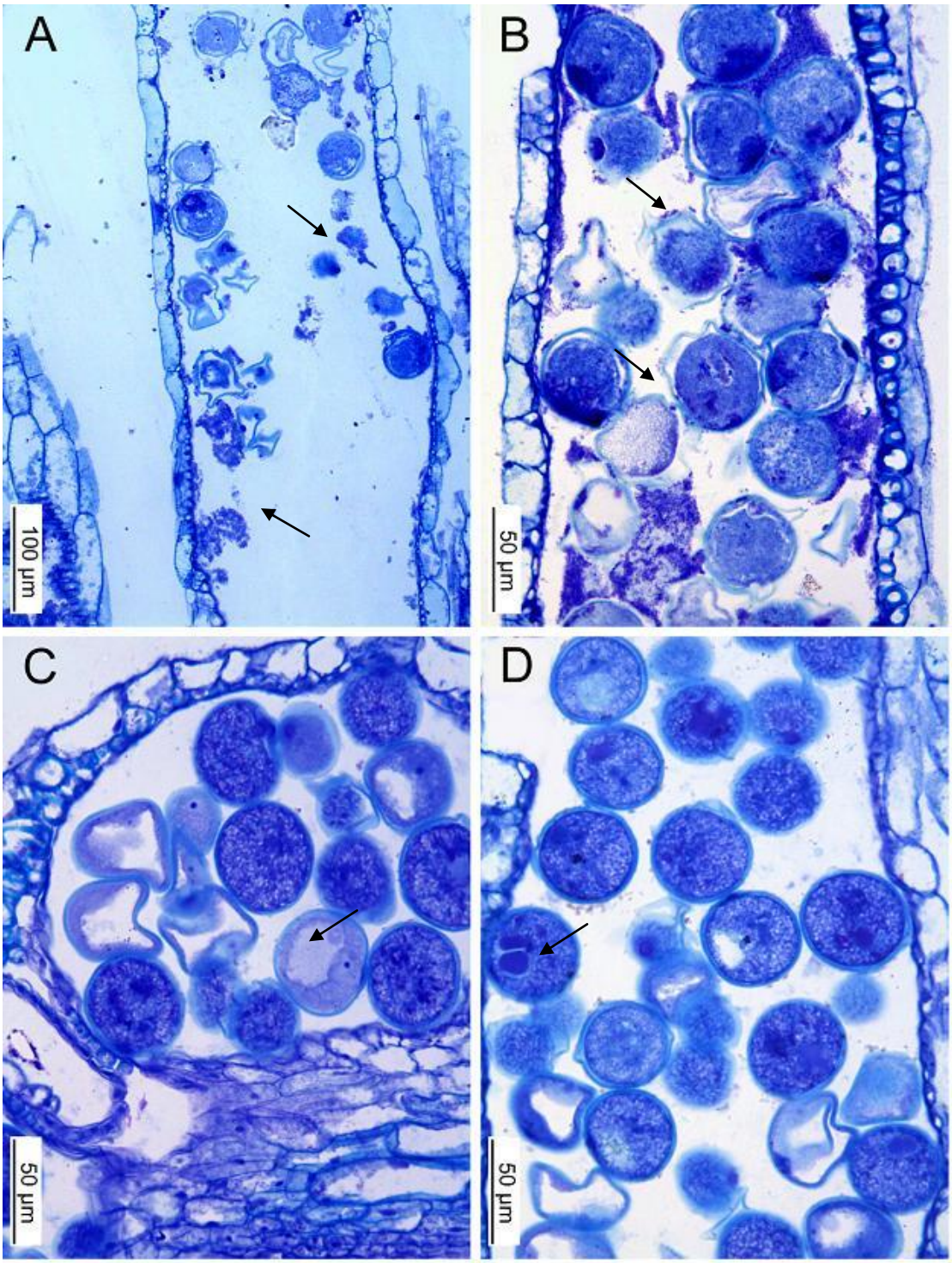

Figure 8: Photomicrographs of anthers from floret at T4 treatment. A: pollen grain debris of anthers from IRGA 422 CL cultivar, scale bar = 100 µm (black arrow). B:collapse of sporoderm, scale bar = 50 µm. C and D: phenolic compounds accumulated inside of pollen grain (black arrow) and vacuolated grain pollen (black arrow) from anther of IRGA 422 CL cultivar, respectively, scale bar = 50 µm.

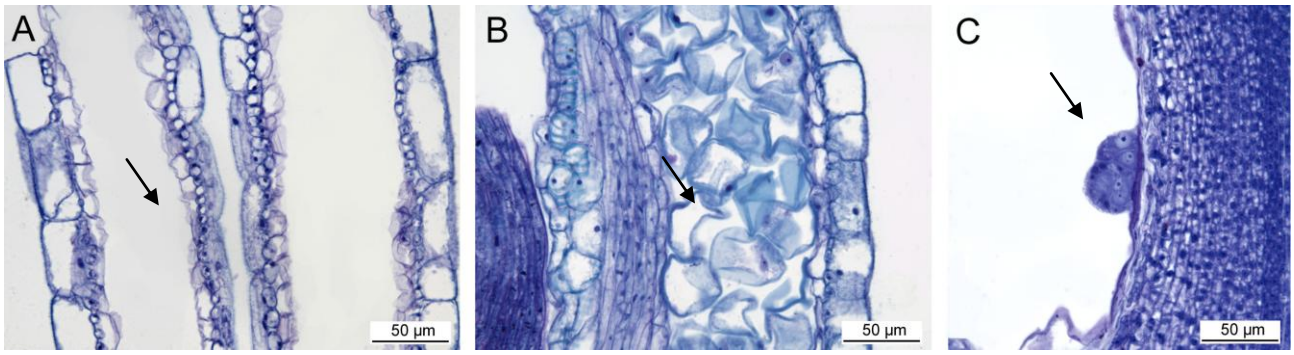

Figure 9: Photomicrographs of anthers and gynoecium from floret at T7 treatment. A: empty anthers, (black arrows), scale bar = 50 µm. B: pollen grains collapsed, (black arrow), scale bar = 50 µm. C: detail of normal antipodes (black arrow), scale bar = 50 µm.

DISCUSSÃO GERAL

Os resultados do presente trabalho permitem concluir que a explicação para a redução significativa na produção de grãos e a alta esterilidade de espiguetas observadas na cultivar IRGA 422 CL após a aplicação do herbicida imazamox na fase reprodutiva da cultura, é de uma complexidade muito maior do que se esperava.

Estudos conduzidos com arroz nos EUA demonstraram que após a aplicação de uma formulação contendo o herbicida imazamox em IPF (Iniciação do primórdio floral) não ocorreram alterações nos índices de produção de grãos da cultivar CL 161(MEINS et al., 2004). A cultivar CL 161 é uma cultivar resistente aos herbicidas inibidores da ALS e por isso este resultado pode estar relacionado ao grau de resistência da cultivar avaliada. O resultado do trabalho de Meins et al. (2004) também pode explicar porque não ocorre alteração deste parâmetro na cultivar PUITÁ INTA CL que possui maior grau de resistência aos herbicidas imidazolinonas em relação a cultivar IRGA 422 CL, ambas avaliadas no presente estudo.

Devido a isso, iniciaram-se estudos relacionados aos efeitos do herbicida imazamox aplicado na fase reprodutiva da cultura do arroz irrigado. Já era de conhecimento geral a existência de diferenças em relação ao nível de resistência entre as duas cultivares estudadas, contudo pouco se sabia sobre o efeito da aplicação deste herbicida sobre cada cultivar.

De maneira geral, a cultivar IRGA 422 CL apresentou alterações significativas em diversos parâmetros avaliados, ao contrário da cultivar PUITÁ INTA CL que não apresentou alterações após a aplicação da formulação contendo o herbicida, independente da época de aplicação. É válido ressaltar que as alterações observadas foram na maioria na estrutura reprodutiva, uma vez que a estrutura vegetativa foi pouco afetada tanto em relação aos parâmetros de crescimento quanto aos parâmetros bioquímicos (folha bandeira e penúltima folha expandida).

O efeito mais pronunciado se deu, em ambos os anos agrícolas (2009/10 e 2010/11) no qual ocorreu redução no rendimento de grãos da cultivar IRGA 422 CL,

em todos os tratamentos submetidos à aplicação do imazamox, quando comparado à testemunha (sem aplicação). Além disso, a esterilidade de espiguetas também foi um dos parâmetros que mais mostrou sensibilidade aos tratamentos. Uma vez que no ano agrícola de 2010/11 a esterilidade chegou a 86,9% em panículas coletadas ao término do cultivo e a 93,9 % quando a avaliação foi realizada em três porções da panícula aos 117 e 123 DAE. Este resultado demonstra o efeito do herbicida imazamox sobre o órgão reprodutivo, pois o elevado percentual de esterilidade de espiguetas observada no presente trabalho pode estar relacionado à redução do rendimento da cultivar também observada em todos os tratamentos. Possivelmente esse herbicida atua nos meristemas alterando a formação das estruturas reprodutivas, fato demonstrado pela alta percentagem de espiguetas estéreis.

No ano agrícola 2010/11, a aplicação do herbicida imazamox, com exceção do tratamento que recebeu a aplicação de 80 g i.a de imazamox na diferenciação do primórdio floral (T2), aumentou o número de dias da emergência à floração. Consequentemente, houve atraso na emissão de panículas. Esse atraso está relacionado ao surgimento de novas panículas dos nós inferiores da planta principal que fez com que houvesse atraso na duração do ciclo da cultura. A duração do ciclo da cultivar IRGA 422 CL no tratamento T2 foi a mesma do tratamento testemunha. Bond e Walker (2011) observaram um atraso na maturidade de cultivares e híbridos de arroz, ambos resistentes aos herbicidas ALS, após a aplicação de imazamox na fase reprodutiva da cultura. No presente estudo, o atraso na emissão de panículas pode ser explicado principalmente pela ação do herbicida imazamox no processo de divisão celular, retardando o crescimento das panículas que estavam em desenvolvimento. É importante ressaltar que a aplicação do herbicida imazamox, proporcionou assincronia do período de florescimento entre os tratamentos que receberam a aplicação de imazamox na cultivar IRGA 422 CL quando comparados à testemunha, dificultando assim o florescimento simultâneo. Além disso, ocorreu emissão de novas panículas provenientes de nós inferiores do colmo as quais surgiram com o avanço do ciclo da cultura em parcelas aspergidas com o herbicida. De forma semelhante, Menezes et al. (2007) encontraram que a maior supressão de panículas emitidas (70%) ocorreu quando o herbicida imazamox foi aspergido na fase do emborrachamento (R2) do arroz vermelho, sendo que 93% das espiguetas

das panículas emitidas eram estéreis. Nesse trabalho, os autores também relataram a ocorrência de emissão de novas panículas oriundas dos nós inferiores. Hensley (2009) também observou que as plantas tratadas com deriva de imazamox na diferenciação da panícula (R1) ou no emborrachamento (R2) apresentaram má formação de folhas e panículas. Além disso, foi observado o brotamento de novas plantas dos nós secundários do colmo principal. Já a cultivar PUITÁ INTA CL não mostrou atraso no desenvolvimento. Sugere-se que a explicação para a diferença de resposta entre as duas cultivares estudadas é a diferença genética oriunda das linhagens utilizadas para o desenvolvimento de cada cultivar. Quando a linhagem utilizada no cruzamento possui maior nível de resistência, como é o caso da linhagem utilizada no desenvolvimento da cultivar PUITÁ INTA CL, consequentemente essa cultivar possui maior resistência ao herbicida quando comparada a uma cultivar que não possui o mesmo gene da linhagem resistente (como é o caso da cultivar IRGA 422 CL estudada no presente trabalho).

Os herbicidas inibidores da ALS possuem como mecanismo de ação primário a paralisação do crescimento da planta devido à inibição da biossíntese dos aminoácidos valina, leucina e isoleucina. Porém as consequências secundárias desses herbicidas ainda não estão bem claras (COBB; READE 2010).

No presente trabalho, como já mencionado anteriormente, com exceção dos parâmetros bioquímicos, não foram observadas alterações na estrutura vegetativa da cultivar IRGA 422 CL. Os resultados bioquímicos de folhas e panículas da cultivar IRGA 422 CL sugerem que a redução de grãos e alta esterilidade de espiguetas não foram causadas pelo leve estresse oxidativo, mas houve uma combinação de fatores que proporcionaram esses resultados. Os resultados do presente estudo sugerem que o herbicida imazamox atua na região meristemática da estrutura reprodutiva da cultivar IRGA 422 CL.

No cultivo 2009/10, na cultivar IRGA 422 CL, foi observado em todos os tratamentos uma elevada viabilidade polínica (>83 %), porém no cultivo 2010/11 os tratamentos T4 e T7 promoveram redução da viabilidade de grãos de pólen na porção mediana da panícula, enquanto que o T2 promoveu redução na porção basal da panícula. Os demais tratamentos mostraram altos percentuais de viabilidade polínica. Através dos resultados observados nas avaliações anatômicas, foi possível

visualizar que os grãos de pólen analisados se encontravam em diferentes estágios de desenvolvimento. Estes resultados sugerem que o método de coloração utilizado na avaliação da viabilidade de grãos de polén não foi eficiente, uma vez que superestimou os resultados de percentual de viabilidade polínica.

A avaliação da morfologia externa das panículas e flores de arroz da cultivar IRGA 422 CL revelou que no tratamento testemunha foram observadas todas as características morfológicas já descritas na literatura (TAKEOKA et al., 1993; FORNARA et al., 2003; ITOH et al., 2005; YOSHIDA; NAGATO, 2011). Porém, nos tratamentos avaliados (T2, T4 e T7) foram observadas alterações na coloração, número e morfologia das flores.

As avaliações anatômicas comprovaram a existência das alterações no órgão reprodutivo da cultivar IRGA 422 CL. De modo geral, tanto o gineceu quanto o androceu mostraram colapso na sua estrutura.

As plantas que receberam a aplicação de imazamox na diferenciação do primórdio floral (T2) mostraram transformações parciais ou totais de estames em carpelos. Estas alterações nos órgãos reprodutivos são semelhantes às alterações homeóticas observadas em Arabidopsis e em plantas de arroz mutante (JACK et al., 1994;. ZHANG et al., 2007; YOSHIDA; NAGATO, 2011). As alterações apresentadas neste tratamento (T2) indicam que ocorreu colapso da estrutura reprodutiva após a aplicação do imazamox e essas alterações contribuíram com a significativa redução na produção de grãos da cultivar IRGA 422 CL.

Nas anteras de flores que receberam a aplicação de imazamox em DPF + 14 dias (T4), foi observado o colapso dos grãos de pólen. Porém, não foram observadas alterações nos carpelos e óvulos destas flores. Apesar da redução na produção de grãos ter sido diminuída significativamente neste tratamento, esse resultado pode estar relacionado à época de aplicação do imazamox, que foi mais tardia quando comparada com o T2 que foi na diferenciação do primórdio floral.

As plantas que receberam a dose fracionada (T7), metade da dose na diferenciação da panícula (PD) + 7 dias e a outra metade em PD + 14 dias, mostraram que o androceu apresentou estrutura colapsada quando comparada ao tratamento testemunha. O colapso da estrutura dos grãos de pólen e a ocorrência de anteras completamente vazias sugere que essas alterações também foram

responsáveis pela redução na produção de grãos e elevada esterilidade de espiguetas na cultivar IRGA 422 CL. Nesse tratamento não foram observadas alterações na gametogênese, onde o estágio inicial da fertilização foi observado.

CONCLUSÃO GERAL

A aplicação do herbicida imazamox na fase reprodutiva da cultura do arroz irrigado causa redução na produção de grãos da cultivar IRGA 422 CL devido a um processo complexo de combinação de fatores. A cultivar PUITA INTA CL não sofre alterações nos parâmetros avaliados no presente estudo.

A aplicação do herbicida imazamox na diferenciação da panícula causa alterações semelhantes às alterações homeóticas em plantas de arroz mutante. As alterações morfológicas e anatômicas observadas na estrutura reprodutiva da cultivar IRGA 422 CL em todos os tratamentos avaliados causam redução na produção de grãos e alta esterilidade de espiguetas desta cultivar.

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APÊNCICE

Apêndice A

Tabela: Tratamentos compostos de diferentes épocas de aplicação fracionada ou total herbicida imazamox em duas cultivares de arroz irrigado.

Época	**DPF[1]**	**DPF + 7 dias[2]**	**DPF + 14 dias[3]**
T1	0	0	0
T2	80 g i.a ha^{-1}	0	0
T3	0	80 g i.a ha^{-1}	0
T4	0	0	80 g i.a ha^{-1}
T5	40 g i.a ha^{-1}	0	40 g i.a ha^{-1}
T6	40 g i.a ha^{-1}	40 g i.a ha^{-1}	0
T7	0	40 g i.a ha^{-1}	40 g i.a ha^{-1}

[1] Diferenciação do primórdio floral
[2] Data da diferenciação do primórdio floral mais 7 dias.
[3] Data da diferenciação do primórdio floral mais 14 dias.

VITA

Bibiana Silveira Moraes é filha de Jose Henrique Machado Moraes e Angelisa Silveira Moraes, nasceu em 26 de agosto de 1981, no município de Tupanciretã, Rio Grande do Sul. No ano de 2001 ingressou no curso de Agronomia junto a Universidade Federal de Santa Maria, através do vestibular. Em outubro de 2006 colou grau, recebendo o título de Engenheira Agrônoma. Durante a graduação, iniciou sua vida científica como estagiária do Departamento de Química, com ênfase em Bioquímica Toxicológica, logo após tornou-se bolsista de iniciação científica FAPERGS e PIBIC. Em 2007, iniciou o curso de mestrado no Programa de Pós-Graduação em Bioquímica Toxicológica da Universidade Federal de Santa Maria/RS, concluindo em agosto de 2008. Após a conclusão do mestrado, ingressou no curso de doutorado no Programa de Pós-Graduação em Agronomia da mesma Instituição, concluindo em março de 2013.