Universidade Estadual de Londrina

RENATA PINHEIRO SOBOTTKA

MODULADORES NATURAIS NA PROTEÇÃO GENÉTICA DE TRIGO (*Triticum turgidum* L. subsp. *durum*) CONTRA *Fusarium* spp. POR TÉCNICA MOLECULAR E LC-MS. APLICAÇÃO DE TECNOLOGIA NIR NA PREDIÇÃO DE COMPOSIÇÃO QUÍMICA DE MILHO (*Zea mays* L.)

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, nível Mestrado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Ciência de Alimentos.

Orientadora: Profa. Dra. Elisa Yoko Hirooka.
Co-Orientador: Dr. Douglas Fernandes Barbin.

Londrina
2014

Catalogação elaborada pela Divisão de Processos Técnicos da Biblioteca Central da Universidade Estadual de Londrina

Dados Internacionais de Catalogação-na-Publicação (CIP)

S677m Sobottka, Renata Pinheiro.
Moduladores naturais na proteção genética de trigo (*Triticum turgidum* L. subsp. *durum*) contra *Fusarium* spp. por técnica molecular e LC-MS. Aplicação de tecnologia NIR na predição de composição química de milho (*Zea mays* L.) / Renata Pinheiro Sobottka. – Londrina, 2014.
102 f. : il.

Orientador: Elisa Yoko Hirooka.
Coorientador: Douglas Fernandes Barbin.
Dissertação (Mestrado em Ciência de Alimentos) – Universidade Estadual de Londrina, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência de Alimentos, 2014.
Inclui bibliografia.

1. Alimentos – Microbiologia – Teses. 2. Alimentos – Análise – Teses. 3. Trigo – Teses. 4. Milho – Teses. 5. Tricotecenos – Teses. 6. Cereais como alimento – Teses. I. Hirooka, Elisa Yoko. II. Barbin, Douglas Fernandes. III. Universidade Estadual de Londrina. Centro de Ciências Agrárias. Programa de Pós-Graduação em Ciência de Alimentos. IV. Título: Aplicação de tecnologia NIR na predição de composição química de milho (*Zea mays* L.). V. Título.

CDU 641:579

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Londrina, 25 de fevereiro de 2014.

Dedico a todos que me apoiaram e incentivaram, em especial à minha família, meu namorado Eduardo e ao meu avô Pinheiro (in memoriam).

AGRADECIMENTOS

À UEL – Universidade Estadual de Londrina e ao Programa de Pós-Graduação em Ciência de Alimentos por oferecer as condições que possibilitaram a concretização desse mestrado.

Á MycSA – unidade de Micologie e Securité des Aliments, do Institut Nationale de Recherche Agronomique (INRA), Bordeaux-Aquitaine, pela oportunidade e condições de trabalho.

À CAPES, pelo fornecimento da bolsa de auxílio.

À minha orientadora Profa. Dra. Elisa Yoko Hirooka, por todos os ensinamentos a mim desprendidos desde o início da graduação, pela amizade, apoio, paciência, disponibilidade, incentivo e pela constante orientação durante a realização deste trabalho.

Ao meu co-orientador Dr. Douglas Fernandes Barbin, da UEL, pelas valiosas contribuições e ensinamentos, pelo incentivo, apoio e auxílio quanto à tecnologia NIR, sendo fundamental nesta etapa final.

Às minhas orientadoras francesas, Laetitia Pinson-Gadais e Vessela Atanasova-Penichon, unidade MycSA, INRA, pela oportunidade de trabalho, pelos ensinamentos e contribuições neste trabalho.

À pesquisadora Dra. Martha Zavariz Miranda, da Embrapa Trigo, pela participação na banca de defesa.

Ao Dr. Wagner Ezequiel Risso, da UEL, pela disponibilização de amostras de milho, pela ajuda, paciência e pela participação na banca de defesa.

Ao Prof. Claudemir Zucareli, da UEL, por ter cedido amostras de milho para realização deste trabalho.

Ao Prof. Ricardo Ralisch, da UEL, pela ajuda e incentivo para realização de intercâmbio na França.

Aos docentes do Programa de Pós-graduação em Ciência de Alimentos da Universidade Estadual de Londrina pelos ensinamentos adquiridos.

À toda a minha família, em especial meus pais Tânia e Orlando, meus irmãos Fernanda e Vitor pelo amor, apoio, carinho, confiança e incentivo.

Ao meu namorado Eduardo pela motivação, carinho, paciência e pelo companheirismo sempre. À toda sua família pelos momentos de alegria, carinho e apoio.

À todos da unidade MyCSA/INRA, em especial à Christine Ducos e à Véronique Pronier, pela amizade, pelos momentos de descontração e pelo auxílio, e ao Carlos e Pilar pela apoio e amizade durante a estadia na França.

A todos os colegas do Laboratório do DCTA/UEL: Cássia Takabayashi, Ana Lúcia Felício, Cleberson, Cintia Handa, Marsilvio Filho, Neusa Cassula, Juliana Almeida pela convivência, pela ajuda e pelos os momentos de descontração.

A todos aqueles que embora não citados participaram de uma alguma forma na realização desse trabalho.

SOBOTTKA, Renata Pinheiro. **Moduladores naturais na proteção genética de trigo (*Triticum turgidum* L. subsp. *durum*) contra *Fusarium* spp. por técnica molecular e LC-MS. Aplicação de tecnologia NIR na predição de composição química de milho (*Zea mays* L.).** 2014. 102 p. Dissertação (Mestrado em Ciência de Alimentos) – Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

O trigo (*Triticum* spp.) e o milho (*Zea mays* L.), pertencentes à Família *Poaceae*, são cereais fundamentais na alimentação mundial devido à importância socioeconômica, sendo amplamente utilizados para o consumo humano e animal. O trabalho avaliou o desenvolvimento fúngico, a produção de tricoteceno (desoxinivalenol, DON) e de moduladores naturais no contexto de proteção genética em cultivares de trigo. Em paralelo, foi avaliada a tecnologia NIR na predição de atividade de água, teor de proteína, umidade e cinzas em milho, visando aplicação de método rápido, capaz de subsidiar a técnica clássica (Capítulo 2). O capítulo 1 foi executado na França e consistiu em estudo com quatro cultivares de trigo com diferente sensibilidade à fusariose, procedendo com e sem inoculo de *Fusarium culmorum*, seguida de análise de grão em dois estágios de desenvolvimento (cinco dias após inoculação e na maturação). A análise em grãos de trigo consistiu na detecção de DON, composição em ácidos fenólicos (solúveis e ligados à parede celular) por LC/MS e determinação do gene *Tri5* por PCR em tempo real. O cultivar de trigo resistente limitou significativamente o desenvolvimento de *Fusarium culmorum* e a contaminação por DON ($p<0,05$). Glumas de trigo de cultivar resistente apresentaram-se ricas em ácido clorogênico e flavonas, sugerindo participação na resistência contra *Fusarium spp.* e produção de DON. Estudo em moduladores naturais com potencial inibidor na biossíntese de tricotecenos deve ser prosseguido visando inocuidade de alimentos, enfocada também na minimização de agrotóxicos. O capítulo 2 avaliou a aplicação da tecnologia NIR na predição de atividade de água (a_w), teor de proteína, umidade e cinzas de milho cultivado sob quatro diferentes populações de planta (densidade, 60, 75, 90 e 105 mil plantas ha^{-1}) sob cinco doses de nitrogênio (0, 60, 120, 180 e 240 kg ha^{-1}). O teor de proteína em milho cultivado sob mesma densidade de planta aumentou com os níveis de adubação (dose de nitrogênio, $p<0,05$). No entanto, não se observou diferença significativa entre grãos de milho tratado com a mesma dose de nitrogênio, mas com diferente densidade de planta ($p<0,05$). As amostras de milho foram digitalizadas na faixa do visível / NIR de 400 – 2500 nm. O melhor coeficiente de predição foi obtido para a proteína (R^2_{cv}=0,90), revelando uma forte relação entre informação espectral e teor de proteína. Os modelos para três parâmetros (proteína, a_w e umidade) apresentaram a razão de erro padrão de calibração e desvio padrão (RPD) entre 2,4 e 4,2. Com exceção de cinzas, os modelos obtidos apresentam perfil adequado para o rastreio de constituintes – proteína, a_w e umidade – em milho, no contexto de monitoramento rápido em planta de processamento industrial.

Palavras-chave: *Triticum* spp., *Zea mays* L., compostos fenólicos, mecanismos naturais de defesa, contaminação fúngica, tricotecenos, PCR em tempo real, modelo preditivo, fertilização nitrogenada, proteína.

SOBOTTKA, Renata Pinheiro. **Natural modulators in genetic protection of wheat (*Triticum turgidum* L. subsp. *durum*) against *Fusarium* sp. evaluated by molecular analysis and LC-MS. Application of NIR technology for prediction of chemical composition in maize (*Zea mays* L.).** 2014. 102 pages. Dissertation (Master Degree in Food Science) – State University of Londrina, Londrina, 2014.

ABSTRACT

Wheat (*Triticum* sp.) and maize (*Zea mays* L.), belonging to the *Poaceae* family, are important cereals on the global socio-economy, being widely used for human and animal consumption. The study evaluated the fungal growth and trichothecene production (deoxynivalenol, DON) in wheat cultivars on the genetic protection context (Chapter 1). In parallel, it was evaluated the application of NIR technology for prediction of water activity, protein, moisture and ash contents in maize, seeking the application of this rapid method being capable for subsidizing the classical technique (Chapter 2). Chapter 1 was executed in France and evaluated four wheat cultivars with different sensitivity to *Fusarium* infection, with and without inoculum of *Fusarium culmorum*, followed by analysis of grains in two stages of development (five days after inoculation and at maturity stage). The analysis of wheat grains was the determination of DON, phenolic acids composition (soluble and bound to the cell wall) by LC/MS and the Tri5 gene determination by real-time PCR. The resistant wheat cultivar significantly limited the development of *Fusarium culmorum* and DON contamination ($p < 0.05$). Glumes of resistant wheat cultivar showed high content on chlorogenic acid and flavones, suggesting involvement on resistance against *Fusarium* sp. and DON production. Study on natural modulators with potential inhibitor on the biosynthesis of trichothecenes should be pursued in order to ensure food safety, also focused on reduction of pesticide application. Chapter 2 evaluated the application of NIR technology for prediction of water activity (aw), protein, moisture and ash contents in maize from four different plant density (60, 75, 90 and 105 thousand plants ha^{-1}) with five doses of nitrogen (0, 60, 120, 180 and 240 kg ha^{-1}). Maize samples from the same plant density had their protein content increased with levels of fertilization (doses of nitrogen, $p < 0.05$). However, no significant difference was observed among maize treated with the same dose of nitrogen, but with different plant density ($p < 0.05$). The maize samples were scanned in the visible / NIR range of 400 - 2500 nm. The best coefficient of prediction was obtained for protein (R^2_{cv}=0.90), which revealed a strong relationship between spectral information and protein content. The models for three parameters (protein, moisture and aw) showed the ratio of standard error of calibration and standard deviation (RPD) between 2.4 and 4.2. Except for ash, the models obtained showed satisfactory for screening purposes of constituents - protein, a_w and moisture - in maize, under the context of rapid monitoring of industrial processing plant.

Keywords: *Triticum* sp., *Zea mays* L., phenolic compounds, natural defense mechanisms, fungal contamination, trichothecenes, Real-Time PCR, predictive model, nitrogen fertilization, protein.

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LISTA DE ABREVIATURAS E SIGLAS

AC.VAN	*Vanillic Acid*
ADON	*Acetil-Deoxinivaleonol*
ANOVA	*Analysis of Variance*
a_w	*water activity*
Benz	*Benzoic Acid*
CHLO	*Chlorogenic Acid*
CL	Concentração Letal
CONAB	Companhia Nacional de Abastecimento
DAD	*Diode Array Detector*
DiFA	*Dímero de Ácido Ferúlico*
DNA	*Deoxyribonucleic Acid*
DON	*Desoxinivaleonol*
Embrapa	Empresa Brasileira de Pesquisa Agropecuária
FER	*Ferulic Acid*
FPP	*Farnesyl Pyrofosfato*
HPLC	*High Performance Liquid Chromotografy*
IAPAR	Instituto Agronômico do Paraná
INRA	*Institut National de la Recherche Agronomique*
LC	*Liquid Chromatografy*
LMT	Limites Máximos Tolerados
LOOCV	*Leave-One-Out Cross-Validation*
LV	*Latent Variables*
MS	*Mass Spectrometry*
MycSA	*Mycologie et Sécurité des Aliments*
N	Nitrogênio
NIR	*Near Infrared*
PCA	*Principal Component Analysis*
P-COUM	*Coumaric Acid*
PCR	*Polymerase Chain Reaction*
PDA	*Potato Dextrose Agar*
PLS-R	*Partial Least Squares Regression*
RDC	Resolução da Diretoria Colegiada

RER	*Ratio of Error Range*
RMSEC	*Root Mean Square Error of Calibration*
RMSECV	*Root Mean Square Error of Cross-Validation*
RMSEP	*Root Mean Square Error of Prediction*
RPD	*Residual Predictive Deviation*
RT	*Real time*
SIN	*Sinapic Acid*
UEL	Universidade Estadual de Londrina
USDA	United States Department of Agriculture
UV	Ultravioleta
UV-VIS	Ultravioleta-visível

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1 INTRODUÇÃO

O trigo (*Triticum* spp.) e o milho (*Zea mays* L.), pertencentes à Família *Poaceae* (Gramíneas), são cereais fundamentais na alimentação mundial devido à importância socioeconômica, sendo amplamente utilizados para o consumo humano e animal.

Com ênfase à fonte de carboidrato, proteína e lipídio na alimentação, a produção de grãos é definida em função de uma série de fatores baseados em cultivar, quantidade de insumos, manejo, condição ambiental, pragas, contaminação microbiana e micotoxinas, capazes de interferir no rendimento e na qualidade de grãos. Portanto, o monitoramento deve abranger toda cadeia produtiva visando segurança de alimento destinado à alimentação humana e animal.

A proteção genética através de cultivar resistente tem sido alternativa mais procurada no controle de fusariose e, conseqüente produção de micotoxinas, com destaque a tricotecenos em grãos de trigo, em especial, o desoxinivalenol (DON). Os compostos fenólicos presentes em todas as plantas desempenham importante papel no mecanismo natural de defesa e resistência, constituindo-se componente natural de parede celular, antimicrobiano ou molécula sinal em resposta à infecção. Estudos sugerem uma relação entre o conteúdo de ácido fenólico (ácido ferúlico e ácido coumárico) em grãos e o nível de resistência à fusariose em trigo (MCKEEHEN *et al.*, 1999; SIRANIDOU *et al.*, 2002).

Outros fatores que influenciam o rendimento e a qualidade de grãos são o manejo da adubação e o arranjo de plantas (densidade e espaçamento). A adubação nitrogenada influi positivamente na produtividade de grãos na cultura de milho, como também aumenta o índice de área foliar, massa de 1.000 grãos, altura de planta, rendimento de biomassa e índice de colheita. A densidade de plantas tem papel fundamental no rendimento da cultura de milho, com pequenas variações influenciando no rendimento final, devido à interferência na interceptação de radiação solar e distribuição de sistema radicular no solo.

O trabalho, dividido em dois capítulos, visa contribuir na cadeia produtiva de trigo (moduladores naturais na proteção genética) e milho (avançar na tecnologia NIR aplicada no controle de qualidade), sendo apresentado em: (i) Capítulo 1: Estudo em grãos obtidos de cultivares de trigo com diferente sensibilidade à fusariose, quantificando o gene codificador da enzima tricodieno

sintetase *(Tri5)* por técnica molecular (PCR em tempo real) e produção de DON / moduladores naturais - ácidos fenólicos (LC/MS). (ii) Capítulo 2: tecnologia NIR na predição de atividade de água, teor de proteína, umidade e cinzas em milho cultivado sob diferente densidade de planta e dose de nitrogênio, visando aplicação de método rápido capaz de subsidiar as respectivas técnicas clássicas.

2 OBJETIVO

2.1 Objetivo Geral

Avaliar o desenvolvimento fúngico e a produção de micotoxinas em cultivares de trigo com sensibilidade diferente à fusariose, bem como estudar os mecanismos naturais de defesa. Além disso, avaliar o emprego da tecnologia NIR na predição de atividade de água, teor de proteína, umidade e cinzas em milho cultivado sob diferentes doses de nitrogênio em cobertura e densidade de plantas.

2.2 Objetivos Específicos

- Identificar componentes naturais moduladores da produção de micotoxinas em grãos de trigo por LC/MS.
- Avaliar a influência de compostos fenólicos moduladores da biossíntese de micotoxinas produzidas por *Fusarium* spp. em grãos de trigo.
- Quantificar o desenvolvimento de *Fusarium* spp. por PCR quantitativo do gene *Tri 5*, implicado na via biosintética dos tricotecenos.
- Avaliar o efeito de diferentes doses de nitrogênio e população de planta nos parâmetros físico-químicos de grãos de milho.
- Comparar os métodos tradicionais com a tecnologia NIR na determinação de atividade de água, proteína, umidade e cinzas em milho.

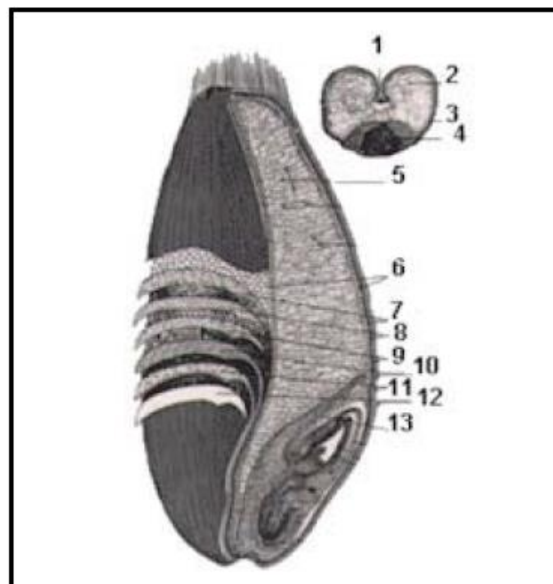
3 REVISÃO DE LITERATURA

3.1 A CULTURA DO TRIGO

O trigo (*Triticum* spp.) pertence à Família Poaceae (Gramíneas), sendo um cereal fundamental na alimentação mundial. *Triticum aestivum* L. é a espécie de trigo mais cultivada em todo o mundo, com *Triticum turgidum* subsp. *durum* a segunda mais importante, destinada para fabricação de massas. Uma das culturas mais antigas no mundo utilizado para o consumo humano, o trigo é originário do Sudoeste Asiático, 10.000 anos a. Ca., sendo introduzido na Índia, China e Europa (OSÓRIO, 1982).

O grão de trigo tem forma oval, sendo sua estrutura dividida em três regiões: gérmen, pericarpo e semente (Figura 1). O gérmen é o embroão de uma nova planta, rico em açúcares e lipídeos, correspondendo a 3% do grão. O pericarpo é a casca ou invólucro protetor da semente, é rico em celulose e correspondente à 18% do grão. E a semente é constituída pela cobertura e endosperma rico em amido e proteínas, sendo a região da qual é extraída a farinha de trigo (NIEVINSKI, 2009).

Figura 1 - Partes do grão de trigo



1-crease, 2-endosperma, 3-farelo, 4-germe, 5-endosperma, 6-aleurona, 7-hialina, 8-testa, 9-células tubulares, 10-células cruzadas, 11-hipoderme, 12-epiderme, 13-germe

Fonte: NIEVINSKI (2009)

É uma cultura de grande importância socioeconômica, com a maior área de cultivo, ocupa-se a segunda posição em quantidade de grãos produzida no mundo. Os principais produtores de trigo nos últimos anos são União Européia, China, Índia, Estados Unidos e Rússia (ABITRIGO, 2011). A área ocupada com o cultivo de trigo *durum* representa somente 8% da área total semeada com trigo e esta espécie não é cultivada no Brasil. A produção brasileira de *T. aestivum* em 2013 foi mais de 5,3 milhões de toneladas de trigo, sendo 22,4% superior à safra anterior. A média de rendimento das lavouras brasileiras de trigo foi de 2.445 quilos por hectare (kg ha^{-1}), sendo o aumento da produtividade em relação à safra 2012 de 45% (CONAB, 2013). Este crescimento foi resultado tanto do cultivo em áreas não tradicionais, quanto no maior investimento do produtor em tecnologias, como cultivares mais resistentes, adubação e defensivos.

A produção da cultura é definida em função de uma série de fatores como, cultivar, quantidade de insumos, especialmente nitrogênio, técnica de manejo (TEIXEIRA FILHO *et al.,* 2007), condição edafoclimáticas, pragas, contaminação microbiana e micotoxinas.

Conforme Reis, Casa e Medeira (2001) os impactos ambientais e climáticos na cultura do trigo, em âmbito geral, acenam para aumento de temperatura e redução de precipitações diárias. Alterações nesses parâmetros devem favorecer a redução da ocorrência e da severidade de varias doenças de cereais de inverno, refletindo na redução dos danos e dos custos de produção, e melhor qualidade de grãos. Em decorrência de alteração climática, novas estratégias deverão ser adotadas visando sustentabilidade no cultivo de trigo, bem como qualidade e segurança alimentar.

3.1.1 Fusariose ou Giberela (*Fusarium* spp.)

A cultura de trigo é afetada por diversas doenças que limitam o rendimento e a qualidade de grãos, com destaque à furasiose (giberela). O principal agente causal é *Gibberella zeae* (Schw.) Petch, forma anamórfica *Fusarium graminearum* (Schwabe), embora outras espécies sejam importantes em determinadas regiões, como *Fusarium culmorum* (W.G. Smith) Sacc. e *Gibberella avenacea* Cook, forma anamórfica *Fusarium avenaceum* (Corda ex Fries)

(BOTTALICO & PERRONE, 2002). Angelotti *et al.* (2006) identificaram *F. graminearum* como principal agente de giberela em trigo no sul do Brasil.

De ocorrência generalizada no mundo, a transmissão da doença ocorre pela semente, sendo os danos manifestados em região tritícola úmida e quente, com nível elevado de precipitação (acima de 48 h de molhamento) no estádio de floração e de maturação do trigo (REIS, 1990; BERGSTROM, 1993; DEL PONTE *et al*., 2004). A disseminação de patógeno decorre de conídios transportados em respingos de chuva a curtas distâncias, com período de suscetibilidade entre o inicio de floração e início de maturação (REIS & CASA, 2007). Lima (2004) enfatiza as epidemias mais recentes de giberela registradas em anos de elevada precipitação pluvial na região sul do Brasil.

A intensidade da fusariose é altamente dependente das condições climáticas, com as epidemias variando de ano para ano (CASA *et al*., 2004). Os períodos de exposição em umidade contínua favorecem a infecção, bem como dias quentes e úmidos, umidade relativa acima de 70% e temperatura elevada superior a 16 °C favorecem o estabelecimento e progresso da infecção por *F. graminearum* em cultivares de trigo no estágio de floração (CLEAR & PATRICK, 1990).

Além de fator climático, provavelmente os resíduos culturais contribuem para o aumento do inóculo e a sobrevivência do patógeno entre as estações de cultivo, exercendo uma maior pressão de inóculo (SCHAAFSMA *et al*., 2001), já que o fungo sobrevive nas sementes e restos culturais de inúmeras gramíneas (REIS & CASA, 1997). Sendo um patógeno com habilidade saprofítica, práticas culturais como rotação de culturas não conduzem a resultado satisfatório, devido ao elevado número de hospedeiros que permitem a sobrevivência do patógeno entre cultivo do trigo ou de outros cereais (REIS & CASA, 1997; RIVADENEIRA, 2001). A partir da década de 90, com a adoção e difusão do sistema plantio direto em grandes áreas cultivadas, a fusariose aumentou de intensidade, não somente no trigo, como em aveia (*Avena sativa* L.), em cevada (*Hordeum vulgare* L.) e em triticale (*Triticum secalotricum* Meister) (REIS; CASA; MEDEIRA, 2001).

Em condição climática favorável, os sinais do patógeno são facilmente observados em espiguetas afetadas com coloração rosa-salmão; essas espiguetas atacadas formam grãos denominados giberelados, com características físicas de chocho, enrugado e de coloração branco-rosada a pardo-clara (LIMA,

2002). O fungo também pode colonizar o sistema radicular e porções basais da planta, causando necrose de tecidos e morte de plântula (SUTTON, 1982). Além de dano direto, com redução do rendimento de grãos, observa-se também perda na qualidade dos grãos infectados e colonizados pelo fungo, associado à produção de micotoxinas (LIMA, 2004).

As estratégias de controle da fusariose baseiam-se no desenvolvimento de cultivares resistentes e uso de fungicidas aplicados na parte aérea durante a fase de floração. Embora a aplicação de fungicidas seja preconizada, dificuldades residem no fato em conhecer o momento ideal de aplicação e na tecnologia utilizada para obtenção de resultados satisfatórios (REIS; CASA; MEDEIRA, 2001; DEL PONTE *et al.,* 2004). Com relação a variedades resistentes, constam poucas informações sobre fontes de resistência genética, sendo a introdução destes genes em materiais de qualidade tecnológica, uma tarefa difícil e de longo prazo (LIMA; FERNANDES; PICININI, 2000).

3.1.2 Resistência à *Fusarium* spp.

A proteção genética empregando cultivares resistentes é uma das alternativas mais procuradas para o controle da enfermidade. O grau de resistência de diferentes cultivares é variável, com a resistência em genótipos extremamente diferentes quanto as demais características de interesse dos melhoristas (OSORIO *et al.,* 1998). Gervais *et al.* (2003), avaliando grandes coleções mundiais de trigo principalmente na China, Japão e Estados Unidos, têm identificado poucas fontes de resistência ao gênero *Fusarium*. As fontes de resistência mais conhecidas são provenientes de três origens: Trigo de primavera da Ásia (Sumai 3, Ning 7840, Nobeokabozu); trigos de primavera do Brasil (Frontana) e trigo de inverno da Europa (Praa 8, Novokrunka). Steiner *et al*. (2004) indicaram que a cultivar Frontana também produz enzimas que degradam desoxynivalenol (DON).

Os mecanismos de resistência para o gênero *Fusarium* em trigo são classificados em morfológicos ou fisiológicos, sendo ambos os mecanismos, conduzidos pela expressão gênica (RUDD *et al*., 2001). Determinadas características morfológicas têm sido associadas com o aumento da doença, a exemplo de genótipos de trigo com aristas, pedúnculo curto e espiga compacta. Os genótipos de porte baixo são mais severamente infectados do que os de porte alto.

Já a resistência fisiológica envolve rotas bioquímicas que produzem compostos inibidores de patógeno pós-infecção (MESTERHAZY, 1995).

Mesterhazy (1995) descreveu cinco mecanismos de resistência à fusariose (giberela). Tipo I: a resistência à infecção inicial, o Tipo II: a resistência à propagação no interior da espiga, Tipo III: redução do acúmulo de DON não correlacionado com as resistências do tipo I e II, Tipo IV: resistência de grãos à infecção (mesmo que a espiga esteja infectada) e Tipo V: tolerância (menor perda de produtividade, mesmo com elevada infecção).

Miller e Arnison (1986) denominaram de resistência do tipo III a baixa produção de DON em determinados cultivares, em comparação com outras cultivares no mesmo ambiente. O baixo nível de DON no grão poderia ser explicado por três possíveis causas: (a) cepas pouco produtoras, (b) degradação enzimática de DON durante o desenvolvimento do grão, ou (c) elevado nível de DON em tecidos que não sejam os grãos na espiga (MILLER & ARNISON, 1986). A resistência à giberela tem sido observada em determinados genótipos desenvolvidos no Sul do Brasil, mas se desconhece o tipo de resistência presente individualmente em cultivares, sendo a pesquisa concentrada nos três primeiros tipos de resistência (LIMA; FERNANDES; PICININI, 2000).

Ainda são desconhecidos os mecanismos bioquímicos e moleculares da resistência a giberela do trigo. Desjardins *et al.* (1996) mostraram que o rompimento de gene codificador de trichodiene sintetase (*Tri5*) em *F. graminearum* reduziu a severidade de doença, enquanto a restauração de gene *Tri5* aumentou a severidade de giberela e na produção de DON. Recentemente, o gene *Tri101* de *F. sporotrichioides,* gene codificador da enzima tricoteceno 3-O-acetiltransferase, foi utilizado em plantas transformadas de trigo e plantas transgênicas, constatando uma redução significativa na severidade da giberela em experimentos em casa de vegetação (OKUBARA *et al.*, 2002).

3.2 TRICOTECENOS

A produção de micotoxinas depende do crescimento fúngico, podendo ocorrer em qualquer época de cultivo, colheita ou estocagem dos alimentos e, permanecem no grão mesmo depois da eliminação de fungos produtores. As condições ótimas para a produção de micotoxinas em grãos infectados são

dependentes do substrato, espécies e isolado ou linhagem de *Fusarium* e, dependente, principalmente, de temperatura e atividade de água (DOOHAN; BRENNAN, COOKE, 2003).

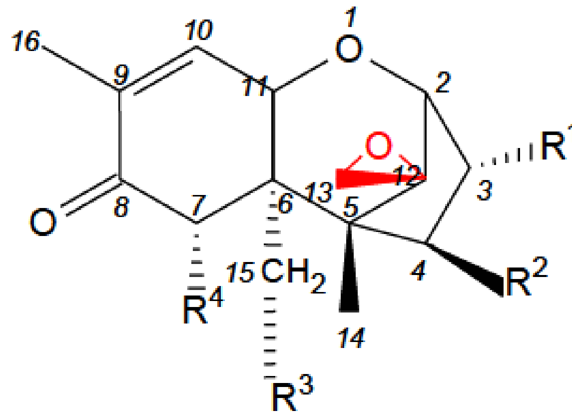
Estudos envolvendo a contaminação de grãos de cevada, trigo, arroz e milho por *F. graminearum* e *F. culmorum* demonstraram que as melhores condições para produção de tricotecenos situam-se entre 25 a 28ºC e atividade de água de 0,97 (GREENHALGH; NEISH; MILLER, 1983; BEATTIE *et al.*, 1998; HOMDORK; FEHRMANN; BECK, 2000).

Os tricotecenos constituem um grupo de 150 metabólitos secundários análogos produzidos por gêneros *Fusarium, Myrothecium, Phomopsis, Stachybotrys, Trichoderma, Trichotecium, Verticimonosporium* (COLE & COX, 1981; UENO, 1983, BETINA, 1989). São sesquiterpenóides caracterizados por uma dupla ligação em posição C-9,10, um anel epóxido em posição C-12,13, responsável pela toxicidade e diversas substituições em C-3, C-4, C-15, C-7 ou C-8.

Ueno (1983) classificou os tricotecenos em quatro grupos em função da estrutura química. Os tricotecenos do tipo A (toxina T-2, toxina HT-2, neosolaniol e diacetoxiscirpenol) não apresentam o grupamento carbonila C_8 conjugada à dupla ligação $C_{9\text{-}10}$, impedindo a absorção de radiação ultravioleta. Os tricotecenos do tipo B (desoxinivalenol e análogos 15- e 3-acetilados e, o nivalenol e análogo acetilado fusarenone X) contêm um grupo carbonila no C_8, portanto a conjugação com a dupla $C_{9\text{-}10}$ permite absorção de radiação ultravioleta. O tipo C caracteriza pelo segundo anel epóxido na posição C-7,8 ou C-9,10, enquanto o tipo D inclui tricotecenos macrocíclicos com um ciclo entre as posições C-4 e C-15.

Contudo, somente os pertencentes ao tipo A ou B têm sido detectados em cereais naturalmente contaminados, sendo os tricotecenos do tipo B mais frequentemente provenientes da fusariose de trigo (BOTTALICO & PERRONE, 2002) (Figura 2).

Figura 2 - Estrutura química de tricoteceno do tipo B



Tricotecenos tipo B	R^1	R^2	R^3	R^4
Desoxinivalenol (DON)	OH	H	OH	OH
3-acetil desoxinivalenol (3-ADON)	$C\text{-}CO\text{-}CH_3$	H	OH	OH
15-acetildesoxinivalenol (15-ADON)	OH	H	$C\text{-}CO\text{-}CH_3$	OH
Nivalenol (NIV)	OH	OH	OH	OH
Fusarenona X (FX)	OH	$C\text{-}CO\text{-}CH_3$	OH	OH

Os tricotecenos são classificados como toxinas gastrintestinais, dermatotoxinas, imunotoxinas, hematotoxinas e genetoxinas. A vomitoxina DON tem gerado preocupação crescente pelo potencial adverso à saúde animal e humana (CREPPY, 2002). A toxicologia animal, bem documentada, focaliza principalmente no sistema imunológico e gastrointestinal. As doses agudas caracterizam por efeitos como diarréia, vômito, leucocitose, hemorragia, choque circulatório e morte, enquanto doses crônicas caracterizam pela recusa alimentar, redução no ganho de peso e absorção de nutrientes e alteração neuroendrócrina e imunológica (LARSEN *et al.*, 2004; PESTKA & SMOLINSKO, 2005).

Em relação à legislação brasileira, foi publicado no Diário Oficial da União – Seção 1, em 30 de dezembro de 2013, a Resolução-RDC nº 59/13 que prorroga até 1º de janeiro de 2017 os prazos para adequação estabelecidos na Resolução-RDC nº 7/11 sobre limites máximos tolerados (LMT) para micotoxinas em alimentos (Tabela 1). Na Europa, o regulamento n°856/2005 de 6 de junho de 2005 estabelece o teor máximo autorizado para fusariotoxinas, em particular DON. O LMT fixado é de 1750 µg kg^{-1} de DON em trigo *durum* e 750 µg kg^{-1} para os produtos de primeira transformação dos cereais como farinhas e macarrão.

Tabela 1 - Limites máximos tolerados (LMT) de desoxinivalenol em alimentos

Vigor a partir de	Alimento	LMT (µg/kg)
janeiro/2014	Trigo e milho em grãos para posterior processamento	3000
	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grão de cevada	1500
	Farinha de trigo, massas, crackers, biscoitos de água e sal, e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada maltada.	1250
janeiro/2016	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo, farelo de arroz, grão de cevada	1000
	Farinha de trigo, massas, crackers, biscoitos de água e sal, e produtos de panificação, cereais e produtos de cereais exceto trigo e incluindo cevada maltada.	750

Fonte: Resoluções - RDC n° 59/13 de 30 de dezembro de 2013 e RDC nº 7 de 18 de fevereiro de 2011.

3.2.1 Biossíntese de Tricotecenos

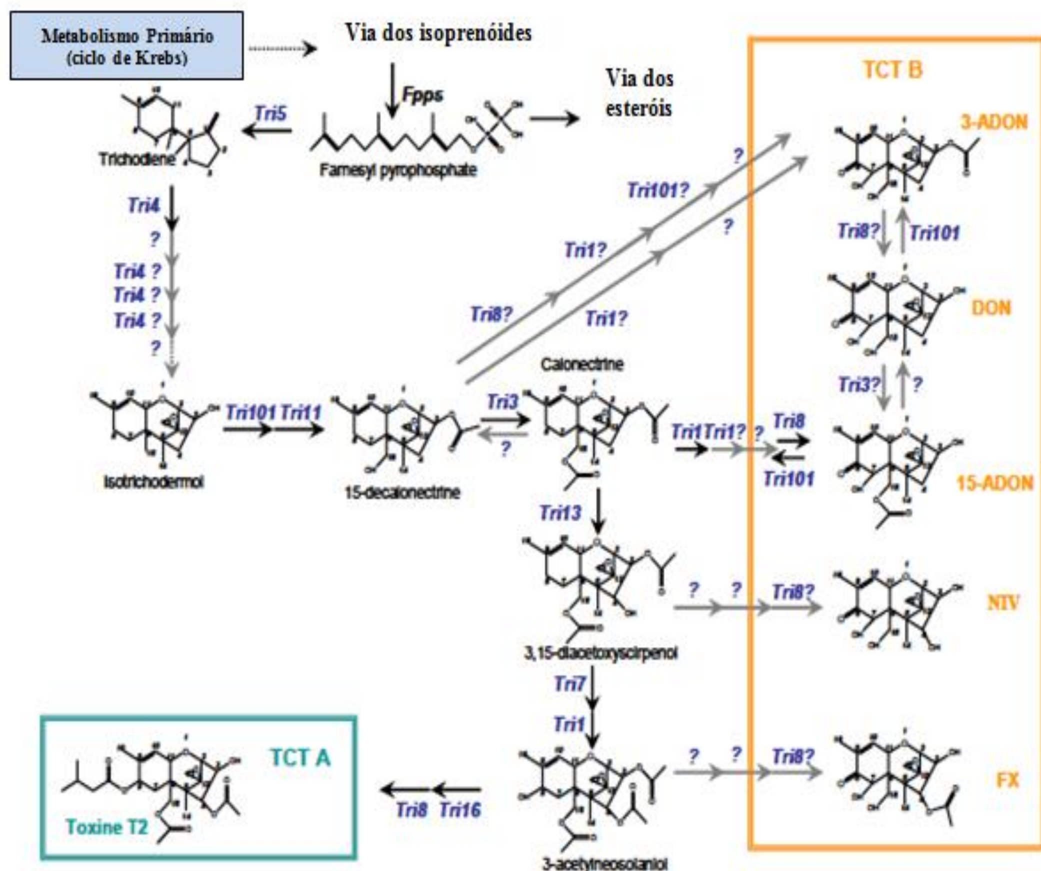
Os tricotecenos derivam do tricodieno, sintetizado a partir do farnesil pirofosfato, produto da via biosintética dos isoprenóides. A biossíntese de tricotecenos a partir do tricodieno envolve numerosas etapas de oxigenação, isomerização, ciclização e esterificação, estabelecidas por meio de experimentos com *G. pulicaris* e *F. culmorum* (DESJARDINS, 2006 ; KIMURA *et al.*, 2007).

A maioria de genes envolvidos na via biossintética dos tricotecenos tem sido identificados e nomeados genes *Tri*, com a atual identificação composta de 16 genes *Tri* para *F. sporotrichioides* e 15 para *F. graminearum* (o gene *Tri16* não é funcional em *F. graminearum*). Entre estes genes, 12 são reagrupados em *cluster* na região de 26-kb de DNA genômica (HOHN *et al.*, 1993a). Os demais genes *Tri* localizam fora do cluster. Adicionais três outros *loci* identificados comportam um locus *Tri101* (KIMURA *et al.*, 1998a ; KIMURA *et al.*, 1998b ; MCCORMICK *et al.*, 1999), outro o *Tri15* (ALEXANDER *et al.*, 2004), e o último composto de dois genes *Tri*, os genes *Tri16* e *Tri1* (BROWN *et al.*, 2003 ; MEEK *et al.*, 2003 ; MCCORMICK *et al.*, 2004).

A seqüência de genes *Tri* é acessível via Internet (Broad Institute, 2003), sendo que a seqüência completa do genoma de *F. graminearum* é disponível, com 11600 genes identificados (CUOMO *et al.*, 2007).

A via biosintética de tricotecenos B (Figura 3) representa todas as possibilidades que conduzem a síntese dos diferentes tricotecenos B para as diferentes espécies de *Fusarium*. As funções de genes *Tri* envolvidos nas diferentes etapas da biossíntese são apresentadas na Tabela 2. O gene *Tri5* codifica a tricodieno sintetase, enzima-chave catalisadora da primeira etapa da via biosintética dos tricotecenos B, responsável por transformar o farnesyl pyrofosfoato em tricodieno (HOHN & DESJARDINS, 1992). A relação entre a expressão de gene *Tri5* e o aumento da produção de DON foi observada em estudos com *Fusarium* (DOOHAN *et al*., 1999 ; COVARELLI *et al*., 2004).

Figura 3 - Via biossintética de tricotecenos e respectivos genes Tri



Fonte: PONTS (2005), DESJARDINS (2006) e BOUTIGNY (2007)

Os genes *Tri4*, *Tri11*, *Tri13* e *Tri1* codificam as enzimas do tipo citocromo P450 envolvido na etapa de oxigenação da via biosintética dos tricotecenos. Os genes *Tri3*, *Tri101*, *Tri8*, *Tri7* e *Tri16* codificam enzimas envolvidas na etapa de acetilação e desacetilação desta via. O gene *Tri12* codifica uma proteína transportadora responsável pela secreção de tricoteceno (ALEXANDER *et*

al., 1999 ; WUCHIYAMA *et al.*, 2000). Os genes *Tri9* e *Tri14* são co-regulatórios da via, com função ainda não determinada.

Os genes *Tri6*, *Tri10* e *Tri15* codificam fatores de transcrição importantes que regulam a expressão de outros genes *Tri*. O gene *Tri6* codifica um fator de transcrição capaz de fixar as seqüências das regiões promotoras de vários genes *Tri* (PROCTOR *et al.*, 1995; HOHN *et al.*, 1999). O gene *Tri10* é um regulador positivo do gene *Tri6* (TAG *et al.*, 2001; PEPLOW *et al.*, 2003). O gene *Tri15* codifica um fator de transcrição que regula negativamente a via de biossíntese dos tricotecenos (ALEXANDER *et al.*, 2004). Em suma, os 12 genes do cluster *Tri* são co-regulados por fatores de transcrição de genes *Tri6* e *Tri10*.

Tabela 2 - Funções dos genes Tri envolvidos na via biossintética dos tricotecenos

Tipo	Gene	Função	Referências Bibliográficas
Sesquiterpeno ciclase	Tri5	Tricodieno sintetase	HOHN & DESJARDINS, 1992; HOHN *et al.*, 1993b
Citocromo P450	Tri4	Tricodieno oxigenase	McCORNICK *et al.* 2006, TOKAI *et al.*, 2007
	Tri11	Isotricoderme 15-oxigenase	ALEXANDER *et al.*, 1998; BROWN *et al.*, 2001
	Tri13*	Calonectrine 4-oxigenase	BROWN *et al.*, 2002; LEE *et al.*, 2002
	Tri1	Triacetoxyscirpenol 8-oxigenase/calocectrine 8-oxigenase	MEEK *et al.*, 2003; McCORNICK *et al.*, 2004
Acetilação/Desacetilação	Tri3	Tricoteceno 15-O-acetiltransferase	McCORNICK *et al.*, 1996 ; BROWN *et al.*, 2001
	Tri101	Tricoteceno 3-O-acetiltransferase	KIMURA *et al.*, 1998a,b; McCORNICK *et al.*, 1999
	Tri8	Tricoteceno 3-O-esterase	BROWN *et al.*, 2001; McCORNICK & ALEXANDER, 2002
	Tri7*	Tricoteceno 4-O-acetiltransferase	BROWN *et al.*, 2001; LEE *et al.*, 2001; LEE *et al.*, 2002
	Tri16**	Tricoteceno 8-O-acetiltransferase	PEPLOW *et al.*, 2003
Regulação	Tri6	Fator de transcrição	PROCTOR *et al.*, 1995; HOHN *et al.*, 1999
	Tri10	Gene regulador	PEPLOW *et al.*, 2003; TAG *et al.*, 2001
	Tri15	Fator de transcrição	ALEXANDER *et al.*, 2004
Transporte	Tri12	Tricoteceno transportador	ALEXANDER *et al.*, 1999; WUCHIYAMA *et al.*, 2000
Genes co-regulados	Tri9	Função indeterminada	BROWN *et al.*, 2001
	Tri14	Função indeterminada	BROWN *et al.*, 2002

*Não funcional em *F. graminearium* (produtor de DON) ** Não funcional em *F. graminearium*

Fonte: DESJARDINS (2006)

3.3 COMPOSTOS FENÓLICOS

Os compostos fenólicos ocorrem em todas as plantas, tendo papel fundamental nos mecanismos de resistência descrito por vários autores (FRIEND, 1981; BELL, 1981; MATERN & KNEUSEL, 1988; NICHOLSON & HAMMERSCHMIDT, 1992; MÉTRAUX & RASKIN, 1993). Estes compostos intervêm nos mecanismos de defesa de planta, seja como constituintes de parede celular quanto compostos antimicrobianos de planta ou induzidos em resposta à infecção como molécula sinal (MÉTRAUX & RASKIN, 1993).

Os compostos fenólicos são aromáticos, contendo pelo menos um grupo carboxílico e um anel fenol, provenientes da via do metabolismo dos fenilpropanóides a partir da fenilalanina derivada da via de chiquimatos. Os ácidos fenólicos são derivados de ácido cinâmico e benzóico, e são precursores de vários compostos secundários como os estilibenos, flavonas, lignina e antocianos (BOUTIGNY, 2007).

Os ácidos fenólicos derivados de ácido cinâmico fluorescem sob UV, o que permite estudar a distribuição histológica por microscopia UV. A técnica foi utilizada por Fulcher *et al.* (1972) em estudo da distribuição de ácido ferúlico na camada de aleurona do trigo. Os ácidos fenólicos ocorrem em concentração mais elevada em camadas de aleurona e camadas externas do trigo, mas estão presentes também no germe (KIM *et al.*, 2006).

Os principais ácidos fenólicos presentes nos grãos de trigo são ácido ferúlico, vanílico, caféico, salicílico, siríngico, coumárico, p-hydroxibenzóico e os aldeídos como a vanilina e siringaldeído (NACZK & SHAHIDI, 2006). O ácido ferúlico ocorre mais abundante nos grãos (KLEPACKA & FORNAL, 2006).

Os ácidos fenólicos ocorrem nos cereais em forma livre ou conjugada (esterificados ou glicosilados), sendo a maioria insolúvel, portanto extraídos da parede celular por hidrólise alcalina (fenólicos insolúveis ligados à parede) (BOUTIGNY, 2007).

Os ácidos fenólicos de parede celular são ligados a cadeias de polissacarídeos. As paredes celulares das camadas externas do trigo são ricas em arabinose e em xilose com quantidade expressiva de glicose e ácidos urônicos, e quantidade pequena de galactose e manose (PARKER; NG; WALDRON, 2005). O ácido ferúlico é o mais frequente, ligado por ligações éster com resíduos de

arabinose das cadeias de arabioxilanas (polímeros de xilose e de arabinose). O ácido cumárico pode ser esterificado à cadeia de arabinoxilana, como o ácido ferúlico, mas aparece mais freqüente associado à lignina (JUNG, 1989).

Diversos isômeros de dímeros de ácido ferúlico foram identificados nas camadas externas de trigo. O isômero 5-5'-DiFA foi isolado a primeira vez das paredes celulares de germe de trigo por Markwalder e Neukom em 1976. Waldron *et al.* (1996) identificaram os isômeros 8-O-4'-DiFA, 8-5'-DiFA e 5-5'-DiFA nas paredes celulares de colmos de trigo. Em grão de trigo, diversos isômeros foram identificados na parede celular: 8-5'-DiFA, 8-O-4'-DiFA, 8-8'-DiFA, 5-5'-DiFA e 4-O-5' DiFA (BUNZEL *et al.*, 2000 ; RENGER & STEINHART, 2000 ; BUNZEL *et al.*, 2001). Na parede celular das camadas externas de trigo (palea e lema), Parker *et al.* (2005) isolaram os isômeros 8-8'-DiFA (forma aryltetralin), 5-8'-DiFA, 5-5'-DiFA, 8-O-4'-DiFA e 5-8'-DiFA (forma benzofurano).

3.3.1 Mecanismo Natural de Resistência em Trigo

A parede celular constitui uma importante barreira física na defesa contra patógenos. Em resposta à infecção, os ácidos fenólicos seriam sintetizados rapidamente pela planta e polimerizados nas paredes celulares (MATERN & KNEUSEL, 1988). Os ácidos fenólicos esterificados aos polissacarídeos consolidam e estabilizam as paredes celulares. Vários estudos sugeriram uma relação entre o conteúdo em ácidos fenólicos (ácido ferúlico e ácido cumárico) dos grãos e o nível de resistência à fusariose em trigo (MCKEEHEN *et al.*, 1999 ; SIRANIDOU *et al.*, 2002) e milho (ASSABGUI *et al.*, 1993).

McKeehen *et al.* (1999) estudaram o conteúdo de ácidos fenólicos de seis variedades de trigo com níveis diferentes de resistência durante o desenvolvimento da planta, da antese à maturação. Um dia após a antese, os ácidos ferúlico e coumárico são os compostos fenólicos mais importantes nos grãos para todas as variedades estudadas, apresentando uma proporção de 3:1 (ácido ferúlico: ácido coumárico). Três dias após a antese, a proporção entre ácidos ferúlico e coumárico é de 18:1 na variedade cv2375. Na maturação, esta proporção é de 62:1, e o ácido sinápico substitui o ácido cumárico na segunda posição dos principais ácidos fenólicos nos grãos.

McCallum e Walker (1991) sugeriram que dois picos de síntese de ácido ferúlico existem e estão associados à diferenciação entre tecidos. Aos 10-12 primeiros dias após a antese, a camada testa se diferencia do pericarpo, depois entre 12-24 dias após a antese, a camada de aleurona se diferencia (SIMMONDS & O'BRIEN, 1981). McKeehen *et al.* (1999) observaram também estes dois períodos de síntese de ácido ferúlico ao longo do desenvolvimento de grãos. Entre 0 e 10 dias após a antese, a síntese de ácido ferúlico na variedade resistente contribuía à resistência da planta, limitando a penetração inicial do fungo por diferenciação dos tecidos externos dos grãos. Em seguida, entre 10 e 25 dias após a antese, a síntese de ácido contribuía no sentido de limitar a proliferação do fungo pela diferenciação da camada de aleurona. Na variedade sensível, o endosperma é mais acessível ao fungo, constituindo um excelente meio para seu crescimento.

Em resposta à infecção por *Fusarium*, foi observado que o conteúdo de ácidos fenólicos aumentava nos grãos de variedades de milho e trigo resistentes à fusariose (REID *et al.*, 1992 ; SIRANIDOU *et al.*, 2002). Em contraste, em grãos de variedade mais sensíveis, Reid *et al.* (1992) observaram que o conteúdo de ácido fenólicos diminuem em resposta à infecção.

Em resposta à infecção, ocorre uma modificação nas paredes celulares através do processo de lignificação. A lignina confere aos vegetais propriedades de impermeabilidade e de resistência aos ataques microbianos, uma vez que constitui um material inerte (SEYER, 2005). Este processo permite estabelecer barreiras mecânicas à invasão de patógenos. Siranidou *et al.* (2002) relataram que existe uma diferenciação entre o conteúdo de lignina das paredes celulares de hospedeiros adjacentes ao local de infecção entre as variedades de trigo resistentes e sensíveis. Além disso, a lignificação diminui a difusão de toxinas secretadas pelo fungo nas células hospedeiras e reduz o fluxo de nutrientes das células hospedeiras em direção ao patógeno (SIRANIDOU *et al.*, 2002).

Como constituintes de paredes celulares, os ácidos fenólicos parecem estar envolvidos nos mecanismos de resistência à penetração do patógeno e na sua proliferação no hospedeiro. A maioria dos patógenos deve degradar a parede celular a fim de invadir as células hospedeiras. Os produtos de degradação das paredes celulares são frequentemente envolvidos nos mecanismos de reconhecimento do patógeno pela planta. A liberação, sob controle enzimático, do ácido ferúlico das paredes vegetais é um fenômeno bastante estudado em fungos

(MATHEW & ABRAHAM, 2004). A atividade feruloilesterase do *Aspergillus niger* permite a liberação de ácido ferúlico nas camadas externas (palea e lema) do grão de trigo (FAULDS & WILLIAMSON, 1995). Topakas *et al.* (2003) purificaram e caracterizaram uma feruloulesterase em *F. oxysporum* capaz de liberar ácido ferúlico destas mesmas camadas em trigo.

Os ácidos fenólicos são considerados fungitóxicos para vários fungos, inclusive *Fusarium* (GUIRAUD *et al.*, 1995). *In vitro*, Guiraud *et al.* (1995) determinaram uma CL_{50} (concentração de inibição de 50% de crescimento fúngico) em torno de 5 mM (ou seja, 970 μg/g) para o ácido ferúlico contra *Fusarium coeruleum, F. moniliforme* ou *F. solani.* Para *F. graminearum* e *F. culmorum*, a CL_{50} de ácido ferúlico é, respectivamente, de 668 μg/g e 329 μg/g (MCKEEHEN *et al.*, 1999). Valores similares de CL_{50} obtidos de vários ácidos fenólicos para *F. graminearum* permitiram classificar os ácidos fenólicos em função de CL_{50}, do menos ao mais inibidor: ácido *p*-hydroxibenzóico < ácido gálico < ácido sinápico < ácido cafeico < ácido siríngico < ácido cumárico < ácido ferúlico (FAVRE, 2004).

Guiraud *et al.* (1995) sugerem que a toxicidade dos ácidos fenólicos contra fungos pode estar ligado às suas propriedades lipofílicas, ou seja sua aptidão em penetrar nas células. Entretanto, a toxicidade dos ácidos fenólicos pode estar ligada à sua interação com diversas enzimas fúngicas intra ou extracelulares, incluindo as fenoloxidases (GUIRAUD *et al.*, 1995). Os ácidos fenólicos são também descritos como potenciais inibidores de atividade hidrolítica (JECU, 1997; EL MODAFAR *et al.*, 2000 ; PAUL *et al.*, 2003) e possuem propriedades antioxidantes (RICE-EVANS *et al.*, 1996). Vários estudos demonstraram o efeito inibidor de muitos compostos antioxidantes no crescimento de fungos, inclusive *Fusarium* (THOMPSON, 1992; THOMPSON *et al.*, 1993 ; THOMPSON, 1996; ETCHEVERRY *et al.*, 2002 ; REYNOSO *et al.*, 2002 ; TORRES *et al.*, 2003).

Considerando os ácidos fenólicos solúveis, são presentes nos cereais em pequenas quantidades. Nas camadas externas (palea e lema) do trigo, o conteúdo de ácidos fenólicos solúveis (livres e esterificados) é em torno de 80 μg g^{-1} (KIM *et al.*, 2006). Estas concentrações em grãos de trigo não permitem atribuir um papel fungitóxico significante aos ácidos fenólicos solúveis. No entanto, Bily (2003) demonstrou que dez dias após a inoculação de *F. graminearum*, o ácido ferúlico livre é presente em maior concentração nos tecidos de grãos de milho infectados. Foi

demonstrado que à 50 µg mL^{-1}, o ácido ferúlico inibe 57% da produção de tricotecenos B produzidos por *F. graminearum* (BILY, 2003). O ácido ferúlico (1 µg mL µg kg^{-1}) inibe a produção de fumonisinas B1 produzidas por *F. verticillioides* em 90% sem afetar o crescimento (BEEKRUM *et al.*, 2003). Isto sugere que os ácidos fenólicos podem ter papel como "anti-micotoxinas", ou seja, eles seriam capazes de limitar a biossíntese de micotoxinas.

3.4 A CULTURA DO MILHO

O milho é uma monocotiledônea pertencente à família Poaceae, gênero *Zea*, cientificamente denominado *Zea mays* L. (FANCELLI; LIMA, 1982). Cereal originado no México e domesticado há cerca de 5 a 10 mil anos nas Américas (WANG *et al.,* 1999; SMITH, 2001; PIPERINO; FLANNERY, 2001) é um dos mais importantes produtos agrícolas mundiais, devido à facilidade de adaptação, podendo ser cultivado tanto em regiões de clima tropical quanto em locais de clima temperado (MAGALHÃES *et al.,* 2003; FORNAZIERI, 1999).

Os maiores produtores mundiais de milho são os Estados Unidos, a China e o Brasil, com 314, 192 e 73 milhões de toneladas de milho (USDA, 2013), respectivamente. O milho é cultivado em todo território nacional, sendo o Estado do Paraná um dos maiores produtores, com uma produção 17 milhões de toneladas nas safras 2012/2013 (CONAB, 2013).

Uma parte da produção no Estado do Paraná deve-se ao cultivo de milho safrinha, iniciado na década de 80 e cultivado em grande escala, a partir dos anos 90. Esta prática tem surgido como alternativa para safra de inverno, principalmente devido à redução da área cultivada com o trigo, obtendo respostas consistentes nos ensaios experimentais conduzidos, principalmente, no Estado de São Paulo devido ao investimento em adubação (COELHO *et al.,* 2006).

O milho possui importância social e econômica e se caracteriza pelos diversos destinos de utilização, estendendo-se desde a agricultura de subsistência e uso na alimentação animal às lavouras e indústrias de alta tecnologia (EMBRAPA MILHO E SORGO, 2008). O milho destinado à alimentação animal (avicultura e suinocultura) representa entre 70 e 80% do milho mundialmente produzido (BRASIL, 2007).

O milho constitui aproximadamente 18% da dieta diária de países em desenvolvimento na América Latina e África, contribuindo com alta qualidade nutricional, representado nos carboidratos (72%), proteínas (9,5%), fibras (9%) e lipídios (4,5%) (CIMMYT, 2009; PAES, 2006). A Tabela 3 apresenta a percentagem de cada constituinte segundo a estrutura física do grão.

Tabela 3 - Percentagem do constituinte total indicado nas estruturas físicas específicas do grão de milho

Fração	% grão	% da parte (base seca)				
		Amido	**Lipídios**	**Proteína**	**Minerais**	**Açucar**
Endosptaerma	82	98	15,4	74	17,9	28,9
Gérmen	11	1,3	82,6	22,4	78,4	69,3
Pericarpo	5	0,6	1,3	2,6	2,9	0,8
Pon	2	0,1	0,7	0,9	0,8	1,0

Fonte: Adaptado a partir de PAES (2006)

A condição climática tropical/subtropical predominante no Brasil (altas temperaturas e umidades) confere ao milho perfil de substrato ideal para propiciar crescimento de fungos e consequente produção de micotoxinas (PEDROSA; DEZEN, 1991).

A minimização do impacto negativo de fungo na produtividade e qualidade de milho requer ampla interação de fatores, direcionado a práticas de manejo, aliado ao monitoramento na cadeia produtiva de milho. Entre fatores interferentes na definição da produtividade de milho enfatizam-se população de planta (espaçamento e densidade de semeadura), época de semeadura relacionada às condições ambientais, cultivar e adubação (ALMEIDA *et al.,* 2000; SILVA; ARGENTA, 2000).

3.4.1 Adubação Nitrogenada

As necessidades nutricionais de qualquer planta são determinadas pela quantidade de nutrientes que esta extrai durante o seu ciclo. Esta extração total dependerá, portanto, do rendimento obtido e da concentração de nutrientes nos grãos e nos restos culturais (EMBRAPA MILHO e SORGO, 2008). Segundo Bull

(1993), as necessidades nutricionais de milho são determinadas pelas quantidades totais de nutrientes absorvidos que permitem estimar as proporções que serão exportadas pela colheita dos grãos e as que poderão ser restituídas ao solo por meio de restos culturais.

O nitrogênio (N) é o nutriente exigido em maior quantidade pela cultura, desde a fase de elongação até o florescimento, variando as recomendações da adubação nitrogenada em cobertura em cultivo de sequeiro para altas produtividades de 50 a 90 kg ha^{-1} de N e, para cultivo irrigado, de 120 a 150 kg ha^{-1} (SOUZA *et al.*, 2003). O N é absorvido pelas plantas, preferencialmente, nas formas de nitrato e amônia. O nitrato pode originar-se da mineralização da matéria orgânica que, contendo os aminoácidos nitrogenados, sofre transformações bioquímicas ou são originados de adubos que contêm este sal. O amônio pode originar-se, no caso do milho, do adubo mineral pelo processo de nitrificação (TANAKA *et al.*, 1997).

A adubação nitrogenada influi positivamente na produtividade de grãos da cultura do milho, como também aumenta o índice de área foliar, massa de 1.000 grãos, altura de plantas, rendimento de biomassa e índice de colheita (ULGER *et al.,* 1987; BULL, 1993). Apesar disso, o manejo da adubação nitrogenada é difícil, por ser o N um elemento que apresenta dinâmica complexa e em virtude do fato da adubação química não apresentar efeito residual (RAIJ, 1991).

Quantidades insuficientes de N não só prejudicam a produção de grãos, mas também aumentam a susceptibilidade da planta à infecção por fungos toxigênicos (BRUNS; ABBAS, 2005). Milho sem adubação nitrogenada apresentou maior infecção fúngica do que o adubado e o excesso de N é também prejudicial, ao incrementar a massa foliar, influencia as condições microclimáticas no interior da cultura, proporcionando menor fluxo de ar e maior umidade relativa, tornando-se ambiente favorável a contaminação e a sobrevivência de fungos (BLANDINO *et al.*, 2008).

O manejo da adubação na cultura de milho, além de influenciar diretamente no desenvolvimento e rendimento de grãos, também interfere na produção de micotoxinas, já que os nutrientes favorecem o desenvolvimento de plantas, e, consequentemente, dificultam a contaminação por fungos (SHIM; WOLOSHIUK, 1999). Assim, a produção de milho está diretamente relacionada com o suprimento de nutrientes, influenciando não só a produtividade, mas também a qualidade de milho (YAMADA *et al.,* 1997).

3.4.2 Densidade de Plantas

A densidade de plantio é definida como o número de plantas por unidade de área. A densidade de plantas tem papel fundamental no rendimento da cultura de milho, pois pequenas variações na densidade influenciam no rendimento final a cultura (CRUZ *et al.*, 2006), já que interfere na eficiência da interceptação da radiação solar e na distribuição do sistema radicular no solo.

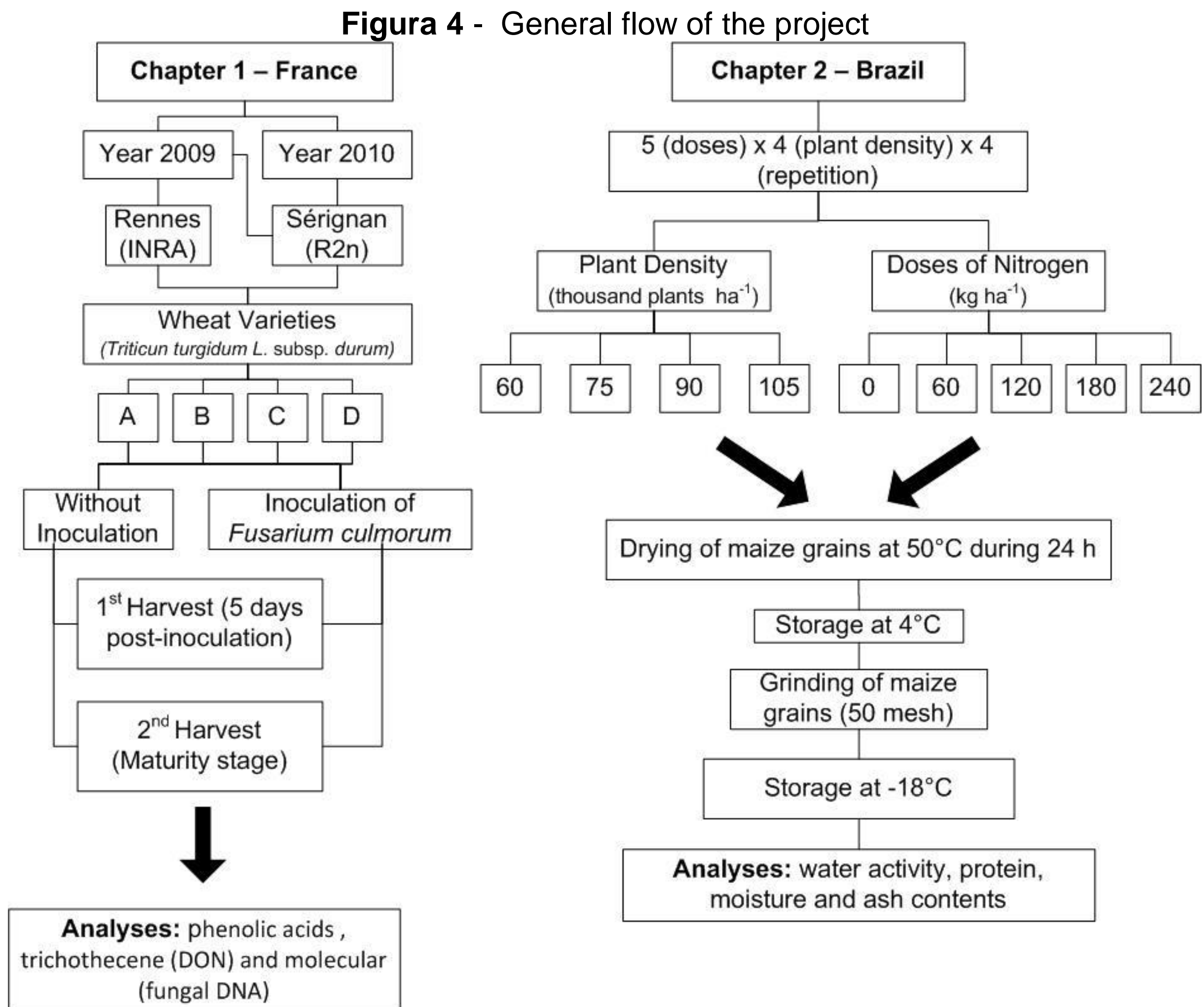
Através do melhoramento genético, híbridos modernos têm mais tolerância a maior densidade de plantas (TOLLENAAR; AGUILERA, 1992). Além disso, a combinação espaçamento entre linhas reduzido e o número de plantas, com adequada utilização de água, luz e nutrientes é uma forma de maximizar a produção de grãos.

Por outro lado, um aumento na densidade de plantas pode gerar maior competição entre indivíduos por água, luz e nutrientes, reduzindo a disponibilidade de fotoassimilados, consequentemente, diminui a síntese e acúmulo de proteínas e absorção de minerais, podendo levar a um aumento nos teores de amido nos grãos de milho (SANGOI; SALVADOR, 1997). Além disso, um aumento na quantidade de plantas por m^2 pode levar a diminuição do fluxo de ar, aumentando a umidade relativa e promovendo a infecção fúngica, além de restringir a atividade fotossintética das folhas, limitando o redirecionamento de fotoassimilados em maior quantidade ao enchimento de grãos (SANGOI *et al.*, 2000). Assim, a escolha da densidade de plantas aliada a adubação nitrogenada adequada é fundamental para reduzir danos causados à cultura de milho sem afetar na produtividade.

4 PLANEJAMENTO EXPERIMENTAL

A Figura 4 apresenta o fluxograma geral do projeto, dividido em dois capítulos. O primeiro foi realizado na França, sendo o campo experimental instalado nas cidades Rennes e Sérignan, seguido de análises laboratoriais na unidade *Mycologie et Securité des Aliments* (MycSA) do *Institut National de la Recherche Agronomique* (INRA Bordeaux-Aquitaine) sob orientação de duas pesquisadoras, Dr[a]. *Laetitia Pinson-Gadais* e Dr[a].*Vessela Atanasova-Penichon*. O segundo foi realizado no Brasil, instalado na Fazenda Panônia, Mauá da Serra, Estado do Paraná, coordenado pelo prof. Dr. *Claudemir Zucarelli*, do Departamento de Agronomia da *Universidade Estadual de Londrina* (UEL). As amostras brasileiras foram analisadas nos laboratórios do Departamento de Ciência e Tecnologia de Alimentos (UEL).

Figura 4 - General flow of the project



5. CHAPTER 1: MOLECULAR BIOLOGY AND LC-MS ON MYCOTOXINS CONTAMINATION IN WHEAT

The field experiment 1 was conducted in two locations in France, which was carried out in Sérignan (R2n) and Rennes (INRA) (Figure 5). The wheat was cultivated in the field conditions in Sérignan, Southern of France, for two consecutive years, 2009 and 2010, while the experiment in Rennes, Northern France, was performed in a polyhouse (2009). Laboratory analysis were performed in the unit *Mycologie et Sécurité des Aliments* (MycSA) of the *Institut National de la Recherche Agronomique* (INRA Bordeaux-Aquitaine), France.

Figure 5 - Localization of field experiment in France

The field experiment involved four wheat (*Triticum turgidum* L. subsp. *durum*) cultivars (A, B, C and D), settled in Sérignan and Rennes in two consecutive years (2009 and 2010). *Fusarium culmorum* was artificially inoculated in wheat plants, and compared with non-inoculated, natural contaminated plant at 5 days post-

inoculation and at maturity stage. Both experimental planning and experimental design are shown in Figure 6 and Figure 7, respectively.

Figure 6 - Experimental planning 1

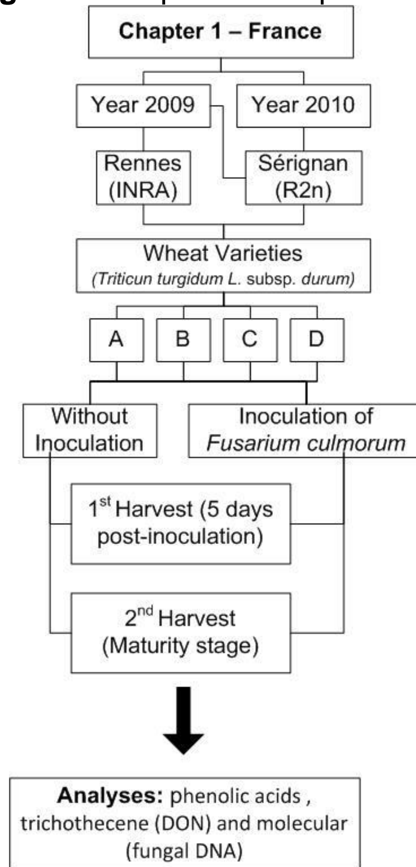
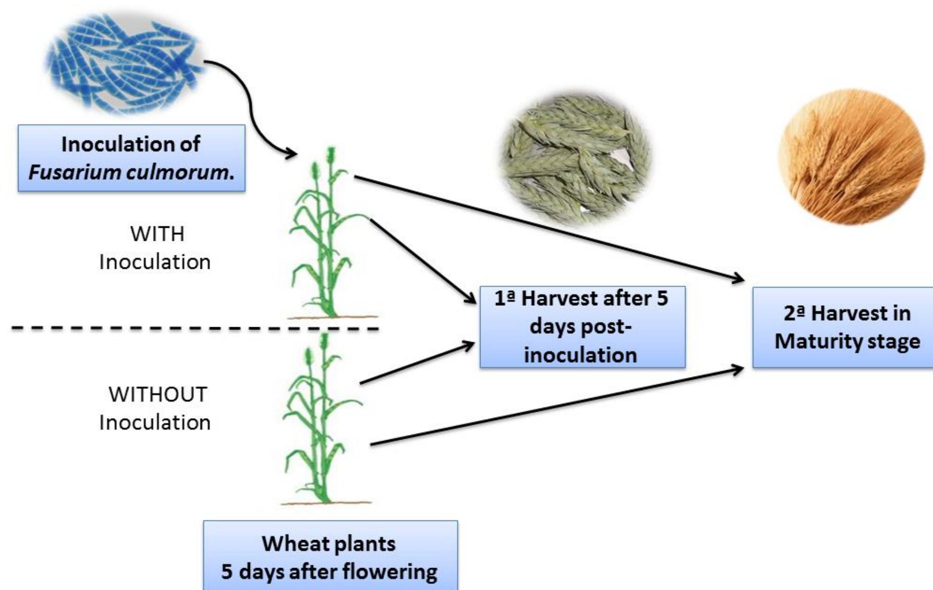


Figure 7 - Experimental design

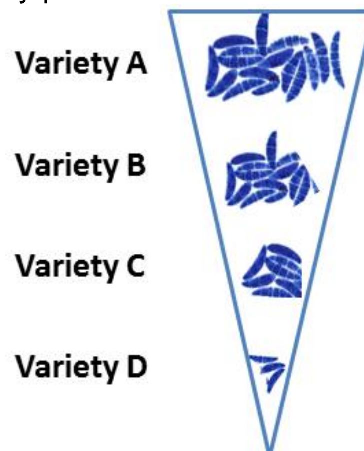


5.1. MATERIAL

5.1.1. Wheat Samples

Four wheat genotypes tested in this study were winter durum wheat varieties (*Triticum turgidum* L. subsp. *durum*), showing different sensitivity against *Fusarium* sp. and trichothecenes contamination in grain. Two varieties (A and B) are considered susceptible for mycotoxins production, while other two varieties (C and D) showed some resistance against trichothecenes production in grain (FAVRE *et al.*, 2004). Figure 8 shows the decreasing susceptibility order against *Fusarium* sp. and its toxins contamination.

Figure 8 - Susceptibility profile of wheat varieties against Fusarium sp.



5.1.2 *Fusarium culmorum* INRA 117 strain

The *Fusarium culmorum* INRA 117 strain, applied in inoculation of wheat plants, was isolated in 1990, Foggia, Italy, and was given by Quirico Migheli (University of Sassari, Italy) for the laboratory collection of de unit *Mycologie et Securité des Aliments* (MycSA) of the INRA Bordeaux-Aquitaine (INRA, Villenave d'Ornon, France).

The INRA 117 strain was selected because it is a strong producer of trichothecenes (DON/ADON chemotype) in liquid cultures (Mycotoxin Synthetic medium: KH_2PO_4, 0.5 g L^{-1}; K_2HPO_4, 0.6 g L^{-1}; $MgSO_4$, 0.017 g L^{-1}; $(NH_4)_2SO_4$, 1 g L^{-1}; glucose, 20 g L^{-1}; biotine, 0.1 mg L^{-1} and 0.1 mL L^{-1} Vogel mineral salts solution)

(VOGEL, 1956). This is a minimal poor medium specifically adapted for mycotoxins production by *Fusarium* spp. Stock cultures were maintained at 4° C on Potato Dextrose Agar (PDA) slants and spore suspension prepared by adding sterile distilled water (6 mL/tube) (BOUTIGNY, 2007).

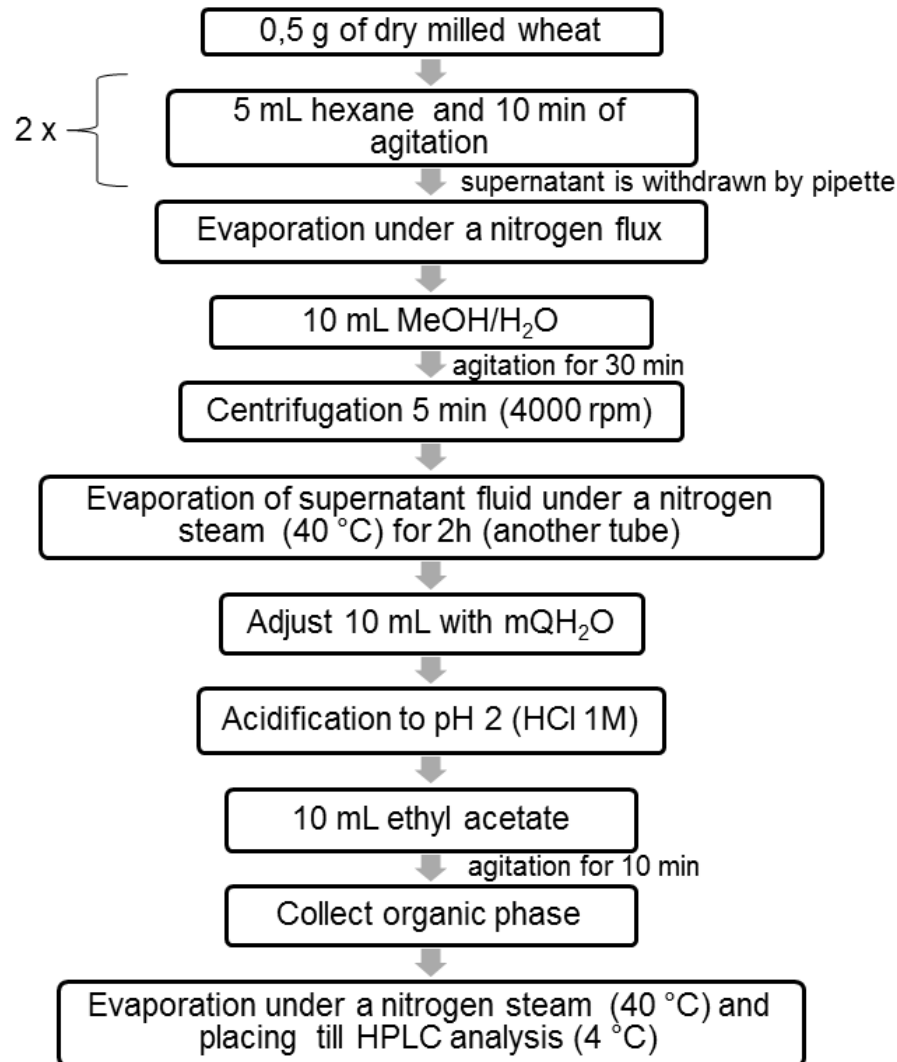
5.2 METHODS

5.2.1 Extraction of Soluble Phenolic Acids

The soluble phenolic acids were extracted according to the protocol of routine procedure carried out in MycSA laboratory, INRA-France adapted on method described by Sosulski *et al.* (1982), Labat *et al.* (2000) and Bily (2003). The dry milled wheat samples (0.5 g) were shaken for 10 min in 5 mL of hexane, then the supernatant fluid was withdrawn by pipette. This first step was performed twice. After evaporation under a nitrogen steam, 10 mL of methanol/water (v/v : 80/20) were added and shaken for 30 min. After centrifugation (2880 x g) for 5 min, the supernatant was collected and evaporated to dryness under a stream of the nitrogen at 40 °C for 2 h. The volume was adjusted to 10 mL with ultrapure water (equipment Milli-Q Water Purification, Millipore, France).

The samples were acidified to pH 2 with hydrochloric acid (1 M) and were extracted with 10 mL of ethyl acetate. After agitation for 10 min, the organic phase was collected and evaporated under a nitrogen steam at 40 °C. Finally, dried samples were dissolved in 200 µL of methanol/water (1:1, v/v) for HPLC-DAD (Diode Array Detector) analysis. The flowchart of soluble phenolic acids extraction is presented in Figure 9.

Figure 9 - Extraction of soluble phenolic acids

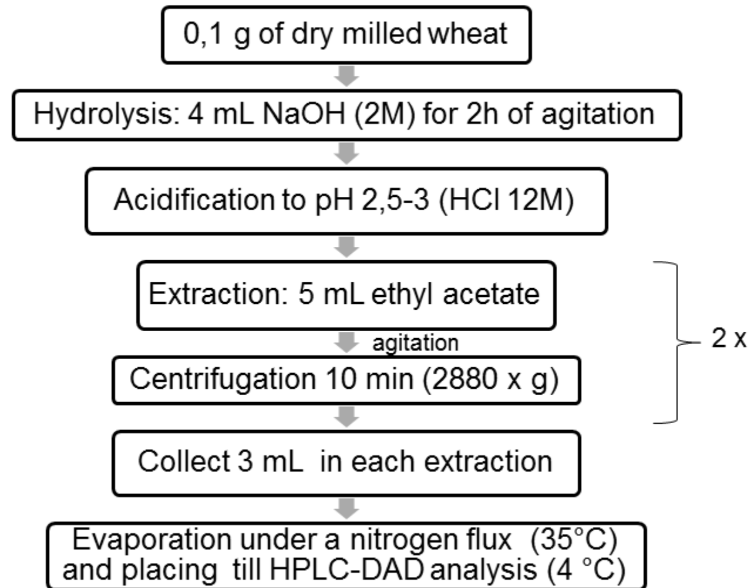


Source: Adapted of BOUTIGNY *et al.* (2010)

5.2.2 Extraction of Insoluble Phenolic Acids

The insoluble phenolic acids were extracted according to the method described by Bily (2003) and Boutigny *et al.* (2010) with some modifications. The insoluble phenolic acids were released from cell walls by alkaline hydrolysis. Dry milled wheat samples (0.1 g) were shaken for 2 h in 4 mL of sodium hydroxide (2 M). Filtrates were acidified to pH 2,5 – 3 with hydrochloric acid (12 M). The samples were extracted twice with 5 mL of ethyl acetate. After centrifugation (2880 x g) for 10 min, 3 mL the ethyl acetate of each extraction was collected and evaporated under a nitrogen steam at 35 °C. Finally, dried samples were dissolved in 200 µL of methanol/water (1:1, v/v) for HPLC-DAD analysis. The flowchart of insoluble phenolic acids extraction is presented in Figure 10.

Figure 10 - Extraction of insoluble phenolic acids



Source: Adapted from BOUTIGNY *et al.* (2010)

5.2.3 HPLC-DAD Analysis of Phenolic Acids

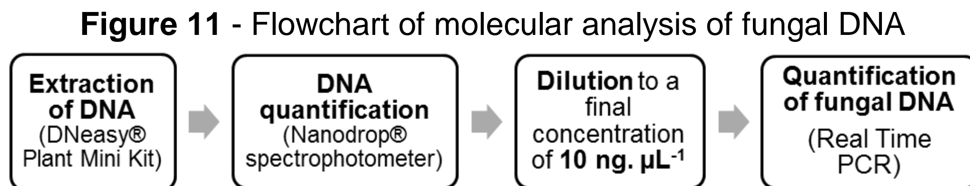
HPLC separation was performed according to a previous procedure described by Kim *et al.* (2006) with some modifications. The method separates 14 phenolic compounds (BOUTIGNY *et al.,* 2007). Separation of phenolic acids was achieved on a Zorbax SB-C18 column (5 µm; 250 mm x 4.6 mm) (Agilent technologies, Palo Alto, USA) maintained at 30 °C. The mobile phase consisted of 2% formic acid in water (v/v) (solvent a) and acetonitrile (solvent b). Phenolic acids was separated by a gradient elution: 5-15% b in 30 min, 15-50% b in 20 min, 50-90% b in 8 min, 90% b for 5 min, 90-5% b in 2 min, and 5% b for 10 min post-run reconditioning. The automatic injection volume was 5 µL. The flow rate was kept at 1 ml min^{-1} for a total run time of 75 min. Peak areas were measured at 260 nm, 280 nm and 320 nm according to the studied phenolic acid (BOUTIGNY *et al*., 2010).

Quantification was performed by using external calibrations with phenolic acid standard solutions prepared from commercial pure powders purchased from Sigma-Aldrich (France). The dimers of ferulic acid were quantified using an external 8-5'-BenDiFA standard (chemically synthesized in INRA-MycSA laboratory, purity > 86%) (BOUTIGNY *et al*., 2010).

5.2.4 DNA Extraction

According to Picot *et al.* (2011), total DNA was extracted from 100 mg of each sample using the DNeasy® Plant Mini Kit in accordance with manufacturer's instructions (Qiagen, France). Grinding was performed with the TissueLyser System (Qiagen-Retsch), using one stainless steel bead in an Eppendorf tube containing 400 µL of AP1 Buffer (Qiagen) for 120 s at 30Hz. After total DNA quantification using a Nanodrop® spectrophotometer (NanoDrop Technology®), each DNA sample was diluted to a final concentration of 10 ng $µL^{-1}$, following quantification of fungal DNA by Real Time PCR (PICOT *et al.*, 2011) (Figure 11).

Figure 11 - Flowchart of molecular analysis of fungal DNA



5.2.5 RT-PCR Analysis

Real Time PCR analysis for quantification of fungal DNA (gene *Tri5*) was performed using a LightCycler® 2.0 system and the LightCycler Software 4.05 (Roche, France) in accordance with Picot *et al.* (2011) and Boutigny *et al.* (2010).

The transcript of the gene Tri5 was evaluated in a final reaction volume of 10 µL using the QuantiTect™ SYBR® Green PCR Kit (Qiagen, France), containing 1 µL of each diluted DNA (concentration of 10 ng $µL^{-1}$), 5 µL of PCR Master Mix, 1 µL of $MgCl_2$ and 0.5 µM each primer. The primer pair used to amplify *Tri5* gene was forward sequence GACCCTAAGCGACTACAG and reverse sequence GTGCTACGGATAAGGTTC (BOUTIGNY *et al.*, 2007). The corresponding melting temperature was at 58°C (BOUTIGNY *et al.*, 2007).

For the amplification of *Tri5* gene, experiments were performed under the following conditions: an initial denaturation step for 15 min at 95°C, then 45 cycles of 15 s in denaturation at 95°C, 20 s annealing at 58°C and 30 s extension until 72°C. Assays for each sample were performed in triplicate (PICOT *et al.*, 2011).

Quantification was determined using standard curves of *Tri5* gene (*F. culmorum* DNA) extracted from pure cultures. Standard curves for the amplification of *Tri5* gene were generated by using serial dilutions ranging from 50 to 0,005 ng μL^{-1}. PCR efficiency always ranged from 95 to 100% while R^2 of standard curves range 0.98 to 0.99 (PICOT *et al.*, 2011; BOUTIGNY *et al.*, 2010).

Absence of non-specific PCR amplification products or primer dimer formation was checked by running melting curves in each run. In addition, each run included positive control (fungal DNA from pure cultures – standard curves) and negative control (without DNA). Quantification of fungal DNA for each sample was expressed as a fungal DNA (ng) to total DNA (ng) ratio (PICOT *et al.*, 2011; BOUTIGNY *et al.*, 2010).

5.2.6 Tricothecenes Analysis (DON)

According to Picot *et al.* (2011), tricothecenes (mainly DON) was extracted by agitating 1 g of milled wheat with 5 mL of acetonitrile/water (86:16) for 1h. After centrifugation, 2 mL of the filtrate were purified using Trichothecene P columns (R-Biopharm, France) before evaporation under a nitrogen steam at 60 °C. Then, dried samples were dissolved in 200 µL of methanol/water (1:1, v/v) before quantification by HPLC-MS.

The HPLC-MS analyses were performed using a QTrap 2000 LC/MS/MS System (Applied Biosystems) equipped with a 1100 series HPLC System (Agilent, France), a Zorbax eclipse XDB C18 column (2.1 mm x 150 mm, 5µm, Agilent, France) and a Turbo Ion Spray ESI source. Solvent A consisted of methanol (100%) and solvent B consisted of methanol/water (10:90, v/v). The flow rate was kept at 0.25 mL min^{-1}. Gradient elution was performed with the following conditions: 8 min held at 10% A, 2 min linear gradient from 10% to 70% A, 2 min linear gradient from 70% to 100% A, 7 min held at 100% A, 1 min linear gradient from 100% to 10% A, 8 min held at 10 % A. The injection volume was 10 µL. Detection was monitored at 230 nm. The electrospray interface was used in the negative ion mode at 400 °C. High concentrated samples were diluted when necessary (PICOT *et al.*, 2011).

Quantification was performed using external calibration with standard solutions DON, 15-ADON and 3-ADON, ranging from 10 to 1000 ng mL^{-1}. The amount of each toxin was expressed in µg of toxin per g of ground wheat.

5.2.7 Statistical Analysis

Statistical analysis was performed using STATISTICA® 7.0 software. The 2009 and 2010 experiments were analyzed separately because a preliminary analysis indicated significant interactions between "year" and other factors. For each year, the effects of variety, inoculation treatments and harvest time were tested on mycotoxin accumulation, fungal DNA and phenolic acids concentration in analysis of variance (ANOVA). Average data were compared by Tukey test with 5% as level of significance.

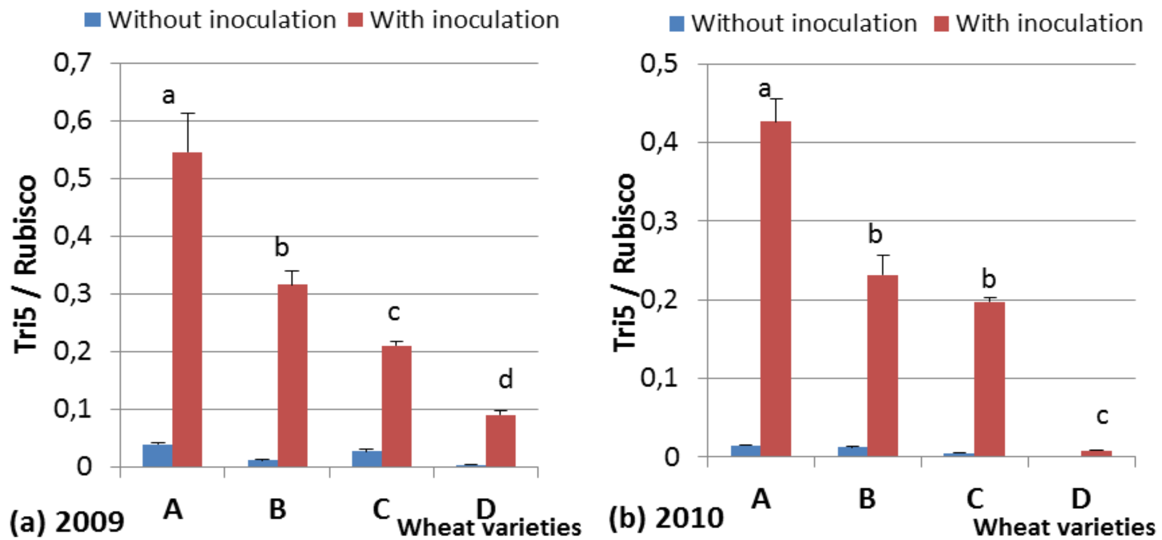
5.3 RESULTS AND DISCUSSION

5.3.1 Contamination with *F. culmorum* and DON

F. culmorum is potentially a DON-producing fungus that can contaminate wheat grains. In order to evaluate the contamination with *F. culmorum* and the possibility of trichothecenes production, the gene *Tri5* was quantified because it encodes the trichodiene synthase, biological catalyst of the first stage of the trichothecene biosynthetic pathway, responsible for transforming the farnesyl pyrofosfoato in trichodiene (HOHN & DESJARDINS, 1992).

Figure 12 shows the quantification of *F. culmorum* expressed as gene *Tri5* (ng) to total DNA (ng) ratio in grains of four wheat varieties analyzed, with or without inoculation, at maturity stage in two consecutive years (2009 and 2010) in Sérignan, where wheat plants were cultivated under field conditions. The four wheat varieties studied had significant difference for fungal contamination ($p<0.05$).

Figure 12 - *F. culmorum* level expressed as gene ratio Tri5 (ng) : total DNA (ng) in wheat varieties at maturity stage in 2009 and 2010, Sérignan-FR



Average values from triplicate measurements.
Average values followed by the different letter differ significantly (Tukey test, p<0.05)

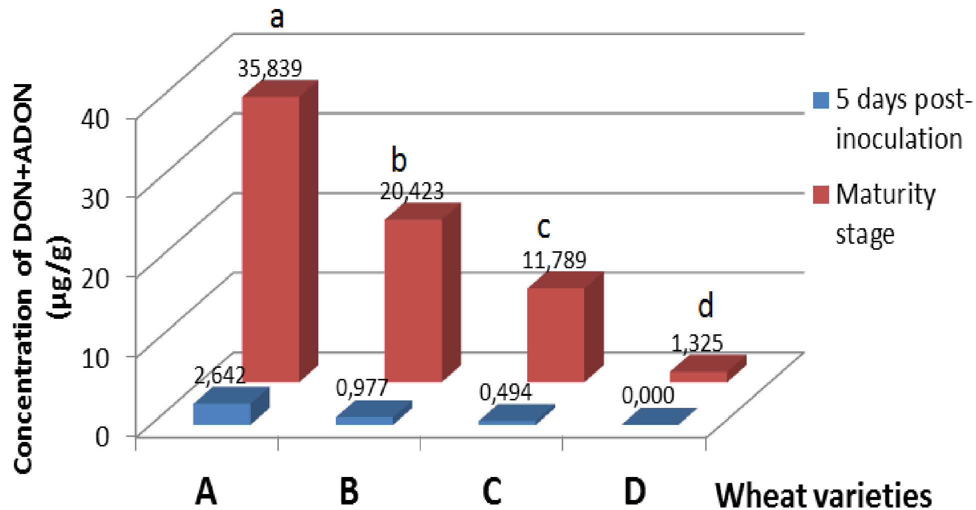
Results showed that variety D limits strongly the development and progression of *Fusarium* sp. (Figure 12). In 2010, the wheat variety A (susceptible) had around 50 times more fungal contamination than the wheat variety D (resistant). The high level of gene *Tri5* in the susceptible variety A can lead to high production of trichothecenes, mainly DON. Fungal DNA (gene *Tri5*) was not detected in wheat grains harvested at 5 days post-inoculation.

Expression of the genes *Tri* encoding first steps of the type B trichothecene biosynthesis pathway has been studied (DESJARDINS, 2006; BOUTIGNY *et al.*, 2009; PONTS *et al.*, 2007). In *F. graminearum*, the gene *Tri6* has been shown to regulate genes from the isoprenoid biosynthetic pathway (SEONG *et al.*, 2009).

The five tricothecenes B (Deoxynivalenol-DON, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, Nivalenol, Fusarenone X) were evaluated, but only DON and its acetylated forms, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol, were quantified. This result is expected because the *F. culmorum* strain used in this experiment is a strong producer of DON and its acetylated forms. However, it could have also natural contamination of others *Fusarium* strains. Concerning the concentration of DON+ADON (the sum of the contents of DON and its acetylated forms, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol), results in Figure 13 and Figure 14 showed that the variety A

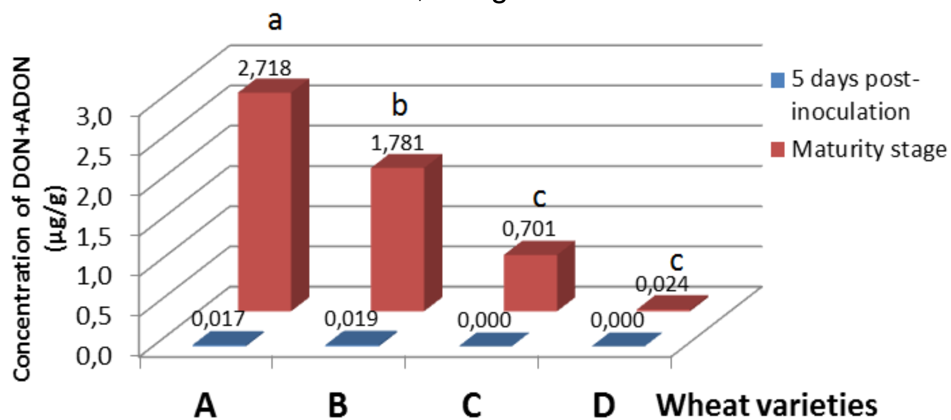
(more susceptible) accumulated more mycotoxins and the variety D had less contamination in both situations, with and without inoculation. In 2010, under experimental conditions, the accumulation of trichothecenes (DON+ADON) followed the classification of wheat varieties according to their susceptibility to *Fusarium* sp..

Figure 13 - DON + ADON (µg/g) level in grains of wheat varieties inoculated in 2010, Sérignan-FR



Average values from duplicate measurements.
Average values followed by the different letter differ significantly (Tukey test, $p<0.05$)

Figure 14 - DON + ADON (µg/g) level in grains of wheat varieties non-inoculated in 2010, Sérignan-FR



Average values from duplicate measurements.
Average values followed by the different letter differ significantly (Tukey test, $p<0.05$)

The results presented in Figure 14 show the mycotoxin concentration from natural contamination with *Fusarium* spp. since no inoculation with *F.culmorum* in these treatments. The varieties A and B had mycotoxin contamination exceeding

the ceiling allowed for DON (1750 μg kg^{-1}) in durum wheat in Europe (Regulation n°856/2005).

The data show high positive correlation (Pearson coefficient r = 0.98) between expression of gene *Tri5* (fungal DNA) and concentration of trichothecenes (DON+ADON) in grains at maturity stage. The higher levels of mycotoxin contamination were obtained in wheat varieties with high levels of *Tri5* gene*,* evidencing *F. culmorum* ability to produce DON/ADON. Therefore, the results indicated a relationship between expression of gene *Tri5* and the increase in DON production, according to other studies with *Fusarium* spp. (DOOHAN *et al.*, 1999; COVARELLI *et al.*, 2004).

The occurrence of *Fusarium* species is largely influenced by climatic conditions (temperature and relative humidity) (PICOT *et al.*, 2011). In this study, the variability in the levels of fungal DNA and mycotoxins in different locations (Sérignan and Rennes) and years (2009 and 2010) may be partly explained by climatic conditions. First, wheat plants were cultivated in Rennes (2009) in a polyhouse where the climatic conditions can be controlled. Differently, in Sérignan, the experiment was installed under field conditions which had natural climatic conditions and allowed to attain more reliable results.

5.3.2 Phenolic Acids Composition

In order to investigate the influence of phenolic compounds (functional components) on the biosynthesis of trichothecenes produced by *Fusarium* spp., the phenolic acids (soluble and insoluble) were evaluated.

According to Figure 15, the resistant variety D did not have higher concentrations of phenolic acids than the others varieties at 5 days post-inoculation and maturity stage for soluble and insoluble compounds. Ferulic acid was the most abundant phenolic acid in accordance with previous studies (BOUTIGNY *et al.*, 2010; KIM *et al.*, 2006; ONYENEHO & HETTIARACHCHY, 1992; KLEPACKA & FORNAL, 2006). McKeehen *et al.* (1999) found ferulic acid, sinapic acid and coumaric acid as predominant phenolic acids in wheat grains of different varieties.

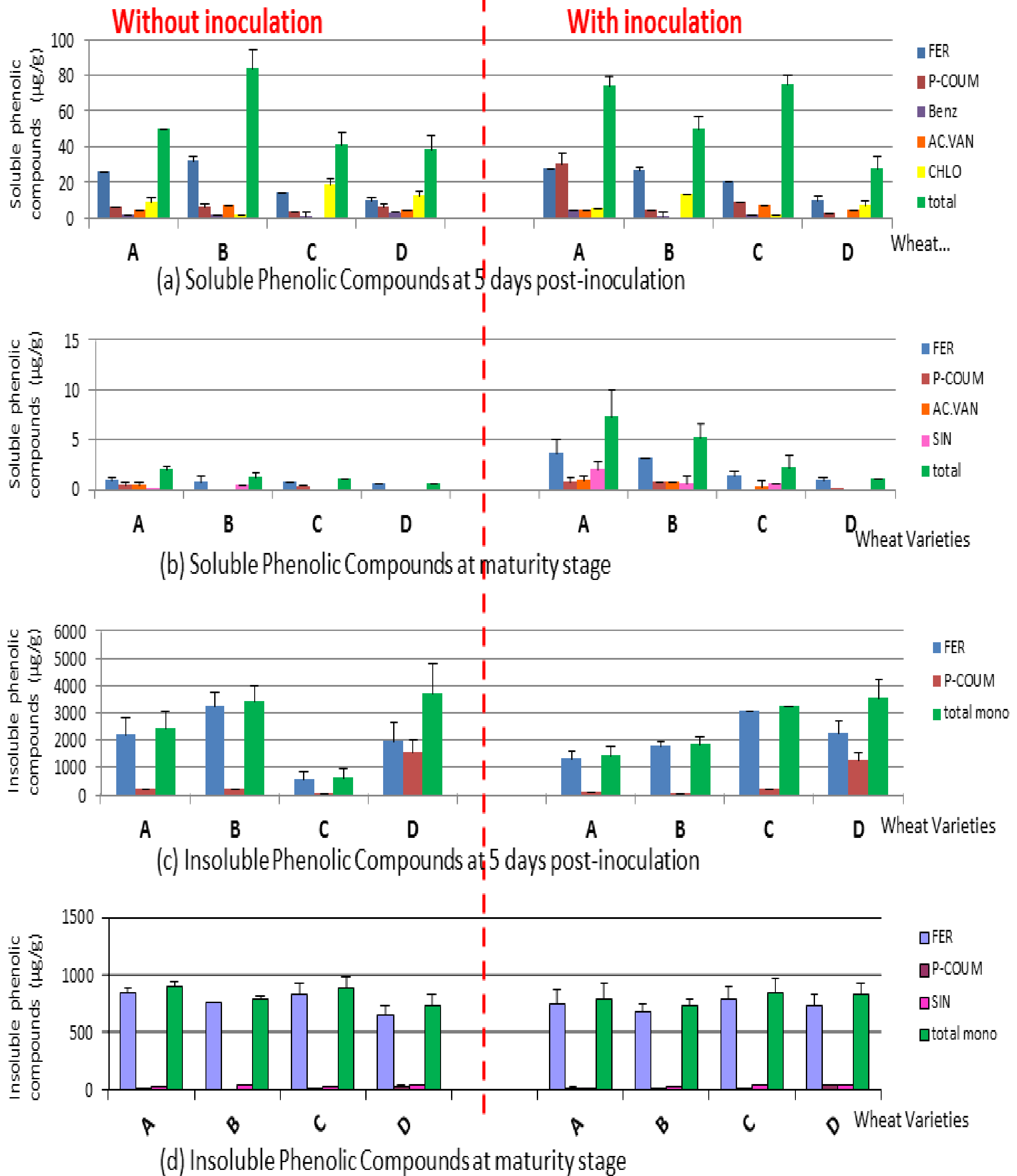
Phenolic acids are considered toxic for various fungi, including *Fusarium* (GUIRAUD *et al.* 1995; FAVRE, 2004). Bily (2003) showed that the 50 mg mL^{-1} of ferulic acid inhibited 57% of the B trichothecenes produced by *F.*

graminearum. Ferulic acid (1 mg mL^{-1}) inhibited the production of fumonisin B1 from *F. verticillioides* (90%) without affecting growth (BEEKRUM *et al*., 2003).

Boutigny *et al.* (2010) showed the natural extract of phenolic acid (ferulic acid) as a potent inhibitor of type B trichothecenes biosynthesis by *F. culmorum in vitro.* Other study described the inhibitory effect of natural phenolic acids extracted from maize germ on type B trichothecene production by *F. graminearum in vitro* (BAKAN *et al*., 2003). In contrast, in this study, it was not found effect of the phenolic acids from wheat grains on the production of trichothecenes.

The content of soluble phenolic acids from wheat grain at maturity stage is too low to account any effect in the grain (Figure 15b). On the other hand, some esterified forms of phenolic acids can be released from cell walls by esterase during infection with *Fusariu*m spp. and then participate in this interaction (FAULDS and WILLIAMSON, 1995). The release of ferulic acid from cell walls, under enzymatic control, is a phenomenon widely studied (MATHEW & ABRAHAM, 2004). The feruloylesterase from *Aspergillus niger* (FAULDS & WILLIAMSON, 1995) and from *F. oxysporum* (TOPAKAS *et al.,* 2003) allow the liberation of ferulic acid from external layers of wheat grains.

Figure 15 - Profile of phenolic compounds in grains of wheat varieties in 2010, Sérignan-FR



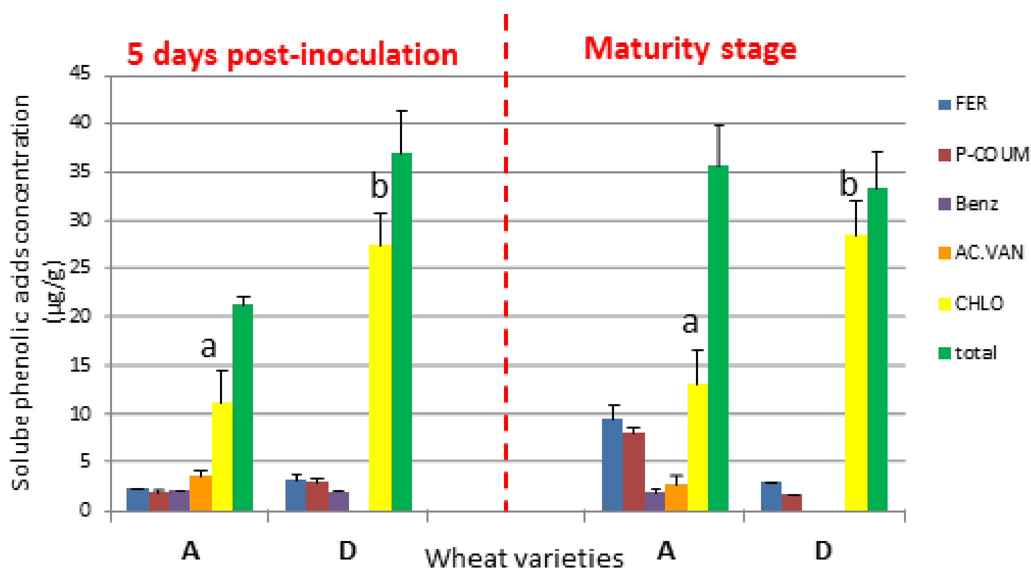
FER: ferulic acid; P-COUM: coumaric acid; Benz: benzoic acid; AC.VAN: vanillic acid; CHLO: chlorogenic acid; SIN: sinapic acid.
Average values from duplicate measurements.
No significant difference at the 5% level of probability by Tukey test.

5.3.2.1 Flavones compounds as a potential inhibitor of trichothecene biosynthesis

Considering that the phenolic compounds composition in wheat grains did not present an influence on trichothecenes biosynthesis in this study, the next step was to study the phenolic compounds from wheat glume (envelope covering the grain). Phenolic compounds analysis of the set (glume + grain) from two varieties (A and D) was performed with the same methodology used for wheat grains. The varieties A and D were chosen due to their different sensitivity to *Fusarium* sp: variety A is susceptible while variety D is resistant.

The Figure 16 shows the phenolic acids concentration from the set (glume + grain) of two varieties (A and D). Chlorogenic acid was found with double concentration in resistant variety D. It can indicate possible influence of this phenolic acid on the mechanism of resistance to *Fusarium* sp. and its toxins contamination. Atanasova-Penichon *et al.* (2012) showed that chlorogenic acid is more abundant in the moderately resistant maize variety, where the *F. graminearum* growth and trichothecene production were drastically reduced. This study in planta indicated that chlorogenic acid may play a role in maize resistance to *Gibberella* ear rot and trichothecene accumulation.

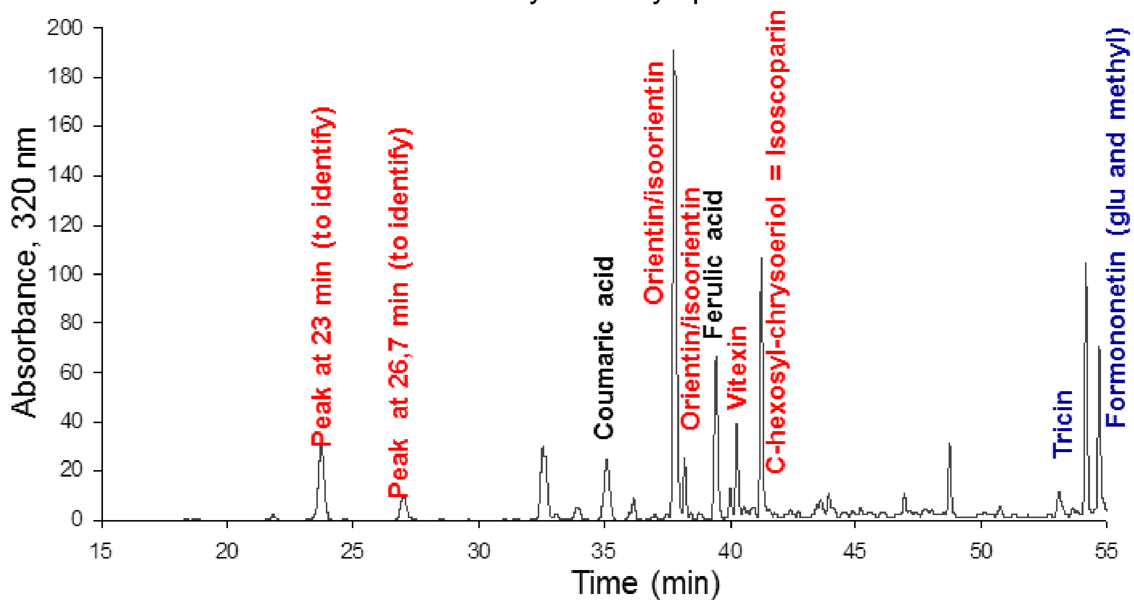
Figure 16 - Phenolic acids level from the set (glume + grain) of wheat varieties



FER: ferulic acid; P-COUM: coumaric acid; Benz: benzoic acid; AC.VAN: vanillic acid; CHLO: chlorogenic acid
Average values from duplicate measurements.
Average values followed by the different letter differ significantly (Tukey test, $p<0.05$)

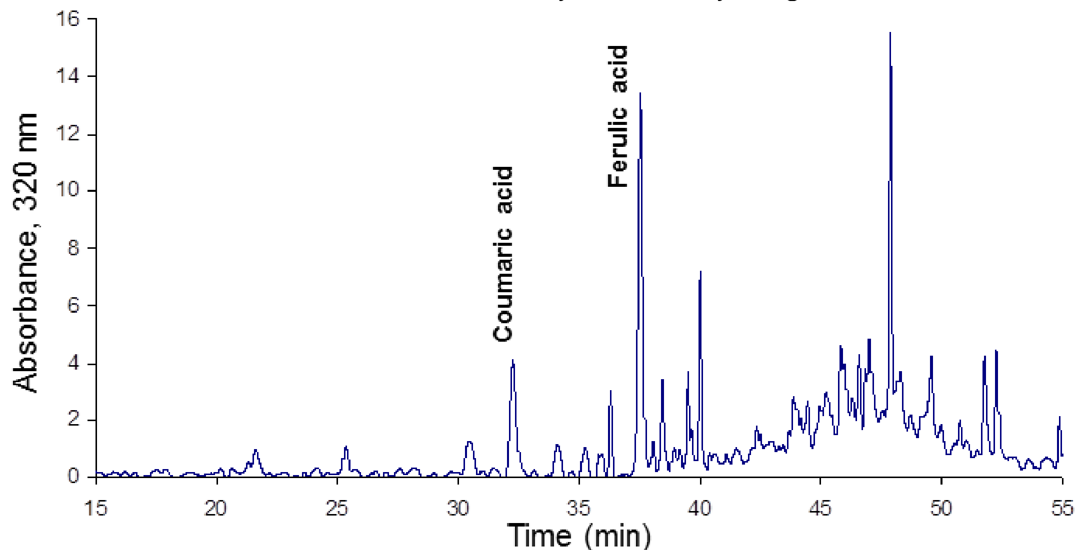
The phenolic acid composition of the set (glume + grain) from the variety resistant D at 5 days post-inoculation and at maturity stage are illustrated in the Figure 17 and 18, respectively. The phenolic acid composition changes over time. At 5 days post-inoculation, the predominant phenolic compounds were flavones, differently at maturity stage coumaric and ferulic acids were predominant. Also, the concentrations of phenolic compounds at 5 days post-inoculation were larger than at maturity stage (see scale).

Figure 17 - HPLC elution profile of phenolic compounds from glume + grain of the resistant wheat variety at 5 days post-inoculation



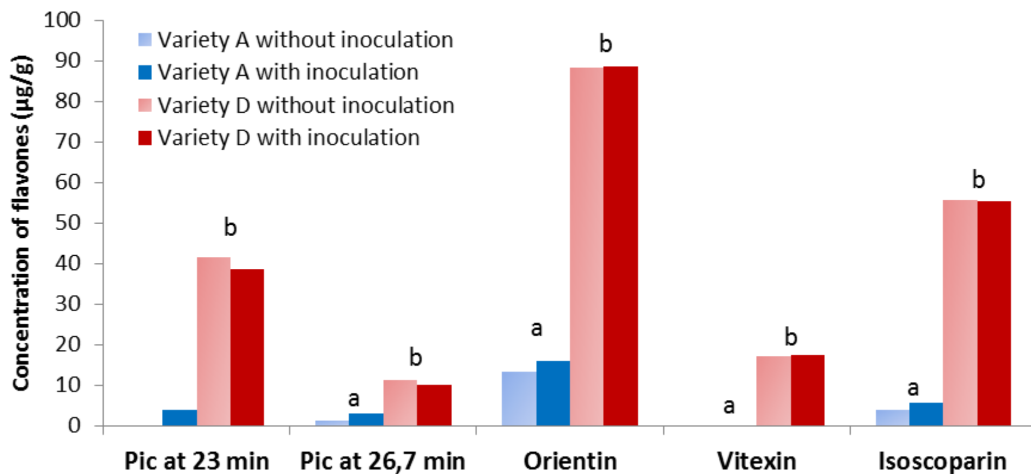
Red: flavones with different concentrations in the varieties A (susceptible) and D (resistant).
Blue: flavones with similar concentration in both varieties (A and D).

Figure 18 - HPLC elution profile of phenolic compounds from glume + grain of the resistant wheat variety at maturity stage



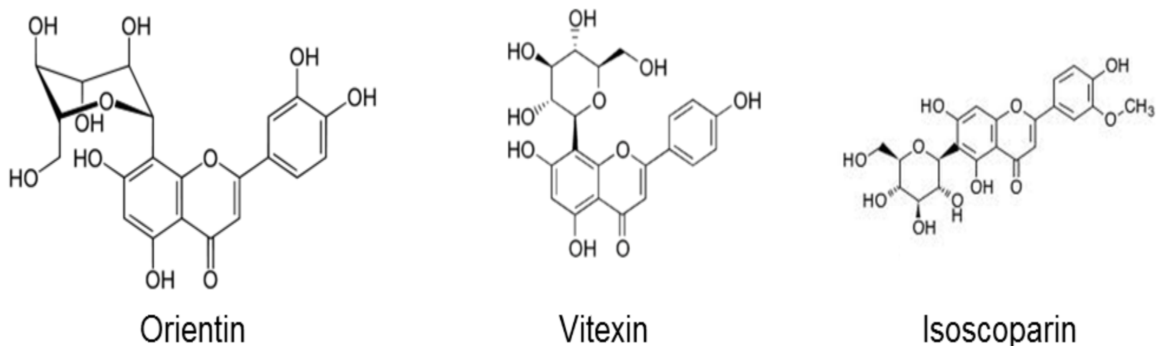
Five flavones compounds were found in the set (glume + grain) from the variety D (resistant) at 5 days post-inoculation with higher concentration than in the variety A (susceptible) (Figure 19). The presence of these flavones was not caused by inoculation of *F. culmorum* since in both situations (with and without inoculation) these compounds were identified with similar concentration (Figure 19 e 20). The flavones Orientin, Vitexin and C-hexosylchrysoeriol (Isoscoparin) were identified (Figure 20), but there are two other peaks at 23 min and 26.7 min that were not identified.

Figure 19 - Flavones from glume of wheat varieties at 5 days post-inoculation



Average values from duplicate measurements.
Average values followed by the different letter differ significantly (Tukey test, $p<0.05$)

Figure 20 - Flavones identified in glume of resistant wheat variety to Fusarium spp



According to the work team knowledge, the identification of flavones molecules in durum wheat variety (resistant to *Fusarium* sp.) had never been described previously. This study could establish the relationship of flavones in glume of wheat grains with the resistance to *Fusarium* sp. and to its toxins, indicating these

flavones as a potential inhibitor of trichothecene biosynthesis, but further investigation is required.

5.4 CONCLUSION

Concerning the expression of *Tri5* (fungal DNA) and DON concentration, the resistant variety D limits strongly the fungal development and the accumulation of its toxins. This variety differs than other ones even at low levels of fungal contamination, indicating the occurrence of a natural mechanism of resistance against *Fusarium* sp..

The *Fusarium* infection does not induce changes on the phenolic compounds composition, which is different at 5 days post-inoculation and at maturity stage. Wheat glumes from resistant variety are riches in chlorogenic acid and flavones that can be linked with resistance to *Fusarium* sp. and to its toxins. Further investigation is required for these molecules as potential inhibitor of trichothecene biosynthesis.

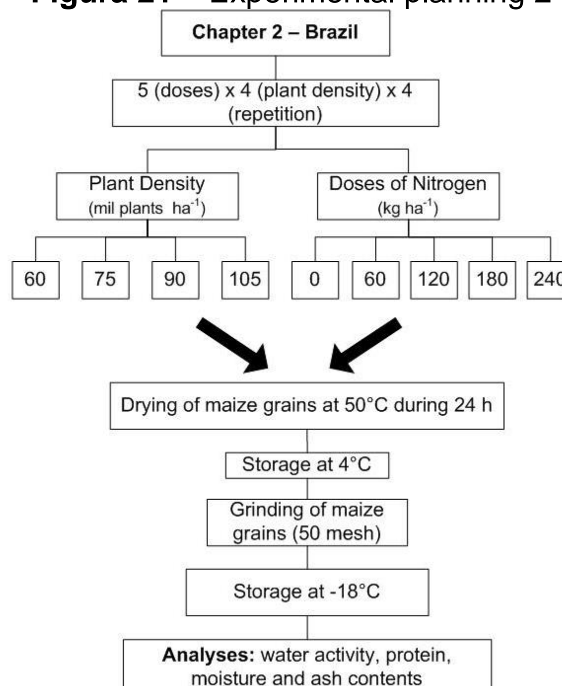
6 CAPÍTULO 2 : TECNOLOGIA NIR VISANDO PREDIÇÃO DE CONSTITUINTES DO MILHO

O campo experimental brasileiro foi instalado na Fazenda Panônia, Mauá da Serra, Estado do Paraná, Brasil, coordenado pelo prof. Dr. *Claudemir Zucarelli*, do Departamento de Agronomia (UEL). O clima é do tipo Cfb, segundo classificação de Köeppen, descrito como clima temperado propriamente dito com verões frescos e sem estação de seca definida (IAPAR, 2012). O solo é caracterizado como Latossolo Vermelho distroférrico (LVdf) (EMBRAPA, 2006).

Conforme, o planejamento experimental apresentado na Figura 21, o experimento envolveu quatro populações de plantas – 60, 75, 90 e 105 mil plantas ha^{-1} – com espaçamento reduzido entre linhas, submetidas a cinco doses de nitrogênio em cobertura 0, 60, 120, 180 e 240 kg ha^{-1} no ano agrícola 2010/2011.

Foram utilizados grãos de milho do híbrido transgênico DKB240YG da empresa Dekalb com tecnologia Yieldgard para controle de lagarta do cartucho (*Spodoptera frugiperda*), lagarta da espiga (*Helicoverpa zea*) e broca do colmo (*Diatraea saccharalis*). Possui ciclo precoce com altura e inserção de espiga baixa, folhas semi-eretas, resistente ao acamamento e ótima qualidade de colmo, recomendado para 70-75 mil plantas ha^{-1}.

Figura 21 – Experimental planning 2



6.1 Article submitted on Journal of Cereal Science

Application of NIR technology for prediction of chemical composition in maize

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6.1.1 Abstract

Near-infrared (NIR) spectroscopy is a rapid and low-cost alternative technology for estimating a wide range of chemical components of biological materials. The objective of this study was to apply NIR spectroscopy to develop predictive models from reference analyses of water activity (a_w), protein, moisture and ash contents for maize from four different plant density – 60, 75, 90 and 105 thousand plants ha^{-1} – and five doses of nitrogen fertilization (0, 60, 120, 180 and 240 kg ha^{-1}). Chemical composition parameters were determined by traditional analytical methods. Maize samples from the same plant density had their protein content increased with doses of nitrogen. No significant difference was observed among maize samples treated with the same dose of nitrogen. Samples were scanned in the visible/NIR range (400 to 2500 nm). Among the different prediction models developed for each parameter, the best coefficient of prediction was obtained for protein (R^2_{cv} = 0.90), which revealed a strong relationship between spectral information and protein content. Except for ash content, models for the assessed parameters showed ratio of standard error of calibration to standard deviation (RPD) between 2.4 and 4.2 indicating that NIR spectra has the potential for screening purposes of maize samples.

Keywords: Maize quality; predictive models; doses of nitrogen; protein

Highlights

> NIR spectroscopy was used for prediction of maize chemical composition. > Maize samples from the same plant density had their protein content increased with doses of nitrogen. > Best coefficient of prediction was obtained for protein content. > NIR spectroscopy has the potential for screening purposes of maize samples

6.1.2 Introduction

Maize is among the main cereals regarding global production of grains, for a wide variety of final applications. Chemical analysis procedures are the most widely accepted reference methods for determining grains composition. However, these methods frequently are destructive, time consuming, laborious, expensive and require large amount of samples. Alternative methods can eliminate these disadvantages and could be used with ease in breeding programs or industry (ORMAN and SCHUMAN, 1991). Near-infrared (NIR) spectroscopy is a well-established alternative as non-destructive technology for measuring constituents of biological materials. Therefore, NIR technology is a rapid and low cost method for estimating a wide range of chemical components and for screening of a large number of samples.

Organic molecules have specific absorption patterns in the near infrared region that can relate to the chemical composition of the material being analyzed. NIR spectrum is influenced by the different vibration modes of the molecules which are caused by their interaction with electromagnetic radiation absorbed at wavelengths between 750 and 2500 nm. The use of chemometrics allows for the extraction of the relevant information contained in the NIR spectra to be used in the development of calibration models that allow the prediction of the composition of food samples (WILLIAMS and NORRIS, 2001).

Applications of NIR spectroscopy technique have been recently reviewed for predicting different attributes of food quality quickly and accurately. An early study by Orman and Schumann (1991) compared NIR calibration methods for predicting protein, oil and starch contents in both whole and ground maize samples. The authors worked within a spectral range of 1100–2500 nm for reflectance and 680–1235 nm in transmittance modes. While the best models were obtained for reflectance spectra of ground samples, it was suggested that the transmittance mode

for whole grains might be more useful because of its greater speed of analysis. Armstrong (2006) developed a rapid single kernel NIR sorting instrument for maize and soybean. Prediction models for moisture of both seed types, and protein contents for soybeans were developed utilizing a spectrometric range from 906 to 1683 nm.

Other studies have been carried out to investigate the applicability of NIR instruments in the analyses of maize grain quality traits: dry matter (WELLE *et al*., 2005), protein, starch, fatty acid composition (BAYE *et al.,* 2006; ORMAN and SCHUMANN, 1991), and carotenoid composition (BERARDO *et al*., 2004). Therefore, the non-destructive nature of this method has been widely used to predict the composition of maize samples (BAYE *et al.,* 2006; TALLADA *et al*., 2009). However, the results obtained were unsatisfactory or limited, requiring further studies on this field for practical applications.

The main objective of this study was to develop predictive models from reference analyses of water activity (a_w), protein, moisture and ash contents for maize from different plant density and doses of nitrogen. Specific objectives were (1) the determination of the chemical composition of maize samples using analytical wet methods, (2) comparison of chemical composition of maize samples grown on different dose of nitrogen and plant densities, (3) acquisition of spectral information in the visible and NIR spectral range for the samples, (4) to build robust PLS-R calibration models to quantitatively relate spectral information and quality attributes, (5) to identify the most significant wavelengths linked to these selected physicochemical attributes of the maize samples.

6.1.3 Material and Methods

Maize samples

Maize samples were obtained from an experimental field in Maua da Serra, State of Paraná, Brazil, during the period between 2010/2011. The maize grain used was a transgenic hybrid, DKB240YG, with YieldGard® technology for control of fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*) and corn borer (*Diatraea saccharalis*) (DEKALB, 2011).

Maize grain samples were harvested from four different plant densities – 60, 75, 90 and 105 thousand plants ha^{-1} – and five doses of nitrogen 0,

60, 120, 180 and 240 kg ha^{-1}, totalizing 79 samples. Samples were dried at 50 °C for 24 hours and stored at 4 °C for further analyses. Maize grains were ground to 50-mesh particle size, aseptically, to avoid samples contamination. Ground grains were stored in plastic bags at -18 °C until chemical analysis and NIR scanning.

Chemical analysis

The chemical parameters analyzed were a_w, protein, moisture and ash content. The water activity was measured on Aqualab equipment (4TE model, Decagon Devices, Pullman, USA) following the manufacturer's specifications. The protein content of ground maize was determined by the Kjeldahl method (AOAC, 1995). Moisture content was determined by weight loss after drying at 105 °C (IAL, 2008; AOAC, 2000) and ash content was determined by weighing the residues of incineration (IAL, 1985; AOAC, 2000).

NIR spectroscopy analysis

Spectral data were collected in reflectance mode and recorded as absorbance (log 1/R) using a XDS Near-Infrared model XM 1100 series – Rapid Content Analyser (Foss NIRSystems, Denmark) over the wavelength range 400–2500 at 2 nm intervals. Approximately 4g of ground maize samples were placed in an individual cell for solid samples (spinning cell) and compacted to eliminate air voids within the sample. Between samples, the sample cell analytical surface was washed with ethanol (70%, v/v), rinsed with distilled water and dried using soft paper tissue.

Data analysis

Chemical analysis

Differences in a_w, moisture, ash and protein content in maize grains from different plant density and nitrogen levels were statistically analyzed using ANOVA, followed by the Tukey multiple-comparison test ($p < 0.05$). Statistical analysis was performed by Statistica software, Version 7.0 (Stat Soft, Inc., Tulsa, OK).

Principal component analysis (PCA)

Multivariate statistical analysis is frequently applied to spectral data for dealing with large complex co-linear information, reducing this original data to a

lower dimension without overlooking useful information. Thus, PCA was applied to the spectral information obtained from the samples. The matrix expression of the PCA model for the spectral data is:

$$\mathbf{A = SV + E} \quad (1)$$

where A is the matrix of spectral information from samples ($n \times w$); S is the score matrix ($n \times p$); V is the eigenvector matrix ($p \times w$); E is a residual matrix ($n \times w$); n is the number of samples; w is the number of wavelengths and p is the number of principal components (PARK *et al.*, 2001).

PCA is commonly applied to the spectral data as an exploratory tool of data structure. In this study, PCA was performed in order to investigate the major external influence of the different factors (plant density and dose of nitrogen) in the spectral information.

Partial least squares regression (PLSR)

PLSR is a procedure used to relate a set of independent variables used as predictors (wavelengths) and the response variables (observations) by reducing a large number of original predictors to a new uncorrelated variable set based on small number of orthogonal factors called latent variables. PLSR was used to develop the calibration models using the NIR spectral information as predictors of the components of interest. The Unscrambler software (version 9.7, CAMO, Trondheim, Norway) was used for creating models (mathematical pre-treatments, selection of wavelengths and PLS factors, outliers determination). In this study, calibration and validation models for the quantitative analysis of the ground maize samples were obtained for a_w, protein, moisture and ash contents.

PLSR was applied to the mean-centered spectral data sets (1050 bands) to establish independent models for each quality attribute and to compare the influence of pre-processing techniques. Calibration results can be optimistic by embracing features accidentally present in the data set that are not representative of the reference measurements. It is suggested that the best protection against overconfidence in regression is to apply the calibration models to an independent set of samples (OSBORNE *et al.*, 1993).

In this study, the dataset was randomly separated into two groups: one for building the calibration model (training set) consisting of 60 samples, and one for validation (prediction set), consisting of the remaining 19 samples. The prediction set was created by randomly selecting 19 samples (approximately 20% of the samples). A t-test was carried out comparing both sets to ensure that it covered appropriately and consistently the whole range of values for each response, since the same set was used for all the pre-processing methods.

PLSR models were built with the training set under full cross validation by using leave-one-out cross-validation (LOOCV) method. Outliers to the model were identified using the common approach of three times standard deviation (CHEN *et al.*, 2005).

The accuracy of the models was assessed comparing the coefficient of determination obtained in the calibration (R^2_C) and cross-validation (R^2_{CV}) models, and the root mean square error of calibration (RMSEC) and cross-validation (RMSECV). Furthermore, in order to evaluate the predictive ability of the calibration models, the ratio of standard error of calibration to standard deviation (RPD) was used (Williams, 1987; Williams and Sobering, 1993). Prediction models with a RPD > 3 are considered suitable for screening purposes, models appropriate for quality control should have RPD > 5, and excellent for all analytical tasks with RPD > 8 (CONZEN, 2006). Other parameters evaluated were the ratio of error range (RER), which represents the ratio of the range of the response values to SECV. Values of RER in the range of 4–8 suggest the possibility of discriminating between high and low response values; values of RER in the range of 8–12 represent the possibility of predicting quantitative data, and RER values greater than 12 indicate good predictability (DAGNEW *et al.*, 2004; PÉREZ-MARIN *et al.*, 2004; WILLIAMS and NORRIS, 2001).

6.1.4 Results and Discussion

Maize samples characterization

Moisture content and a_w are important traits regarding quality of grains and should be controlled during storage to avoid the growing of molds and its effect on maize quality. All samples were stored in controlled temperature and humidity conditions, and the moisture content values were not significantly different

for all maize samples, with results between 12.0 and 13.3%. Results for a_w values varied between 0.54 and 0.64, which is below the optimum level for fungal growth (CASTRO *et al.*, 2002). Ash content values were not significantly different for all samples, which varied between 0.97 and 1.35%.

The results of protein content revealed a broad range of variability and are summarized in Table 4.

Table 4 - Averages values for protein content in maize samples (%) according to doses of nitrogen and plant density

Doses of nitrogen ($kg\ ha^{-1}$)	Plant density (thousand plants ha^{-1})			
	60	**75**	**90**	**105**
0	6.89 ^{aA}	5.74 ^{aA}	5.67 ^{aA}	5.59 ^{aA}
60	7.44 ^{abA}	6.60 ^{abA}	6.84 ^{abA}	6.33 ^{abA}
120	8.27 ^{abA}	7.64 ^{bcA}	7.35 ^{bA}	7.32 ^{bcA}
180	8.74 ^{bA}	8.24 ^{cA}	8.10 ^{bA}	7.69 ^{bcA}
240	8.92 ^{bA}	8.25 ^{cA}	8.44 ^{bA}	8.01 ^{cA}

*Means with different letters (small letters for columns and capital letters for rows) are significantly different (Tukey's Test, $p<0.05$)

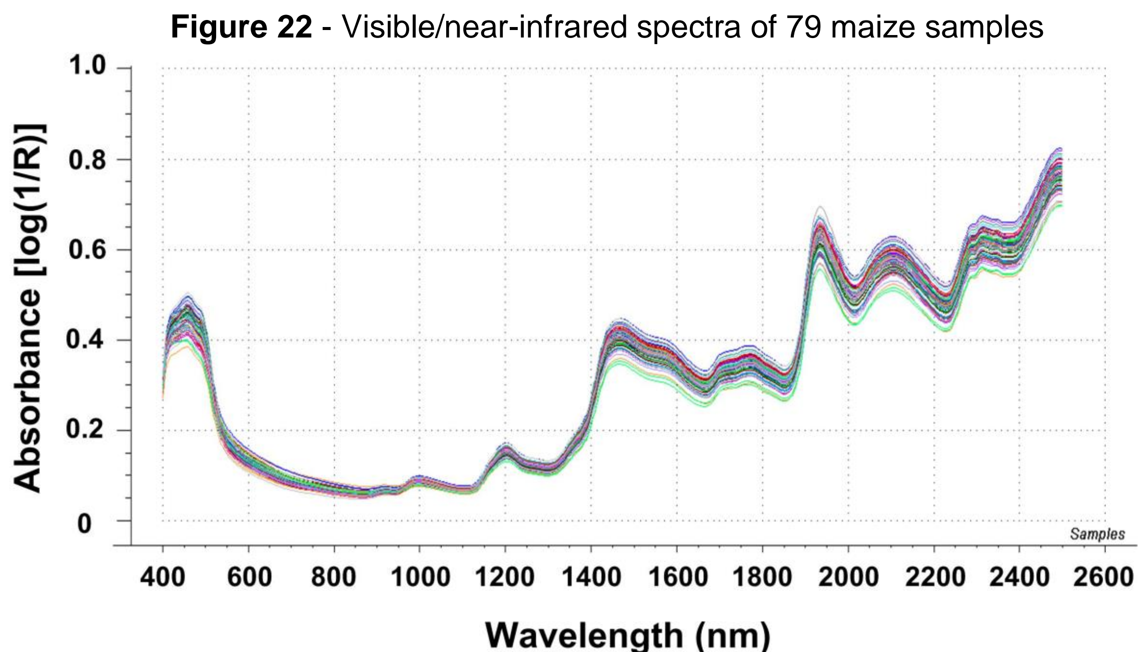
Maize samples from the same plant density had their protein content increased with levels of fertilization (doses of nitrogen). It can be seen that for population of 60 thousand plants ha^{-1}, the level of protein content increases (from 6.89 to 8.92%) with the increase of nitrogen doses in maize plants. This effect can be observed for any plant density. This result is in accordance with Ferreira *et al.* (2001) who observed that doses of nitrogen from 0 to 210 kg ha^{-1} increased the protein content in maize grain from 75 to 105 g kg^{-1}. Samples from the three levels of nitrogen (120, 180 and 240 kg ha^{-1}) did not show significant difference on average values of protein content among them for any plant density tested. However, it could be observed a tendency of decrease in protein content of maize samples with the increase of plant density. This variation could be caused by the competition among plant for water, light and nutrients which can reduce the availability of photoassimilates to supply the plant demand, consequently, the formation and accumulation of protein in grains decreased (SANGOI and SALVADOR, 1997).

Spectral analysis

Typical untreated spectra for the maize ground samples are shown in Figure 22. The NIR spectrum contains information from chemical constituents of the sample and direct interpretation of the absorbance values is difficult for complex samples such as kernels (WILLIAMS and NORRIS, 2001). The NIR spectra showed clear differences from 1400 to 2500 nm that could be related to the internal chemical composition of the kernels.

It can be observed a broad absorption peak in the visible region between 410 and 490 nm associated to the blue colour. Some wide local absorption maxima are distinguishable in the region of 1200, 1450, 1940, 2100 and 2300 nm of the NIR region. Peaks located at 1450 and 1940 nm correspond to O-H stretching and combination bands that could be attributed to water absorption, while the region around 1200 nm is associated to C-H stretching second overtone. The peak located around 2100 nm is related with O-H deformation and C-O stretching associated to starch, whereas the 2300 nm maxima could be due to N-H stretching of amino acids (OSBORNE and FEARN, 1986).

Figure 22 - Visible/near-infrared spectra of 79 maize samples



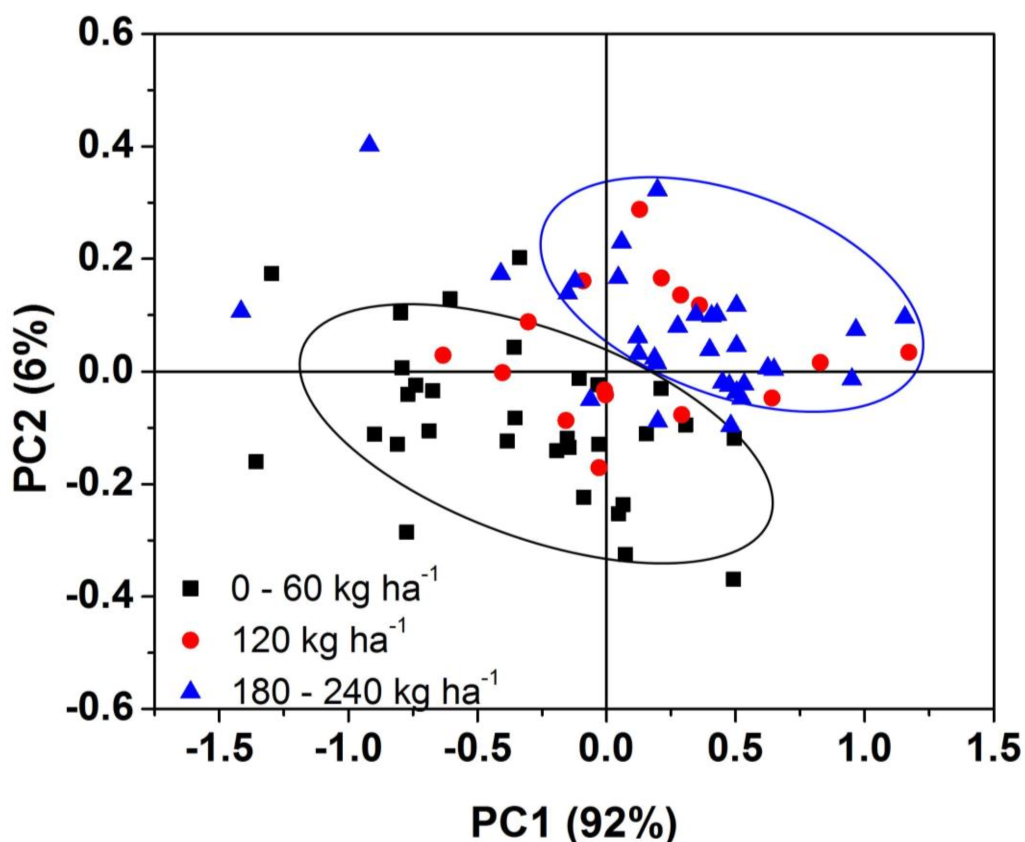
PCA

PCA was carried out with a full (leave one out) cross-validation on the spectral data to identify the qualitative discrimination in the spectra among the samples. The original reflectance spectral data matrix was rearranged to a reduced

number of variables where the samples were located according to their PCA scores instead of reflectance intensities. The first three principal components were responsible for 92%, 6% and 1%, respectively, of the total variance among the examined samples. The interpretation of the results of PCA is usually carried out by visualization of its PC scores (Figure 23).

Figure 23 show that samples that were not treated with nitrogen are clustered in the negative part of PC1 and PC2, while samples with high doses of nitrogen tend to cluster in the positive part of PC1 and PC2. In addition, samples with intermediate doses of nitrogen are scattered in between these two regions. These results support the data presented in Figure 23, indicating that the nitrogen amount is a major influence in the spectral information of the samples.

Figure 23 - Score plot of the first two principal components for spectral data of maize samples



PLSR

In order to evaluate the predictive ability of the calibration models, parameters of prediction are shown in Table 5. Pre-processing methods (MSC, SNV)

were tested and did not improve the quality of the models. Thus, prediction models were developed using the raw spectra. The coefficient of prediction values obtained for the calibration (R^2_C) and full cross-validation (R^2_{CV}) models were comparable in terms of prediction performance. Following the guidelines for interpretation of modeling results that were given by Williams and Norris (2001) and using the coefficient of determination (R^2) as cited by Kovalenko *et al.* (2006), protein content showed the best R^2_{CV} value (0.90), indicating the strong effect of this component in the spectra. The results showed R^2_{CV} of 0.80 and 0.83 for a_w and moisture content, respectively. Ash content was the parameter which was predicted with the least accuracy (R^2_{CV} of 0.65), although the results were satisfactory.

In addition, it was not observed large number of latent variables, indicating that overfitting did not occur. Hence, results have shown that it is feasible to use the raw spectra extracted from ground maize samples to build prediction models for the studied set. Except for ash content prediction, models for the other three parameters showed RPD between 2.4 and 4.2 indicating that the obtained models are satisfactory for screening purposes (CONZEN, 2006).

Table 5 - Prediction models results

	Protein content	a_w	**Moisture content**	**Ash content**
Average values (%)	7.42	0.58	12.40	1.11
Standard Deviation	1.14	0.05	0.80	0.14
Max	9.45	0.81	16.78	1.44
Min	4.77	0.51	11.27	0.85
Range	4.68	0.30	5.51	0.59
LV	7	5	4	6
RMSEC	0.27	0.02	0.17	0.07
RMSECV	0.33	0.02	0.19	0.09
RER	14.17	15.11	29.02	6.55
RPD	3.44	2.40	4.22	1.53
R^2_C	0.93	0.87	0.86	0.73
R^2_{CV}	0.90	0.80	0.83	0.65

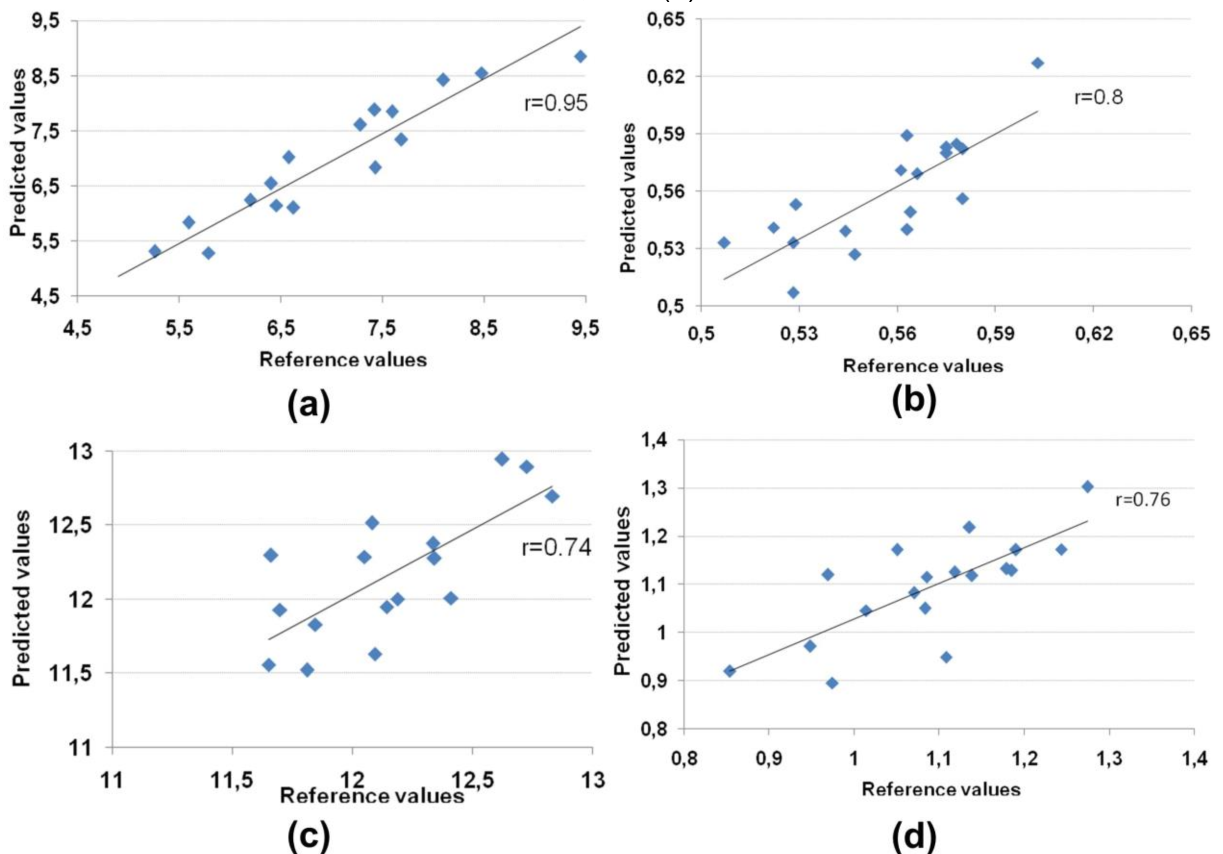
RMSEC: root mean square error of calibration; RPD: the residual predictive deviation, LV:latent variables.

The reported results are in accordance with Orman and Schumann (1991) and Baye *et al.* (2006) who showed a R^2 of 0.97 for predicting protein in maize. Results obtained show an improvement compared to others studies that

reported the estimation of protein content in maize grain by NIR. Tallada *et al*. (2009) reported R^2_{CV} and SECV values of 0.64 and 0.70, respectively, for crude protein calibration models.

Results obtained for the independent set of samples (validation set) are presented in Figure 24. Correlation coeficients (*r*) values (0.95, 0.8, 0.76 and 0.74 for protein, aw, ash and moisture, respectively) support the quality of the prediction models obtained, confirming the applicability of the models for further analyses. However, it is important to emphasize that the models should be confirmed in a larger number of samples, in order to be validated for routine applications.

Figure 24 - Predicted versus reference values for (a) protein content, (b) aw, (c) moisture content, and (d) ash content



6.1.5 Conclusion

The traditional methods used for determination of maize compositional attributes have the disadvantage of being laborious and time-consuming, thus not suitable for assessment of large number of samples in fast-

paced large-scale processing plants. It would be advantageous if a single device could provide immediate and simultaneous determination of maize composition.

The results reported in this study indicate that the NIR spectra of maize samples are highly influenced by chemical parameters such as protein contents. Scrutiny of the spectra has indicated influence by chemical composition parameters. Some wavelengths identified are related to water and other chemical components of the samples. Identifying most important wavelengths allows for the later introduction of cheaper multispectral NIR instruments for the desired application.

High quality PLSR models were obtained for protein content from the spectral signature of the samples. The results have shown that NIR spectroscopy has the potential to be used for predictive analysis and analytical purposes on maize, since it has been shown its sensitivity to chemical composition changes in maize. Furthermore, the NIR technology in combination with PLS regression provides a prospective fast and chemical-free method to estimate the chemical composition in maize. The technique can be implemented as a key component of computer-integrated manufacturing, offering a number of potential advantages, including the elimination of subjective judgment and the creation of product data in a real time for documentation, traceability, and labelling.

6.1.6 Acknowledgements

The authors gratefully acknowledge the financial support from the Coordination for the Improvement of Higher Education Personnel (CAPES) strategic research initiative under the Brazilian Ministry of Education, project number 23038.019085/2009-14. Professor Elisa Yoko Hirooka is a CNPq research fellow.

7. CONCLUSÃO GERAL

O trabalho contribuiu tanto para a cadeia produtiva do trigo quanto a do milho. A qualidade de grãos tanto nutricional quanto sanitária é influenciada por diversos fatores como manejo da cultura, material genético, condições edafoclimáticas, aplicação de produtos químicos, adubação entre outros. Maior investigação na proteção genética de grãos através de estudos com moduladores naturais de defesa da planta contra patógenos torna-se necessária, visando inocuidade de alimentos e minimização do uso de produtos químicos.

Além disso, a tecnologia NIR apresenta-se promissora para fins analíticos e de predição da composição química de grãos ao estar associada a regressão PLS, sendo capaz de subsidiar os métodos tradicionais.

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ANEXOS

ANEXO A – CONFIRMAÇÃO DA SUBMISSÃO DE ARTIGO NO JOURNAL OF CEREAL SCIENCE

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ANEXO B – GUIA DE NORMAS PARA AUTORES DO JOURNAL OF CEREAL SCIENCE

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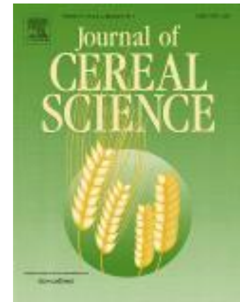

ISSN: 0733-5210

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DESCRIPTION

The *Journal of Cereal Science* was established in 1983 to provide an International forum for the publication of original research papers of high standing covering all aspects of **cereal science** related to the functional and nutritional quality of **cereal grains** and their products.

The journal also publishes concise and critical review articles appraising the status and future directions of specific areas of cereal science and short rapid communications that present news of important advances in research. The journal aims at topicality and at providing comprehensive coverage of progress in the field.

Papers submitted to *Journal of Cereal Science* should not be purely descriptive or confirmatory in nature, but should be innovative and offer new insights into cereal science research.

Research Areas Include:

- **Composition** and analysis of cereal grains in relation to **quality** in end use
- Morphology, biochemistry, and biophysics of cereal grains relevant to **functional** and **nutritional characteristics**
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INTRODUCTION

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