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QUANTITATIVE PCR (qPCR) FOR *Fusarium* TOXIN ANALYSIS IN BARLEY GRAINS

PCR QUANTITATIVA (qPCR) PARA ANÁLISE DE TOXINAS DE *Fusarium* EM GRÃOS DE CEVADA

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Orientadora: Profª. Drª. Liliana de Oliveira Rocha

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RESUMO

Os principais fungos contaminantes da cevada, pertencem ao complexo de espécies *Fusarium sambucinum* (CEFSAM), composto por representantes capazes de produzir micotoxinas, principalmente os tricotecenos do tipo B (desoxinivalenol/DON e nivalenol/ NIV), zearalenona (ZEN) e eniatinas (ENNs). Devido à importância do CEFSAM, métodos rápidos para estimar a ocorrência de DON, NIV, ZEN e outras toxinas vêm sendo cada vez mais relatados na literatura. Nesse sentido, a técnica de PCR quantitativo (qPCR) se mostra promissora para a triagem de micotoxinas em alimentos, por meio da quantificação de genes-chave das vias biossintéticas destes compostos. O objetivo deste estudo foi avaliar a ocorrência de *F.graminearum sensu latu* (*s.l.*) e *F. poae*, bem como de suas micotoxinas (DON, ZEN – *F. graminearum*, NIV, e ENNs – *F. poae*) em grãos de cevada cultivados no Brasil e correlacioná-las com a quantificação de genes-chave envolvidos nas vias biossintéticas de cada uma das micotoxinas citadas, visando o uso da qPCR como um método rápido para estimar a ocorrência de fungos e micotoxinas nas amostras. Foram utilizadas 53 amostras de cevada para análise da micobiota, perfil das micotoxinas (DON, NIV, ZEN e ENNs) por UPLC-MS/MS e quantificação dos genes *TRI12* (genótipo 15-ADON, presente em cepas produtoras de DON) *TRI12/NIV* (genótipo NIV, presente em cepas produtoras de NIV), *ZEB1* e *ESYN1* por qPCR. *EF1-α* foi utilizado como gene normalizador. As análises estatísticas de correlação entre concentração dos genes detectados, de micotoxinas e do nível de contaminação por *F. graminearum s.l.* e *F. poae* foram feitas por meio do software Prism versão 9 (GraphPad, 2022, v. 9.3.1). As micotoxinas mais detectadas foram ENNs, seguidas de DON, ZEN e NIV. Considerando o isolamento, 83% das amostras se encontraram contaminadas por *F. graminearum s.l.* e 51% por *F. poae*. A validação do ensaio de qPCR apresentou resultados satisfatórios para a quantificação dos genes *TRI12/15-ADON, TRI12/NIV, ZEB1* e *ESYN1.* Não foram obtidas correlações significativas entre *TRI12* e nivalenol e *ZEB1* e ZEN, possivelmente, pela baixa ocorrência destas micotoxinas nas amostras. Por outro lado, *TRI12*/15-ADON e *ESYN1* foram positivamente correlacionados aos níveis de DON e ENNs, respectivamente. Ademais, os genes *TRI12/15-ADON* e *ZEB1* foram significantemente correlacionados à ocorrência de *F. graminearum s.l.*, já *TRI12/NIV* e *ESYN1* foram positivamente associados a *F. poae*. Com isso, sugere-se que a qPCR pode ser considerada uma ferramenta promissora para estimar o perfil de contaminação fúngica e a ocorrência de micotoxinas em amostras altamente contaminadas, sendo um método rápido e que possibilita avaliação de um maior número de amostras quando comparado com as metodologias de quantificação convencionais.

Palavras-chave: micotoxinas, fungos toxigênicos, qPCR, métodos analíticos alternativos, *Fusarium*.

ABSTRACT

The *Fusarium sambucinum* species complex (CEFSAM) is composed of species able to produce various mycotoxins, including type B trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV), as well as zearalenone (ZEN) and enniatins (ENNs). Due to the importance of the CEFSAM, rapid methods to estimate their occurrence and the occurrence of DON, NIV, ZEN, and other toxins have been increasingly reported in the literature. Among these, the quantitative PCR (qPCR) technique has shown promising results for screening mycotoxins in foodstuffs by quantifying key genes involved in mycotoxin biosynthetic pathways. Therefore, the objectives of this study were to evaluate the occurrence of *F.graminearum sensu latu* (*s.l.*) and *F. poae*, as well as their mycotoxins (DON, ZEN – *F. graminearum*, NIV, and ENNs – *F. poae*) in barley grains from Brazil and correlate these results with the quantification of key genes involved in the biosynthetic pathways of DON, NIV, ZEN and ENNs. Fifty-three barley samples were used for the analyses of mycobiota, mycotoxin levels (DON, NIV, ZEN and ENNs) by UPLC-MS/MS and quantification of *TRI12* genes (15-ADON genotype, for DON-producing strains) *TRI12/NIV* (NIV genotype, for NIV-producing strains), *ZEB1* and *ESYN1* by qPCR*. EF1-α* was used as the reference gene. Correlation tests between the quantified genes and mycotoxin levels; the quantified genes and the occurrence of *F. graminearum s.l.* and *F. poae* were performed using Prism software v.9. The most detected mycotoxins were ENNs, followed by DON, ZEN and NIV. Considering the mycobiota, 83% of the samples were contaminated by *F. graminearum s.l.* and 51% by *F. poae*. The qPCR assay showed satisfactory results for quantifying the *TRI12/15-ADON, TRI12/NIV, ZEB1* and *ESYN1 genes*. No significant correlations were observed between *TRI12* and nivalenol and *ZEB1* and ZEN, possibly due to the low occurrence of these mycotoxins in barley grains. On the other hand, *TRI12*/15-ADON and *ESYN1* were positively correlated with DON and ENNs levels, respectively. Furthermore, *TRI12*/15-ADON and *ZEB1* were significantly associated with the occurrence of *F. graminearum s.l.* and *TRI12/NIV,* as well as *ESYN1* to *F. poae*. Based on the results, qPCR is a promising tool to estimate the contamination of fungal strains able to produce a specific mycotoxin and the occurrence of mycotoxins in highly contaminated samples, with the advantages of bringing fast results with a higher sample throughput when compared to conventional quantification methodologies.

Keywords: mycotoxins, toxigenic fungi, qPCR, alternative analytical methods, *Fusarium*.

SUMÁRIO

1. Introdução

Cereais constituem o grupo de alimentos mais produzidos no mundo, ficando à frente de culturas como a de cana-de-açúcar, ovos e aves (FAO, 2020). O comércio destes representa 17% do consumo global, sendo que até 2030 este parâmetro deve aumentar em 1%. Estima-se também, que a sua produção cresça em 336 Mt nos próximos dez anos, e que os preços de produtos abaixem (OECD; FAO, 2021).

A cevada (*Hordeum vulgare* L.) é o quarto cereal mais cultivado no mundo e, atualmente é produzida em mais de 100 países. Esta apresenta adaptabilidade a diversas condições climáticas, como o frio e seca, além disso, não necessita de um solo rico em nutrientes para seu cultivo, sendo considerada mais tolerante a condições climáticas adversas do que o trigo (ZHANG; LI, 2009).

O malte, utilizado como matéria prima para a produção de bebidas alcoólicas, é um dos subprodutos da cevada que apresenta elevada relevância para o mercado. Entretanto, o seu uso para ração animal também é importante, pois os grãos são ricos em proteínas, fibras e vitaminas (BADEA; WIJEKOON, 2021; LANGRIDGE, 2018).

Como outros cereais, a cevada é suscetível a infecções por diversos micro-organismos nos períodos de pré e pós-colheita. No campo, o cereal pode ser acometido por fungos patogênicos, destacando os gêneros *Claviceps*, *Ustilago* e *Fusarium*, capazes de ocasionar, principalmente, infecções de origem floral (MURRAY; BRENNAN, 2010).

O gênero *Fusarium* é considerado um dos principais patógenos da cevada, ocasionando a doença denominada giberela ou *Fusarium Head Blight* (FHB). A infecção se inicia nas inflorescências, podendo destruí-las. Quando ocorre a formação de grãos, estes apresentam baixo rendimento, tornam-se “chochos”, rosados e, usualmente contaminados por micotoxinas. Desta forma, a infecção por *Fusarium* representa um risco para a indústria de alimentos, devido à redução da qualidade e da segurança destes grãos destinados ao consumo humano ou animal (JANSSEN; LIU; FELS-KLERX, 2018; FERNANDO et al., 2021).

Os principais representantes do gênero *Fusarium* capazes de ocasionar a giberela, pertencem ao complexo de espécies *Fusarium sambucinum* (CEFSAM), que é composto por seis clados distintos. O principal clado associado à doença é o Graminearum, também conhecido por *F. graminearum sensu lato* (*s.l.*), que contêm

23 espécies filogenéticas, dentre as quais *F. graminearum sensu stricto* (*s.s.*) se destaca, sendo considerada o patógeno predominantemente causador de FHB (OSBORNE; STEIN, 2007; LARABA et al., 2021). Outro clado denominado Sambucinum, possui como um de seus representantes a espécie *Fusarium poae*, que vem ganhando destaque, devido a sua associação com a giberela em regiões quentes e secas e produção de diacetoxiscirpenol (DAS), nivalenol (NIV) e eniatinas (ENNs) (VALVERDE-BOGANTES et al., 2019).

Micotoxinas são metabólitos secundários produzidos por fungos filamentosos e que são tóxicos para animais vertebrados (XUE et al., 2019). O CEFSAM é capaz de produzir diversas micotoxinas, como por exemplo a zearalenona (ZEN), ENNs, NIV, desoxinivalenol (DON) e seus intermediários acetilados 15-ADON e 3-ADON, emgrãos contaminados por estes fungos (MUNKVOLD; PROCTOR; MORETTI, 2021).

Os tricotecenos são micotoxinas constituídas por anéis tricíclicos que possuem um grupamento epóxido nas posições 12 e 13 da estrutura, que proporciona os efeitos tóxicos relacionados às moléculas. Em *Fusarium*, o tricotecenos são divididos nos tipos A e B, sendo DON, seus intermediários acetilados e NIV classificados como tipo B, por apresentarem uma oxigenação no C-8 da molécula principal (PROCTOR et al., 2018).

DON é também denominado “vomitoxina”, devido ao efeito emético ocasionado em animais. A sua toxicidade se deve, primariamente, à inibição da síntese proteica, citotoxicidade e alterações imunológicas (KNUTSEN et al., 2017; WANG et al., 2021). Os metabólitos intermediários de DON, 3-ADON e 15-ADON apresentam toxidez semelhante à DON, que envolve o estresse oxidativo das células (citotoxicidade). Estudos *in vitro* envolvendo o epitélio gástrico, indicam maior toxicidade de 15-ADON quando comparado à 3-ADON. Ademais, quando em conjunto, foi observado o efeito sinérgico destes precursores acetilados, ou seja, maior efeito tóxico em caso de co-ocorrência (PINTON et al., 2012; YANG et al., 2017; JUAN-GARCÍA et al., 2019).

NIV é considerada uma “micotoxina emergente”, com evidências de elevada incidência desta em alimentos, mas com dados toxicológicos escassos. Estudos demonstram potencial citotoxicidade e hematotoxicidade, com testes de genotoxicidade inconclusivos (NAGASHIMA, 2018; ZINGALES; FERNÁNDEZ-FRANZÓN; RUIZ, 2021). Considerando as ENNs, estas são ciclohexadepsipeptídeos

e também denominadas “micotoxinas emergentes”. Estudos demonstram que possuem potencial genotóxico, podem ocasionar toxicidade aguda no intestino, citotoxicidade e imunotoxicidade (MARANGHI et al., 2018; CIMBALO et al., 2021; FRAEYMAN et al., 2017; BERTERO et al., 2020).

ZEN é uma lactona do ácido resorcílico, com afinidade aos receptores de estrogênio, sendo capaz de ocasionar danos nos órgãos reprodutivos, como carcinomas, hiperplasia do útero e diminuição de fertilidade. Estudos também já demonstraram efeitos genotóxicos, carcinogênicos e imunotóxicos (RAI; DAS, TRIPATHI; 2019; ROPEJKO, TWARUZEK, 2021).

Devido aos potenciais efeitos tóxicos das micotoxinas, estudos sobre a ocorrência, toxicidade e métodos de análise são cruciais para avaliar o risco da exposição humana e contribuem para o desenvolvimento de estratégias de controle. Neste sentido, limites máximos permitidos destas substâncias em alimentos foram estabelecidos por diversos países, a fim de subsidiar as relações econômicas e assegurar alimentos com qualidade. No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA), pela RDC nº 722 e IN nº 160 define os limites máximos para diversas micotoxinas em alimentos e bebidas. Dentre as toxinas produzidas pelo CEFSAM, apenas duas delas se encontram representadas: DON e ZEN. Em grãos de cevada, o nível máximo permitido para DON é 1000 µg/kg e para ZEN este se aplica a cereais de forma geral, sendo este 100 µg/kg (BRASIL, 2022).

A fim de analisar os níveis de micotoxinas em amostras, o desenvolvimento de métodos analíticos adequados é essencial (CAI; MCLAUGHLING; ZHANG, 2020). As abordagens analíticas convencionais para determinação de micotoxinas sãobaseadas em cromatografia líquida e detectores UV ou fluorescência. Apesar destesmétodos serem robustos, a maior desvantagem está relacionada com a análise de uma única ou de uma classe de micotoxinas; ademais, as técnicas de preparo de amostras são laboriosas e o custo dos equipamentos elevados (ANFOSSI; GIOVANNOLI. BAGGIANI, 2016). Com o advento da cromatografia líquida associadaao detector de massas, houve a possibilidade de análise de múltiplas micotoxinas e, ao mesmo tempo, menor tempo para o preparo das amostras (GONZÁLEZ-JARTÍN et al., 2021). No entanto, os custos de aquisição, manutenção e treinamento para uso dos equipamentos, associados às múltiplas etapas de análise e necessidade de tratamento de resíduos químicos são alguns dos obstáculos para o amplo uso destes

métodos (ANFOSSI; GIOVANNOLI; BAGGIANI, 2016; CAI; MCLAUGHLING; ZHANG, 2020).

Por outro lado, métodos rápidos para a análise de micotoxinas em numerosas amostras podem ser uma alternativa para inferir a ocorrência destes contaminantes em diversas matrizes alimentares. Vale ressaltar que estes não devem ser utilizados em detrimento dos métodos convencionais, mas como uma ferramenta complementar e útil para a triagem inicial da contaminação por micotoxinas em amostras. As vantagens incluem a reduzida geração de resíduos tóxicos e menor tempo para obtenção dos resultados (AGRIOPOULOU; STAMATELOPOULOU; VARZAKAS, 2020; YANG et al., 2020; GONZALEZ-JARTÍN et al., 2021).

Dentre os métodos rápidos, a espectroscopia de infravermelho próximo (NIR) associada a ferramentas quimiométricas, se mostrou promissora quando aplicada em amostras de cevada naturalmente contaminadas por DON e ENNs (CARAMÊS et al., 2020; CARAMÊS et al., 2022). Métodos baseados em ensaios imunoenzimáticos, como o ELISA (*Enzyme Linked Immunosorbent Assay*) também têm sido utilizados com sucesso durante as últimas décadas, resultando em kits comerciais para análise de micotoxinas (NOLAN et al., 2019).

Adicionalmente, métodos moleculares, como a PCR quantitativa (qPCR), vêm sendo cada vez mais relatados na literatura. Esta pode ser aplicada na quantificação de genes *housekeeping* de *Fusarium*, onde são obtidos dados de biomassa das espécies alvo nas amostras de grãos (SARLIN et al., 2006; KUMAR et al., 2015; HIETANIEMI et al., 2016; BILSKA et al., 2018; SCHÖNEBERG et al., 2018; SOHLBERG et al., 2022).

É importante mencionar que, os métodos clássicos para contagem, isolamento e identificação de fungos potencialmente toxigênicos também requerem períodos prolongados de análise, são laboriosos e dependem de técnicos treinados para identificação adequada. O desenvolvimento de métodos que independem de cultivo e posterior análise são relevantes para a celeridade e precisão dos resultados obtidos (HAFEZ et al., 2020).

Uma estratégia adequada para analisar a ocorrência de fungos toxigênicos é por meio da quantificação de genes-chave relacionados às vias biossintéticas de micotoxinas. Neste sentido, a qPCR também pode ser empregada para relacionar a

quantificação destes genes com os níveis de micotoxinas em grãos, a fim de obter resultados rápidos acerca da contaminação das amostras (ATOUI et al., 2012).

Neste âmbito, a qPCR pode ser uma alternativa promissora para a estimativa de micotoxinas e da biomassa fúngica capaz de produzi-las em amostras de grãos (TRALAMAZZA; BRAGHINI; CORRÊA, 2016; GÓRAL et al., 2022). Portanto, o objetivo principal deste estudo foi avaliar o uso da qPCR para estimar a ocorrência de tricotecenos do tipo B, ENNs e ZEN e dos fungos produtores *F. graminearum sensu latu* (*s.l.*) e *F. poae* em amostras de cevada brasileira naturalmente contaminadas.

2. Objetivos

2.1. Objetivo geral

O objetivo geral deste estudo foi a quantificação dos genes *TRI12* (15-ADON e NIV), *ZEB1* (ZEN) e *ESYN1* (ENNs) em amostras de cevada cultivada no Brasil e correlacioná-los com os níveis destas micotoxinas na cevada como também com os níveis de contaminação por *F. graminearum s.l.* e *Fusarium poae* nas amostras, visando avaliar o uso da qPCR como uma técnica de triagem para as referidas micotoxinas e para a contaminação fúngica.

2.2. Objetivos específicos

- Padronizar o ensaio de qPCR elaborando curvas padrão a partir de DNA de culturas puras de fungos como também de cevada artificialmente contaminada;
- Quantificar os genes *TRI12* (genótipos 15-ADON e NIV), *ZEB1* (ZEN) e *ESYN1* (ENNs) em 53 amostras de cevada cultivada no Brasil por meio de qPCR;
- Analisar a ocorrência de *F. graminearum s.l.* e *Fusarium poae* nas amostras;
- Realizar análises de correlação de Pearson (r) entre a concentração de DNA dos genes-chave da via biossintética de DON, NIV, ENNs e ZEN e os respectivos níveis destas toxinas nas amostras;
- Realizar análises de correlação de Pearson (r) entre a concentração de DNA *TRI12* (15-ADON) e *ZEB1* e a contaminação de *F. graminearum s.l.* nas amostras;
- Realizar análises de correlação de Pearson (r) entre a concentração de DNA *ESYN1*, como também *TRI12* (NIV), e a contaminação de *F. poae* nas amostras.

ARTIGO CIENTÍFICO

Quantitative PCR (qPCR) for rapid screening of high levels of *Fusarium* toxins in barley grains

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Quantitative PCR (qPCR) for rapid screening of high levels of *Fusarium* toxins in barley grains

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Abstract

Representatives of the *Fusarium sambucinum* species complex (FSAMSC) are among the primary contaminants of cereals, including barley. These fungi present the ability to produce mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEN) and enniatins (ENNs). Due to their importance, alternative methods for screening these mycotoxins in samples have been reported in the literature. In this regard, quantitative PCR (qPCR) enables the quantification of key genes involved in mycotoxin biosynthetic pathways. The aim of this study was to validate a qPCR assay to estimate the occurrence of DON, NIV, ENNs and ZEN and their producing fungi (*F. graminearum sensu latu* (*s.l.*) and *F. poae*) in naturally contaminated Brazilian barley samples, seeking the possibility of this technique's use as a green, cheaper, and quick screening analysis method. For this purpose, 53 barley samples were selected for the analyses of the mycobiota, mycotoxin levels (DON, NIV, ZEN and ENNs) by UPLC-MS/MS and quantification of *TRI12*/15-ADON (15-ADON genotypes, for DON-producing strains) *TRI12*/NIV (NIV genotype, for NIV-producing strains), *ZEB1* and *ESYN1* by qPCR, with *EF1-α* as reference gene. Pearson correlation analysis was employed using Prism software version 9. The qPCR method validation presented adequate results and was within the expected standards. ENNs were the most frequent mycotoxins, followed by DON, ZEN and NIV. 83% of the samples were contaminated by *F. graminearum s.l.* and 51% by *F.* poae. The quantification of *ESYN1, TRI12*/15-ADON, *TRI12*/NIV and *ZEB1* was successfully performed by qPCR. No significant correlations were observed between *TRI12*/NIV and nivalenol and *ZEB1* and ZEN,

possibly due to these mycotoxins' low occurrence and levels in barley grains. Significant correlations were observed between *TRI12*/15-ADON and DON, as well as between *ESYN1* and ENNs. Given the isolation data, *TRI12*/15-ADON and *ZEB1* showed a positive correlation with *F. graminearum s.l.,* whereas *ESYN1* and *TRI12*/NIV showed a positive correlation with *F. poae.* Based on the results, qPCR is a possible tool to estimate high levels of *Fusarium* mycotoxins, as well as contamination by their producing fungi in grains.

Keywords: mycotoxins, toxigenic fungi, qPCR, alternative analytical methods, *Fusarium*.

Highlights

- Barley samples were highly contaminated by DON and ENNs
- Highly mycotoxin-contaminated samples were correlated with gene yield
- *F. graminearum s.l.* occurrence was correlated with *TRI12*/15-ADON and *ZEB1*
- *F. poae* occurrence was correlated with *TRI12*/NIV and *ESYN1*
- qPCR can be helpful for estimating high levels of mycotoxins and fungal biomass

1. Introduction

Barley (*Hordeum vulgare* L.) is the world's fourth most widely grown cereal. It is cultivated in over 100 countries due to its high tolerance to adverse climatic conditions, including cold and drought (Badea & Wijekoon, 2021). In 2022, the barley world's production achieved 151,624 million metric tons (MT), an increase from the 146,143 million MT obtained in 2021 (USDA, 2023). Although malting is this cereal's high-value usage, animal feed represents its primary use because of the nutritional value of the grains (Blake et al., 2010; Langridge, 2018).

Throughout its cultivation period, barley is susceptible to various infections. Among them, *Fusarium* Head Blight (FHB) is a significant fungal disease that causes a reduction of grain yield and quality, leading to discolored and decreased-size kernels; moreover, the grains are generally contaminated with high levels of mycotoxins, causing rejection of the product at marketing. Therefore, infection by *Fusarium* constitutes a risk for barley industry, considering the yield and safety losses it can provide (Janssen et al., 2018; Fernando et al., 2021).

Species belonging to the *Fusarium sambucinum* species complex (FSAMSC) are commonly associated with FHB, highlighting *F. graminearum sensu stricto* (*s.s.*), which is considered the predominant FHB pathogen, especially in warmer to temperate regions of the world (Osborne & Stein, 2007). Additionally, *F. poae* and other species are associated with FHB. Although some studies have reported that *F. poae* is found more frequently in temperate climates and that they are more pathogenic at lower temperatures (i.e. 20 °C) (Osborne & Stein, 2007), other surveys have observed an increased occurrence of *F. poae* in small grain cereals due to dry weather conditions, especially over the last years (Beccari et al., 2017; Iwase et al., 2020; Karlsson et al., 2021; Pinheiro et al., 2021; Valverde-Bogantes et al., 2019).

These fungi also produce several mycotoxins, toxic secondary metabolites harmful to vertebrate animals (Xue et al., 2019). A wide range of these compounds is produced by species within the FSAMSC, including zearalenone (ZEN), ENNs, and type A and B trichothecenes such as diacetoxyscirpenol (DAS) nivalenol (NIV), deoxynivalenol (DON) as well as its acetylated precursors (15-ADON and 3-ADON) (Munkvold et al., 2021). *F. graminearum s.s.* produces mainly DON, its acetylated forms, and ZEN, whereas *F. poae* can produce DAS, NIV, and ENNs (Munkvold et al., 2021), highlighting that there is evidence for a strong association between NIV and *F. poae* occurrence in small grains cereals (Schöneberg et al., 2018).

Concerning mycotoxin toxicity, ZEN presents a high affinity to estrogen

receptors, having the potential to cause damage to the reproductive system (Rai et al., 2019; Ropejko & Twaruzek, 2021)., ENNs are considered “emerging mycotoxins” due to their high incidence and lack of regulation. They have been reported to be genotoxic, cytotoxic, and immunotoxic (Bertero et al., 2020; Cimbalo et al., 2021).

Among type B trichothecenes, NIV is also known as an “emerging mycotoxin” with cytotoxic and hematotoxic effects (Nagashima, 2018; Zingales et al., 2021). DON is known as “vomitoxin” because of its emetic effect on animals. It displays the ability to inhibit protein synthesis, and it is considered cytotoxic (Wang et al., 2021). DON’s acetylated precursors, 15-ADON and 3-ADON, present similar toxicity to DON, with 15-ADON being more toxic than 3-ADON to gastric epithelial cells (Yang et al., 2017; Juan-García et al., 2019).

Considering the risks these toxins offer to human and animal health, strategies for their control in food and feedstuff are crucial. The maximum levels established by governmental agencies are important for managing mycotoxin contamination throughout the food chain (Daou et al., 2021).

Until 2003, 100 countries set maximum levels for mycotoxins; no other studies have been developed since then (FAO, 2003). In Europe, the maximum levels for DON and ZEN in unprocessed cereals are 1250 µg/kg and 100 µg/kg, respectively (EC, 2006). In Brazil, the National Health Surveillance Agency allowed the maximum levels of 1000 µg/kg for DON and 100 µg/kg for ZEN in cereals (Anvisa, 2022). Thereare no regulations for ENNs and NIV in food worldwide; some government authorities,such as the European Food Safety Authority, have raised concerns regarding human exposure to these toxins. Nevertheless, more studies on their occurrence and toxicological data are necessary for setting the maximum allowed levels for these toxins (CONTAM, 2013; CONTAM, 2014).

In light of the health problems associated with mycotoxin exposure and the worldwide regulations on these compounds, monitoring their levels in foodstuffs is necessary; therefore, various analytical methods have been developed, ranging from highly sophisticated techniques for the determination of multiple mycotoxins, such as liquid chromatography coupled with mass spectrometry (LC-MS), to rapid methods for screening mycotoxins, aiming to obtain quick answers for straightforward decisions in the food industry (Lattanzio et al., 2019; Agriopoulou et al., 2020; Singh & Mehta, 2020).

Methods based on LC-MS are the most frequently reported since it allows the analysis of multiple mycotoxins, require less time for sample preparation and give

reliable results (González-Jartín et al., 2021). However, some hindrances include high costs for equipment acquisition and maintenance, the need for a trained analyst and waste treatment, all of which can restrict its use (Anfossi et al., 2016; Cai et al., 2016).

In this context, novel methods for detecting mycotoxins in food have been increasingly described in the literature to provide simpler, faster, and more sustainable alternatives to traditional methods (Agriopoulou et al., 2020; Yang et al., 2020). Near Infrared (NIR) spectroscopy has shown promising applications (Caramês et al., 2020; Tyska et al., 2021; Caramês et al., 2022). Immunoassays, such as ELISA (Enzyme-Linked Immunosorbent Assay) and Lateral Flow Immunoassays (LFI), have been widely used, ensuing in the selling of kits for mycotoxin analysis (Nolan et al., 2019).

Previous studies have shown that DNA-based methods, such as quantitative PCR (qPCR), are promising tools for evaluating the occurrence of toxigenic fungi and estimating the levels of mycotoxins in food ecosystems. This is possible due to the quantification of key genes involved in the biosynthetic pathways of mycotoxins that could be correlated with a specific group of toxigenic fungi and their produced mycotoxin (Rodriguez et al., 2012a; Tralamazza et al., 2016; Vogelgsang et al., 2019; Góral et al., 2022).

The primary purpose of this study was to verify the use of qPCR as a green, cheaper, and quicker technique for screening both DON, NIV, ZEN, and ENNs, as well as their producing fungi in barley grain samples. The specific objectives were: i) to assess the occurrence of *F. graminearum s.l.* and *F. poae*; and ii) to estimate the levels of DON, ZEN, NIV and ENNs in barley grain samples using qPCR by quantifying the key genes involved in trichothecene production (*TRI12*: 15-ADON and NIV genotypes), ZEN and ENN production (*ZEB1* and *ESYN1,* respectively).

2. Materials and Methods

2.1. Barley samples

Fifty-three barley grain samples were collected by the Brazilian Agricultural Research Corporation (EMBRAPA) from Brazil's Southeast and South regions. Twenty-five of them were harvested from Taquarivaí (São Paulo), sixteen from Passo Fundo (Rio Grande do Sul), and twelve from Victor Graeff (Rio Grande do Sul) (Figure 1). The samples were obtained after the cleaning and drying (60 ºC) stages of the production. A grain auger from different points of the bulk batches was used, and a 5 kg sample was retrieved. Subsequently, grains were homogenized, and the sample size was reduced into portions of 1 kg and stored at 4 °C in polyethylene bags. At last, for long-term use, samples were stored at -20ºC (Piacentini et al., 2018; Iwase et al., 2020).

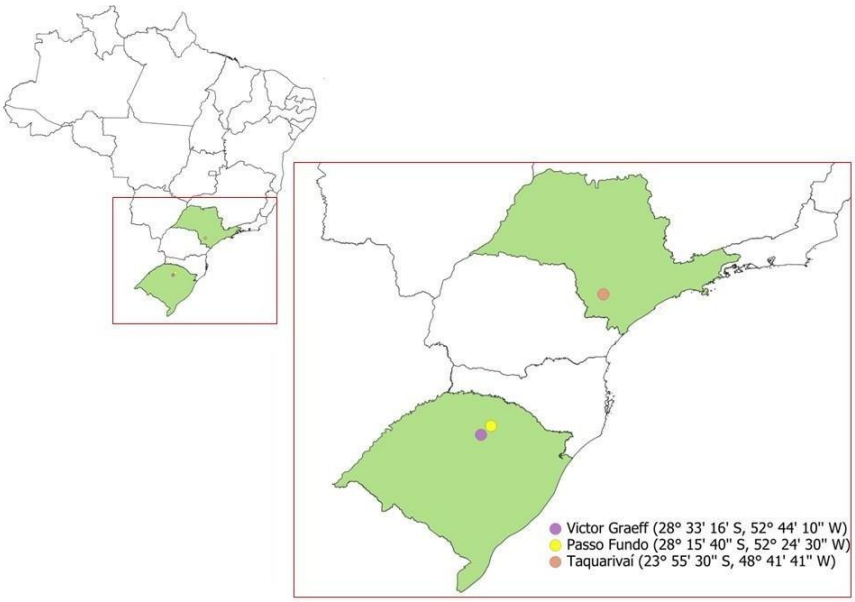


Figure 1. Map of Brazil, indicating the sites where barley samples were collected and their respective coordinates.

2.2. Mycotoxin analysis using reference method based on UPLC-MS/MS

The QuEChERS method described by Anastassiades et al. (2003) and Zachariasova et al. (2010) was used for mycotoxin extraction, with some modifications. Firstly, grain samples were milled and homogenized. Then, a 50 mL polypropylene tube was filled with 2 mg of each sample, 10 mL of 0.1% formic acid and 10 mL of acetonitrile. Next, the mixture was vigorously agitated for 20 minutes, followed by the addition of 4 g $MgSO_4$ and 1 g NaCl. The supernatant was then diluted with deionized

water (1:1) and filtered (0.2 µm nylon membrane filter).

The detection and quantification of DON, NIV, ZEN, ENN B, ENN B1, ENN A, and ENN A1 were carried out by the UPLC-MS/MS (Bolechová et al., 2015), using Acquity UPLC (Waters) with the XEVO TQ MS system (Waters) and a C_{18} column (5 mm × 2.1 mm × 1.7 µm, Acquity UPLC BEH). The mobile phase was employed in the following gradient elution conditions: H_2O + HCOOH 0.1% (v/v) (mobile phase A) and NH_4HCO_2 + HCOOH 0.1% (v/v) (mobile phase B), with a flow rate of 0.4 mL/min and an injection volume of 2.5 µl. The start of the elution was at 0 min with 10 % mobile phase B, which increased linearly to 20% (0.3 min) and to 99.5% (4.5 min). This condition was held until 7.1 min and then turned back to 10% of phase B. Finally, the column's initial condition was rebalanced for 10 min before other following injections.

MS/MS parameters are described in Table 1, which was carried out in the MRM mode ESI (ion- [M + H] + mode; source temperature: 150 °C; desolvation temperature: 450 °C; cone nitrogen gas flow: 15 L/h; desolvation gas flow: 700 L/h; capillary voltage 3 kV). Data processing was performed with MassLynx™ version 4.1. software (Waters, USA). Selectivity, recovery and extended uncertainty were obtained by spiking barley samples with non-detectable mycotoxin levels at three different toxin concentrations, each with six replicates. The limits of detection (LOD) (minimum concentration of each mycotoxin in the spiked sample; signal-to-noise ratio = 3), limits of quantification (LOQ) (signal-to-noise ratio = 10) and other method validation parameters are described in Table 2 (Iwase et al., 2020).

Table 1. Parameters applied in the UPLC-MS/MS analysis.

Mycotoxin	RT (min)	Precursor ion (*m/z*)	Cone voltage (V)	Product ion 1 *(m/z)*	Collision energy (eV)	Product ion 2 *(m/z)*	Collision energy (eV)
NIV	0.79	313.0	26	125.0	12	177.0	13
DON	1.04	297.1	20	203.0	20	231.1	20
ENN B	4.27	640.4	50	196.1	25	214.1	25
ENN B1	4.38	654.4	50	196.1	30	214.2	30
ENN A	4.54	682.5	50	210.3	27	228.3	27
ENN A1	4.46	668.5	50	210.2	27	228.2	27

Table 2. Method validation parameters for each mycotoxin that was analyzed.

Mycotoxin	Spike level (µg/kg)	Recovery (%) ± RSD	LOD (µg/kg)	LOQ (µg/kg)
Deoxynivalenol	50,0	75,0 ± 5,0	15,0	50,0
	100,0	78,0 ± 8,0		
	500,0	85,0 ± 10,3		
Enniatin B	5,0	96,0 ± 12,0	1,5	5,0
	10,0	101,0 ± 14,0		
	50,0	114,0 ± 7,0		
Enniatin B1	5,0	107,0 ± 10,0	1,5	5,0
	10,0	99,0 ± 6,0		
	50,0	105,0 ± 5,0		
Enniatin A1	5,0	96,0 ± 7,0	1,5	5,0
	10,0	95,0 ± 4,0		
	50,0	99,0 ± 3,0		
Nivalenol	50,0	50,0 ± 2,0	24,0	80,0
	160,0	51,0 ± 8,0		
	800,0	50,0 ± 8,0		
Zearalenone	5,0	82,0 ± 44,0	3,0	10,0
	10,0	86,0 ± 15,0		
	50,0	102,0 ± 7,0		

2.3. Occurrence of *Fusarium graminearum sensu lato* (*s.l.*) and *Fusarium poae* in barley samples

Primarily, subsamples (100 g) of barley grains were obtained, which were disinfected with a chlorine solution (0.4%) for 1 minute and then rinsed with distilled sterile water. Next, 50 grains were apportioned, transferred to PDA (potato dextrose agar) medium plates (10 grains/plate), and incubated for five days at 25ºC. After this period, infected grains of each sample were counted, and the results were expressed in percentages (Pitt & Hocking, 2009). *Fusarium* species were then transferred into PDA and CLA (carnation leaf agar) media for morphological identification of *F. graminearum sensu lato (s.l.*) and *F. poae* (Leslie & Summerell, 2006).

The morphological identification of *F. graminearum s.l.* and *F. poae* was confirmed using *RPB1* locus (O'Donnell et al., 2010; Iwase et al., 2020); at least one strain was identified morphologically as *F. graminearum s.l.,* from each barley sample, were subjected to sequencing. The identification of 50 isolates of *F. graminaerum s.l.* was confirmed based on sequencing. Regarding *F. poae,* due to its typical morphological characteristics (Leslie & Summerell, 2006), only five isolates were sequenced. All sequences were submitted to NCBI.

Fusarium species were grown into PDA for five days at 25ºC. DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was used for extraction; primer design and reaction conditions were conducted according to O'Donnell et al. (2007) and O'Donnell et al. (2010). Sequencing was carried out in an Applied Biosystems® 3500 Genetic Analyzer

(Applied Biosystems, Foster City, CA, USA) and analyzed in the Geneious v. 6.0.6 (Biomatters, Auckland, New Zealand) software. Sequences were submitted to BLAST (Basic Local Alignment Search Tool from the National Centre for Biotechnology Information-NCBI).

2.4. qPCR for quantification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1*

2.4.1. Experiment validation

2.4.1.1. Amplification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* from pure fungal cultures

Two fungal strains, one *F. graminearum s.s.* (FML41) and one *F. poae* (FML59) obtained from the Food Microbiology Laboratory I (State University of Campinas) were used for the first step of the qPCR experiment validation. DNA extraction of both strains was performed using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), as previously described and quantified by band observation in agarose gel at 1% and by spectrophotometry (Biochrom, Cambridge, United Kingdom).

Conventional PCR reactions were performed to verify the presence of the genes *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* in the strains. For *TRI12*/15-ADON and *TRI12*/NIV, a multiplex assay was employed utilizing the primers described by Ward et al. (2002). Whereas for *ZEB1* and *ESYN1*, two distinct reactions were performed with the FPEsyn1_F, FPEsyn1_R, ZEB1_F and ZEB1_R primers obtained in this study (Table 3).

Then, four 5-point qPCR standard curves, one for each target gene, were constructed to assess the performance of the assays (Svec et al., 2015). For this purpose, five-fold dilution series of fungal DNA with its starting concentration at 80 ng/µL were performed. All points were amplified in triplicates in the CFX 384 Touch Real-Time PCR System (Bio-Rad, Hercules, USA), with three no-template controls (NTCs) for each assay. Every curve was elaborated considering the genes each strain portrayed, as previously observed by conventional PCR. Reaction efficiency ($E = 10^{(-1/slope)-}1$), R^2, and slope were calculated for all curves by the CFX Mastro Softwareat version 1 (Bio-Rad, Hercules, USA), and melting curves from each assay were also determined.

Table 3. Quantitative PCR primers utilized in this study for target gene amplification.

Target gene	Primer	Primer sequence (5'-3')	Tm (ºC)	Reference
ESYN1	FPEsyn1_F	ACAAAGCGGTCGAGTCTGTA	59,04	Obtained in this study
	FPEsyn1_R	CGTGAACCTTGGCCTTTTCA	58,97	
ZEB1	ZEB1_F	GGACTCAGTTTTGCCTCGTC	58,85	Obtained in this study
	ZEB1_R	GAAATGGGTCGCAGCTTTGA	59,12	
TRI12 (15-ADON)	15-ADON_F	CTCACAGACCATGTTCCGCA	60,3	Obtained in this study
	15-ADON_R	TGGAGATGACTGCGAATCGG	59,9	
TRI12 (NIV)	NIV_F	CTCCTCGTTGTATCTGGCCC	59,9	Obtained in this study
	NIV_R	CCAACGCCAATCCCCTTCTA	59,7	
EF-1α (grain)	Hor1F	TCTCTGGGTTTGAGGGTGAC	78,5	Nicolaisen et al., 2009
	Hor2R	TCTCTGGGTTTGAGGGTGAC	78,5	

2.4.1.2. Amplification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* from barley grain samples

Initially, three plugs (0.5 cm^2) from the fungal strains described in item 2.4.1.1. were used to contaminate 400 mg of barley grains each. Then, after five days at 25ºC, those grains were used for DNA extraction. Grains were ground, with 100 mg placed into microtubes containing six ceramic beads each. The following steps of the extraction were conducted according to item 2.4.1.1.

Those procedures were performed to elaborate standard curves for *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* in barley. They were posteriorly employed to quantify target DNA in the naturally contaminated samples. Additionally, the matrix effect of each assay was calculated to check whether the food matrix would influence the DNA's recovery when compared to the quantification of DNA analyzed from a pure culture. The formula was based on previous studies, as described below (Svec et al., 2015; Pizzutti et al., 2016).

$$Matrix\ Effect\ (\%): \left[\frac{(\text{slope standard curve with fungal DNA}) - (slope\ standard\ curve\ with\ barley\ DNA)}{slope\ standard\ curve\ with\ fungal\ DNA}\right] 100$$

For the 5-point standard curves, five-fold dilution series of artificially contaminated barley DNA with a starting concentration of 80 ng/µL were performed. Points were amplified in triplicates in the CFX 384 Touch Real-Time PCR System (Bio-Rad, Hercules, USA). Additionally, three NTCs were used for each assay, and amplification was accomplished by employing the primers shown in Table 3. For all assays, R^2, reaction efficiency ($E = 10^{(-1/slope)-}1$), the slope and melting curves were determined by the CFX Mastro Software at version 1 (Bio-Rad, Hercules, USA).

2.4.1.3. Amplification of *EF1- α* from a barley grain sample

An assay targeting the *EF1- α* gene from barley grains was also performed for quantifying barley DNA in the samples and posterior DNA yield normalization. First, DNA was extracted from a grain sample following the steps described in items 2.4.1.1. and 2.4.1.2. Then, a standard curve was constructed by performing a five-folddilution series of barley DNA with its starting concentration at 50 ng/µL. Points were amplified in triplicates, with three NTCs in the CFX 384 Touch Real-Time PCR System(Bio-Rad, Hercules, USA). For this purpose, the Hor1F and Hor2R primers (Table 3) were used. The slope, reaction efficiency ($E = 10^{(-1/slope)-}1$), R^2 and melting curves regarding the assay were obtained from the CFX Mastro Software at version 1 (Bio-Rad, Hercules, USA).

2.4.2. qPCR assay for analyses of naturally contaminated barley samples

To quantify *TRI12/*15-ADON, *TRI12/*NIV, *ZEB1*, *ESYN1* and *EF1- α* in 53 naturally contaminated barley samples, the DNA was extracted according to items 2.4.1.1 and 2.4.1.2. All samples were amplified in duplicates with two NTC for each targeted gene in the CFX 384 Touch Real-Time PCR System (Bio-Rad, Hercules, USA). Next, five qPCR assays, one for every target gene, were performed on the samples. Each qPCR reaction had a final volume of 6,25 µL, which consisted of 1X of SYBR® Green PCR Master Mix (Thermofisher, São Paulo, Brazil), 0,4 µM of each primer (Table 3), DEPC treated water (Thermofisher, São Paulo, Brazil) and 1,5 µL of sample DNA diluted at 1:10. Thermocycling parameters were based on Nielsen et al. (2012).

The obtained Ct values were used for DNA quantification, performed by their interpolation with the standard curves constructed for each gene in barley (item 2.4.1.2.). After obtaining target gene concentrations for all samples, the DNA yield from *TRI12/*15-ADON, *TRI12/*NIV, *ZEB1* and *ESYN1* was normalized from nanograms to pg fungal DNA/ng grain DNA (*EF1- α*) (Nicolaisen et al., 2009; Kulik; Treder; Zaluski, 2015). Finally, those levels were applied for correlation analyses with mycotoxin concentrations and the occurrence of the fungi.

2.5. Statistical analysis

Pearson correlation analysis was performed among concentrations of *TRI12/*15-ADON, *TRI12/*NIV, *ZEB1* and *ESYN1* and their respective mycotoxin levels. Other analyses were also carried out between the occurrence of *F. graminearum s.l.*,

TRI12/15-ADON and *ZEB1* yield, and *F. poae* occurrence*, TRI12/*NIV and *ESYN1* concentrations. All analyses were executed in GraphPad Prism software at version 9 (GraphPad, 2022, v. 9), considering only *p*-values of $p < 0.05$ as statistically significant.

3. Results

3.1. Mycotoxin analysis

Of the 53 analyzed samples in this study, 85% were contaminated with at least one *Fusarium* mycotoxin. It is relevant to mention that ENN A was detected in only one sample and, therefore, not included in the analysis.

Overall, the most frequent mycotoxins were ENNs (85%), followed by DON (64%), ZEN (30%) and NIV (25%). Additionally, among the samples containing ENNs, all of them were contaminated with ENN B, 78% with ENN B1 and 58% with ENN A1. Considering the samples from São Paulo, 92% contained ENN B, 60% DON, 56% ENN B1, 40% ENN A1, 28% NIV and 20% ZEN. As for the ones from Rio Grande do Sul, 79% presented ENN B, 75% ENN B1, 68% DON, 57% ENN A1, 39% ZEN and 21% NIV (Figure 2). It was noticeable that Rio Grande do Sul showed a higher occurrence of mycotoxins, except for ENN B and NIV.

Additionally, 72% of the samples were found to be contaminated with at least two of the mycotoxins analyzed; nevertheless, the co-occurrence of all of the toxins was found in only 9% of the samples; DON, NIV and ENNs (ENN B, B1 and A1) co-occurred in 15% of the samples; DON, ZEN and ENNs in 17%; DON and ENNs in 8%; DON, ZEN, ENN B and ENN B1 in 4%; DON, ENN B and ENN B1 in 4%; DON and ENN B in 8%; ENN B and ENN B1 in 8% of the samples (Figure 3).

Mycotoxin minimum and maximum levels, as well as their mean, are shown in Table 4. ENN B, the most frequent mycotoxin in this study, also exhibited the overall highest level detected (7459.25 µg/kg). Conversely, ENN A1 presented the lowest level detected (5.39 µg/kg). In addition, 23% and 17% of the samples presented DON and ZEN levels above the maximum limits established by Brazilian and European regulations, respectively (1000 and 1250 µg/kg for DON; 100 µg/kg for ZEN) (EC, 2006; Anvisa, 2022).

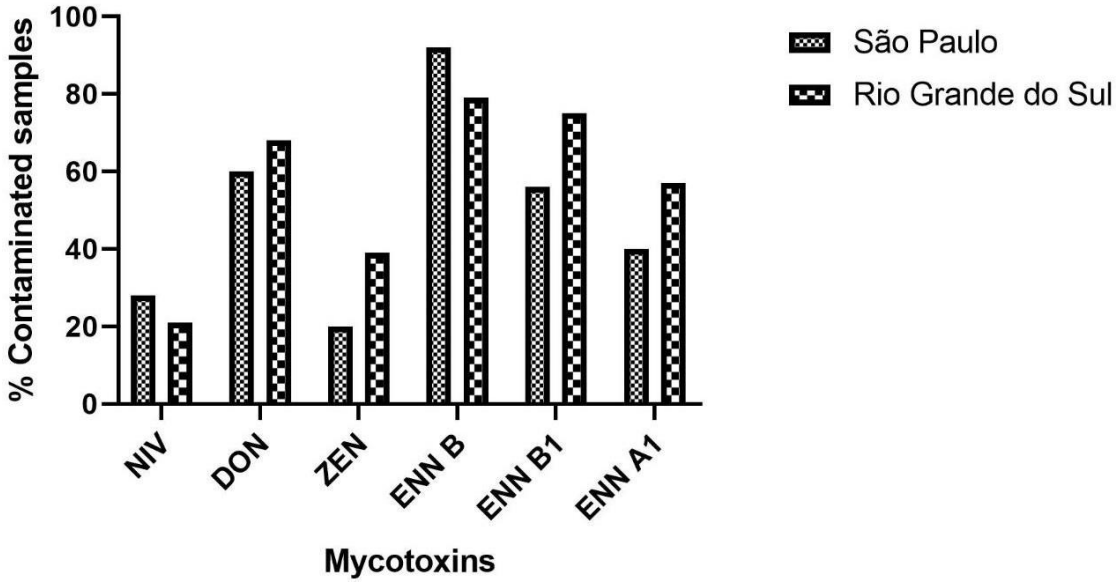


Figure 2. Occurrence of the mycotoxins NIV, DON, ZEN, ENN B, ENN B1 and ENN A1 in barley samples from São Paulo and Rio Grande do Sul.

Table 4. Maximum, minimum and mean levels (µg/kg) of each mycotoxin analyzed in barley samples.

	São Paulo		Rio Grande do Sul	
Mycotoxins	Range (min.-max.)	Mean	Range (min.-max.)	Mean
NIV	ND - 2121.13	438.27	ND - 2074.41	668.89
DON	ND - 2478.71	153.53	ND - 909.38	82.96
ZEN	ND - 57.87	9.62	ND - 556.16	86.73
ENN B	ND - 5269.1	665.01	ND - 7459.25	1434.15
ENN B1	ND - 936.45	133.09	ND - 1390.25	293.12
ENN A1	ND - 111.99	14.24	ND - 141.55	31.34

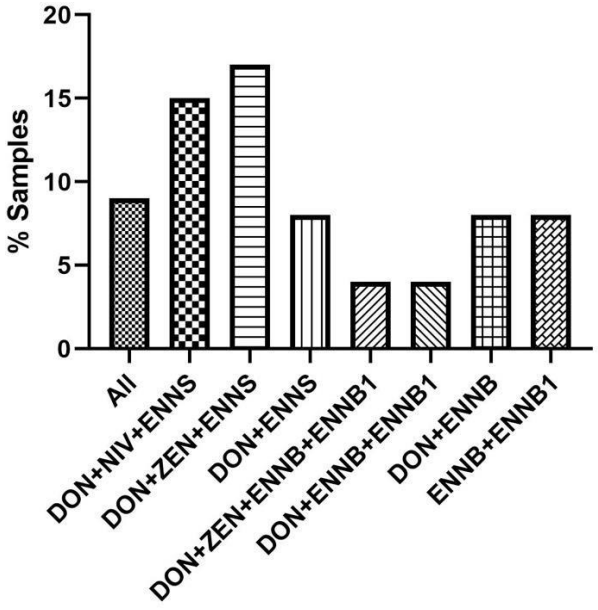


Figure 3. Co-occurrence of mycotoxins in barley samples.

3.2. Occurrence of *Fusarium graminearum sensu latu* (*s.l.*) and *Fusarium poae* in barley samples

Mycobiota demonstrated that 83% of the samples were contaminated by *F. graminearum s.l.*, with infections ranging from 2% to 90%, and 51% were contaminated by *F. poae,* with infections ranging from 2% to 20%. Other *Fusarium* species belonging to the *F. fujikuroi* species complex (FFSC), *F. incarnatum-equiseti* species complex (FIESC), and *F. tricinctum* species complex (FTSC) were isolated but in lower frequency (Table 5).

In the State of Sao Paulo, 54% of the samples were contaminated by *F. graminearum s.l.*, with infections ranging from 10% to 2%; and 15% were contaminated by *F. poae,* with infections ranging from 12% to 0%. Other *Fusarium* species complexes were recovered, including FFSC (20%) and FIESC (16%).

In the State of Rio Grande do Sul, 100% of the samples were contaminated by *F. graminearum s.l.*, with infections ranging from 90% to 2%, and 73% of the samples were contaminated by *F. poae,* with infections ranging from 20% to 2%. Other *Fusarium* species complexes were recovered, including FFSC (1%) and FTSC (12%).

To confirm the morphological identification, five isolates of *F. poae* and at least one *F. graminearum s.l.* isolate from each barley sample were submitted to sequencing

analysis. Among the 50 isolates belonging to the *F. graminearum s.l.*, 80% were identified as *F. graminearum s.s.* (15-ADON genotype), 10% as *F. meridionale* and 10% as *F. cortaderiae*.

Table 5. Occurrence of *Fusarium* spp. isolated from barley samples.

	Frequency (%)	
Fungi	São Paulo	Rio Grande do Sul
F. graminearum s.l.[a]	54	100
F. poae	15	73
FFSC	20	1
FIESC	16	0
FTSC	0	12

FFSC: *Fusarium fujikuroi* species complex; FIESC: *Fusarium incarnatum-equiseti* species complex; FTSC: *Fusarium tricinctum* species complex.

[a] *Sensu Lato*

3.3. qPCR for quantification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1*

3.3.1. Experiment validation

3.3.1.1. Amplification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* from pure fungal cultures

The conventional PCR results showed that the selected *F. graminearum s.s.* (FML41) strain presented the *TRI12*/15-ADON genotype, the predominant within this species (Aoki et al., 2012; Arruda et al., 2021). This strain also portrayed the *ZEB1* gene, indicating that the isolate may produce ZEN (Munkvold et al., 2021). Moreover, the *F. poae* strain (FML59) showed the *TRI12*/NIV genotype and the *ESYN1* gene, suggesting it is a potential NIV and ENN producer, corroborating with the mycotoxin production profile (Munkvold et al., 2021).

In addition, the *TRI12*/15-ADON and *ZEB1* standard curves were constructed with FML41 DNA; the *TRI12*/NIV and *ESYN1* curves were constructed with FML59 DNA (Supplementary Figure 1A-1D). Reactions' efficiency varied from 99.6% (*ZEB1*) to 105.4% (*TRI12*/NIV), which is within the accepted range of 90%-110% (Svec et al., 2015). R^2 were ≥ 0.9 and therefore considered adequate (Kitchen et al., 2010). Lastly, the melting curves exhibit a single peak each (Supplementary Figure 2A-2D), indicating the absence of primer dimers and other interferants in the reactions (Bustin

et al., 2009). Therefore, these results demonstrated satisfactory assay performance in amplifying the *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* genes.

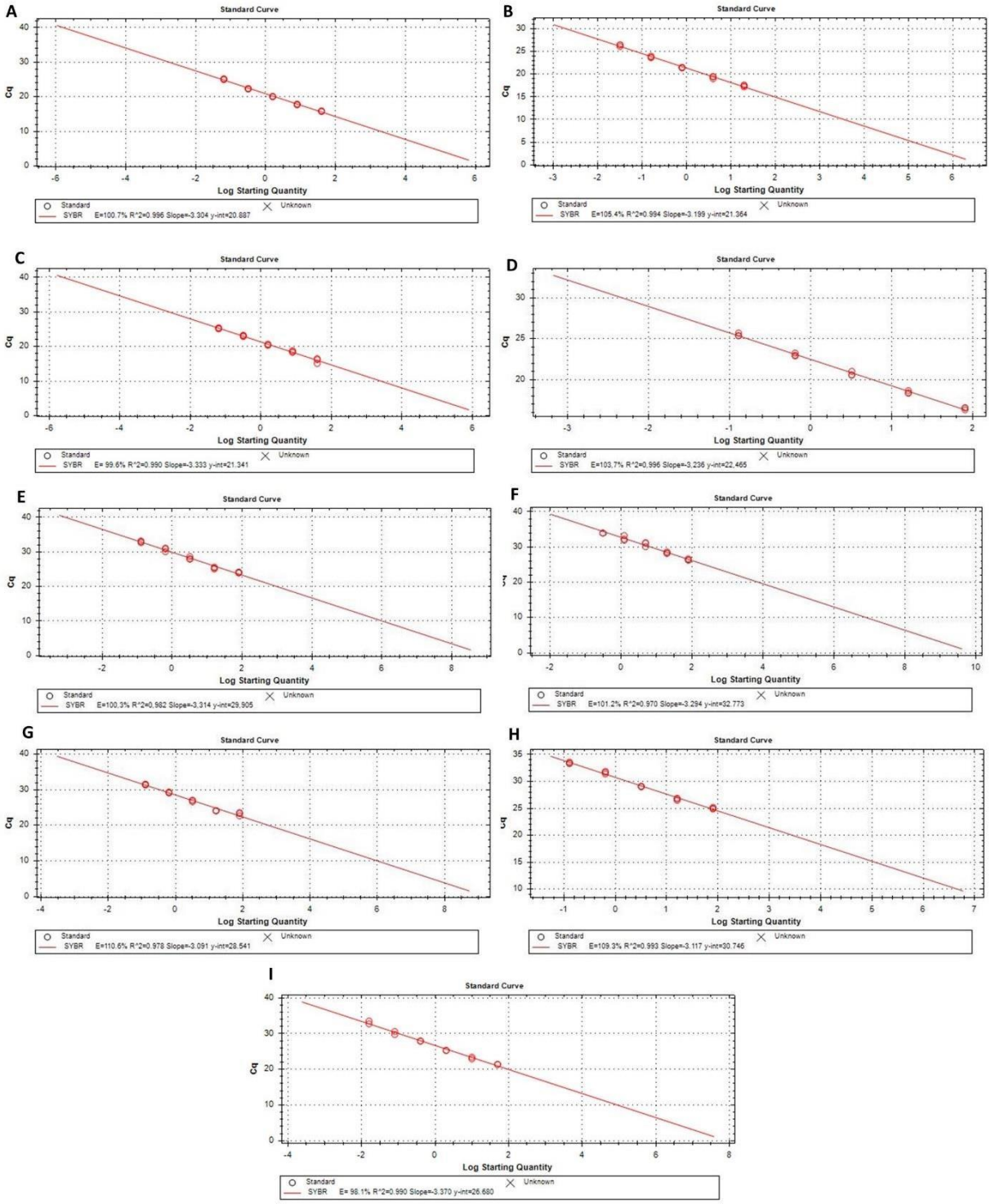
3.3.1.2. Amplification of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* from barley grain samples

According to the results obtained from the conventional PCR assay, FML41 (*F. graminearum s.s.*) was used to contaminate barley grains for later DNA extraction and elaboration of the curves regarding *TRI12*/15-ADON and *ZEB1*. For *TRI12*/NIV and *ESYN1* curves, FML59 (*F. poae*) was used to contaminate barley grains and extract DNA for standard curves (Supplementary Figure 1E-1H). The efficiencies of the reactions ranged from 100.3% (*TRI12*/15-ADON) to 110.6% (*ZEB1*), and R^2 values were ≥ 0.9, which are both within the acceptable range (Kitchen et al., 2010; Svec et al., 2015). Additionally, no non-specific amplification was detected in the melting curve analysis (Supplementary Figure 2E-2H).

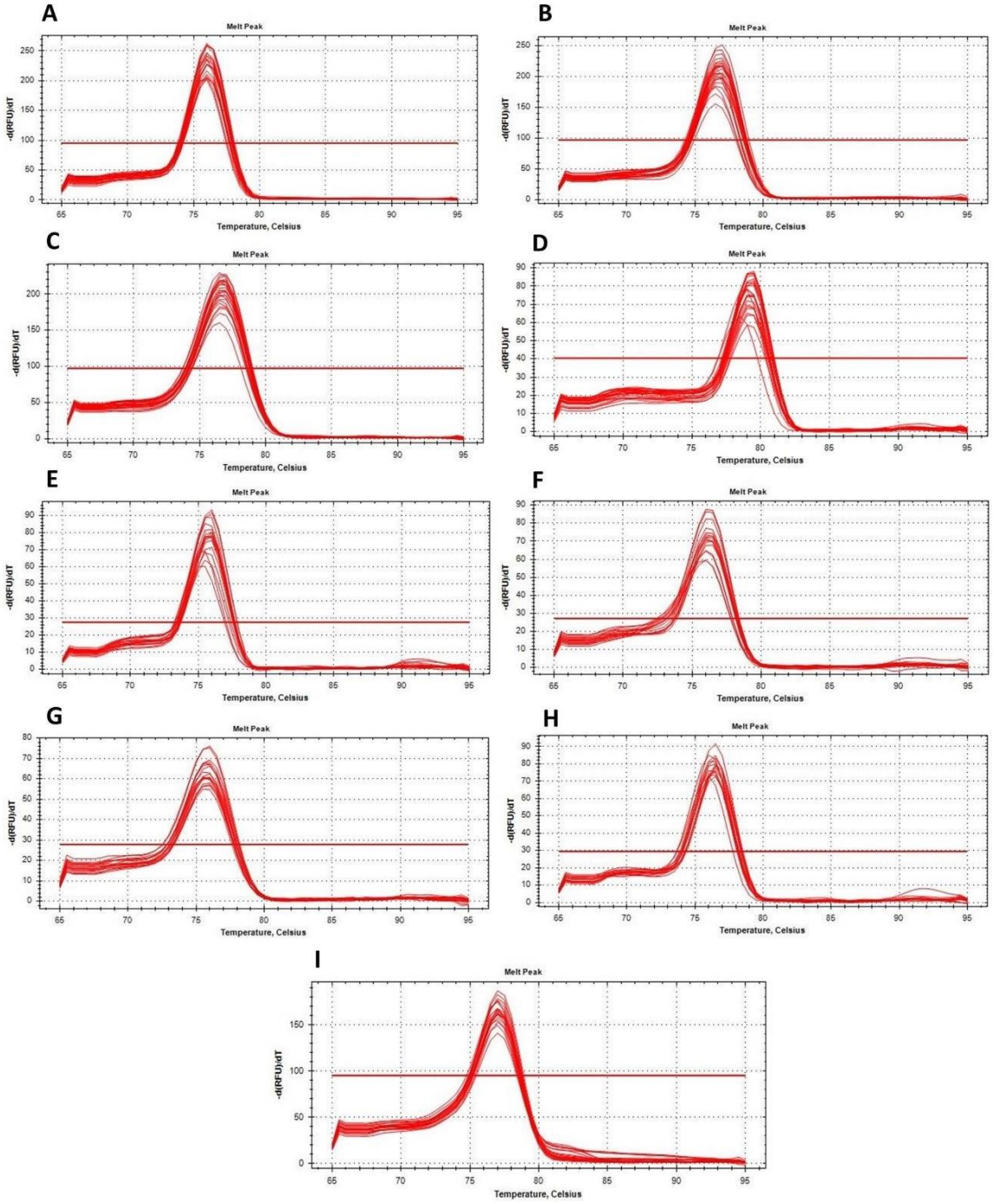
The matrix effects obtained in this study for *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* were -0.3%, -2.97%, 7.26% and 3.68%, respectively. A positive matrix effect indicates suppression in the quantification, and negative values suggest an enhancement might occur (Zhou et al., 2017). However, since the matrix effects reported here are <20%, they were not considered significant (SANTE, 2020).

3.3.1.3. Amplification of *EF1- α* from barley grain samples

The assay targeting *EF1- α* in barley resulted in the standard curve shown in Supplementary Figure 1I. Reaction's efficiency was 98.1% and within the adequate range of 90%-110% (Svec et al., 2015). In addition, R^2 was 0.99, which is also considered appropriate (Kitchen et al., 2010). Moreover, the melting curve obtained exhibited only one peak, suggesting no primer dimers and other interferants were present in the reaction (Supplementary Figure 2I) (Bustin et al., 2009). Altogether, these parameters described suggested a proper DNA quantification, enabling the DNA quantification based on pg of fungal DNA/ng of grain DNA.



Supplementary Figure 1. Standard curves obtained in the analysis, with their efficiency, slope and R^2 values. A-D: Curves retrieved from pure culture DNA. E-H: Curves retrieved from artificially contaminated barley DNA. A: *TRI12*/15-ADON; B: *TRI12*/NIV; C: *ZEB1;* D: *ESYN1;* E: *TRI12*/15-ADON; F: *TRI12*/NIV; G: *ESYN1*; H: *ZEB1*; I: *EF1-α* retrieved from barley DNA.



Supplementary Figure 2. Melting curves obtained in the analysis, each containing a single melt peak. A-D: Curves retrieved from pure culture DNA. E-H: Curves retrieved from artificially contaminated barley DNA. A: *TRI12*/15-ADON; B: *ZEB1*; C: *ESYN1*; D: *TRI12*/NIV; E: *TRI12*/15-ADON; F: *ZEB1*; G: *ESYN1*; H: *TRI12*/NIV; I: *EF1-α*.

3.3.2. qPCR assay for analyses of naturally contaminated barley samples

The genes *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1, ESYN1* and *EF1- α* were quantified in barley by interpolating Ct values of each sample with the standard curves. Then, fungal DNA yield was normalized with the amount of grain DNA from every

sample, resulting in DNA concentrations expressed as pg fungal DNA/ng grain DNA (pg/ng). As a result, fungal DNA was present in all samples, with levels ranging from 2.48 pg/ng (*TRI12*/NIV) to 6333.12 pg/ng (*ESYN1*) (Table 6). Furthermore, most of the DNA recovered was from Rio Grande do Sul, matching the mycotoxin analysis and the isolation results.

Table 6. Maximum, minimum and mean levels (pg fungal DNA/ng grain DNA) of each gene analyzed in this study quantified in barley samples.

Gene	Range (min.-max.)	Mean
TRI12 (15-ADON)	2.80 - 3674.18	530.74
TRI12 (NIV)	2.48 - 1704.50	150.16
ESYN1	46.22 - 6333.12	978.31
ZEB1	12.46 - 1421.76	347.63

3.4. Statistical analysis

3.4.1. Correlations among *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* yield and mycotoxin levels

Correlation analysis was performed between *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* yield and mycotoxin levels. In this regard, no significant association was found among *ZEB1* and ZEN, as well as *TRI12*/NIV and nivalenol (data not shown). This may be because both toxins were detected in low levels and incidence, hindering the statistical analysis (Alisaac et al., 2019; Islam et al., 2021).

In contrast, ENN B, ENN B1 and ENN A1 levels were significantly correlated to *ESYN1* yield in samples. Moreover, *TRI12*/15-ADON concentrations were also positively associated with DON levels (Table 7). Statistical analysis was feasible in this case, considering the higher frequency of these toxins in the samples. Altogether, these results suggest that the qPCR assays described in this study could be used for screening DON and ENNs in highly contaminated barley samples.

Table 7. Pearson correlation analysis (r) results among *ESYN1* yield and ENN B, ENN B1 and ENN A1 levels as well as between *TRI12*/15-ADON yield and DON levels ($p < 0.05$).

	ESYN1	*TRI12*/15-ADON
ENN B	0.8241	-
ENN B1	0.8610	-
ENN A1	0.8414	-
DON	-	0.8999

3.4.2. Correlation among the *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* yield and the occurrence of *F. graminearum s.l.* and *F. poae*

The association between the yield of *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* and the occurrence of *F. graminearum s.l.* and *F. poae* was also assessed. These two groups of fungi were selected for correlation tests because they were the most detected species.

F. graminearum s.l. was significantly correlated with *ZEB1* and *TRI12*/15-ADON yield, with a greater association with the first. Additionally, *TRI12*/NIV and *ESYN1* were positively correlated to *F. poae* incidence (Table 8), indicating that the qPCR based on these genes might also be helpful in inferring fungal contamination in barley grains. It is essential to highlight that sequencing analysis revealed more than *F. graminearum s.l.* species, most of the identified isolates belonged to the *F. graminearum s.s.* 15-ADON genotype, which corroborates with the results of the correlation test. Other species within *F. graminearum s.l.* may also produce ZEN, which may explain the stronger correlation obtained.

Table 8. Pearson correlation analysis (r) results among the occurrence of *F. graminearum s.l.* and *F. poae* and *ESYN1, TRI12*/15-ADON, *TRI12*/NIV and *ZEB1* yield ($p < 0.05$).

	% F. graminearum s l.	*% F. poae*
ZEB1	0.9162	-
TRI12 (15-ADON)	0.8931	-
TRI12 (NIV)	-	0.8457
ESYN1	-	0.9093

4. Discussion

The current study has shown the potential of qPCR for screening mycotoxin contamination and the occurrence of related *Fusarium* species in barley grains based on mycotoxin biosynthetic genes. Positive correlations were found between the levels of ENNs and *ESYN1*, as well as between DON levels and *TRI12*/15-ADON. Moreover, the occurrence of *F. graminearum s.l.* and *F. poae* was significantly correlated to DNA levels, with the first being associated with *TRI12*/15-ADON and *ZEB1* and the latter with *TRI12*/NIV and *ESYN1*.

Barley grain samples were mainly contaminated by *F. graminearum s.l.*, followed by *F. poae* and other *Fusarium* species, as previously demonstrated by various studies (Garmendia et al., 2018; Nogueira et al., 2018; Iwase et al., 2020; Pereira et al., 2021). The high occurrence and levels of DON and ENNs corroborated the mycobiota results (Munkvold et al., 2021). Therefore, qPCR assays based on the quantification of key genes involved in mycotoxin production were tested to check whether this method could be used to estimate mycotoxin and fungal contamination.

The validation of qPCR assays showed adequate results, which included efficiency tests, specificity based on melting curves, and matrix effect. The last test was performed to evaluate whether the food matrix could affect the quantification of *TRI12*/15-ADON, *TRI12*/NIV, *ESYN1* and *ZEB1*.

The matrix effect is defined by the Directorate-General for Health and Food Safety (SANTE) 11312/2021 as “an influence of one or more co-extracted compounds from the sample on the measurement of the analyte concentration or mass”. Regarding qPCR, multiple components that are part of a food matrix may interfere with the target DNA quantification (Svec et al., 2015). Therefore, matrix effect assessment could be an important step in such studies to avoid potential enhancement or suppression in DNA quantification.

So far, studies targeting mycotoxin biosynthetic genes based on qPCR, such as *omt-1* (aflatoxins), *idh* (patulin), *otanpsPN* (ochratoxin A) and *TRI12* (DON and NIV) in foodstuffs have not evaluated the matrix effect on the DNA quantification (Rodríguez et al., 2011a; Rodríguez et al., 2011b; Rodríguez et al., 2012b; Tralamazza et al., 2016, Góral et al., 2022). In our study, the matrix effect was below 20% for all tested genes, which is considered non-significant (SANTE, 2020).

On this subject, *TRI12*/15-ADON, *TRI12*/NIV, *ZEB1* and *ESYN1* yields were obtained in pg fungal DNA/ng grain DNA (pg/ng) with no need for further normalization.

The levels ranged from 2.48 pg/ng (*TRI12*/NIV) to 6,333.12 pg/ng (*ESYN1*). These results are consistent with mycotoxin frequency, where ENNs were the most detected, and NIV was the least detected in barley samples. Higher gene quantifications, levels of mycotoxins, and fungal contamination were retrieved from samples from Rio Grande do Sul, probably due to the State's extensive cereal production and favorable climatic conditions for FHB development (Del Ponte et al., 2015; Mallmann et al., 2017).

F. graminearum s.s. is considered the prevailing FHB pathogen worldwide (Osborne & Stein, 2007; Scoz et al., 2009; Astolfi et al., 2011). In this study, 83% of the samples were contaminated by *F. graminearum s.l.* and 51% by *F. poae*; the latter has also been reported as a FHB pathogen (Pereira et al., 2021). Based on sequencing results of a subset of isolates, *F. graminearum s.s.* was the most frequent species in our samples. Similar results, showing a higher frequency of *F. graminearum s.s.* and lower of *F. poae*, were obtained in barley samples from Argentina and Uruguay, suggesting these two species are considerably adapted for colonizing this crop in South America (Garmendia et al., 2018; Nogueira et al., 2018).

A positive correlation was observed between *TRI12*/15-ADON yield and *F. graminearum s.l.* incidence in this study. Correlations between this gene and the occurrence/biomass of *F. graminearum s.l* main species (*F. graminearum s.s.*) have been previously reported (Nielsen et al., 2012; Vogelgsang et al., 2019) since 15-ADON is the most prevalent genotype within this species (Aoki et al., 2012; Arruda et al., 2021).

Moreover, no former research has associated *ZEB1* levels with the occurrence of *F. graminearum s.l.* However, quantification of another gene from the ZEN biosynthetic pathway (*PKS4*) has been successfully applied to detect and quantify ZEN-producing *Fusarium* species, including *F. graminearum s.s.* (Meng et al., 2010). In addition, significant correlations between this species' DNA yield and ZEN levels have been described (Fredlund et al., 2008; Vogelgsang et al., 2019; Birr et al., 2020; Gavrilova et al., 2021). This suggests that the presence of *F. graminearum s.l.* is associated with ZEN contamination and, therefore, with the occurrence of this mycotoxin biosynthetic pathway key genes, including *ZEB1*, as reported in this study.

Positive correlations between *TRI12*/NIV and *ESYN1* levels and the occurrence of *F. poae* were obtained in this work. Different results were described by another study, which showed a non-significant correlation between NIV genotype yield and *F. poae* incidence. This lack of association may have occurred due to the primers

employed, which were specifically designed for *F. graminearum s.l.* (Vogelgsang et al., 2019). Additionally, it is important to highlight that *F. poae* has been reported as one of the main NIV producers (Schöneberg et al., 2018; Islam et al., 2021; Yli-Mattila et al., 2022; Senatore et al., 2023).

Other studies attempting to correlate *F. poae* DNA yield and ENN levels didn't obtain significant relationships (Orlando et al., 2019; Yli-Mattila et al., 2022), which may be due to the less considerable contribution of *F. poae* to ENN contamination when other efficient ENN-producing species are present (Stępień & Waśkiewicz, 2013; Gautier et al., 2020). However, even if at lower levels, *F. poae* is a well-known ENN producer and therefore portrays the *ESYN1* gene, which supports the positive correlations reported in our study (Stępień & Waśkiewicz, 2013).

Considering the correlations between DNA yield and mycotoxin levels, no significant relationships were found among *TRI12*/NIV and nivalenol concentrations, as well as between *ZEB1* and ZEN levels. Both mycotoxins were detected in low incidence and levels in this study, which may be the reason behind such results. Other studies have presented the same issues in associating fungal DNA yield with less detected toxins (Alisaac et al., 2019; Islam et al., 2021). In addition, although the correlation between the levels of *ZEB1* and ZEN was insignificant in the current study, an association between *PKS13* yield, a ZEN biosynthetic pathway gene, and ZEN content has been reported in the literature (Atoui et al., 2012).

Significant correlations were demonstrated between ENN levels and *ESYN1* yield, as previously described by Kulik et al. (2011). Nevertheless, the authors found a better association between *ESYN1* from *F. avenaceum/F. tricinctum* and ENN levels than among *ESYN1* from *F. poae* and this mycotoxin content. Another study assessing *F. avenaceum*, *F. tricinctum* and *F. poae* occurrence through *EF1-α* quantification by qPCR found only an association of *F. avenaceum* and *F. tricinctum* DNA with ENN content (Orlando et al., 2019).

These stronger associations among DNA from *F. avenaceum* and *F. tricinctum* and ENNs may occur because both are considered the most efficient ENN-producing species in various hosts (Stępień & Waśkiewicz, 2013; Gautier et al., 2020). In this study, the occurrence of *F. poae* was correlated with *ESYN1* incidence, probably because this species was found in 51% of the barley samples, indicating that this species may contribute to the occurrence of ENN in barley samples.

Another significant correlation was obtained between *TRI12*/15-ADON yield and the levels of DON. Previous studies also got such close associations (Nielsen et al.,

2012; Vogelgsang et al., 2019). Additionally, *TRI5*, the gene that encodes the first enzyme for trichothecene biosynthesis (Proctor et al., 2020), has also shown positive correlations with DON levels (Schiro et al., 2019; Ducos et al., 2021). Similar results were also reported by Góral et al. (2022), which found significant correlations between the DNA of *F. culmorum* (3-ADON and NIV genotypes) and FHB incidence and toxin levels in wheat.

The current study suggests that qPCR assays based on quantifying mycotoxin biosynthetic genes are useful for detecting toxigenic fungal biomass and screening high mycotoxin content in grain samples (Rodríguez et al., 2015). In this regard, other works have also successfully developed such assays for estimating aflatoxins (AF), patulin (PAT), ochratoxin A (OTA) and fumonisins (FUMs) as well as their respective producing fungi in food matrixes (Rodríguez et al., 2012a; van Rensburg et al., 2015; Preiser et al., 2015; Tannous et al., 2015; Hussien et al., 2017). Additionally, this technique provides results on the occurrence of toxigenic fungi and mycotoxins and enables high sample throughput and reduced analysis time, which is highly advantageous (Rodríguez et al., 2015).

5. Conclusions

The quantification of the *TRI12* (15-ADON and NIV genotypes) *ZEB1* and *ESYN1* was successfully performed in barley. Significant correlations between DNA levels and mycotoxin contamination were only obtained among DON and ENNs since ZEN and NIV were detected in lower frequencies and levels. Additionally, *TRI12/*15-ADON and *ZEB1* were positively correlated to the occurrence of *F. graminearum s.l.;* and *TRI12/*NIV as well as *ESYN1* were significantly associated with *F. poae* incidence. These results demonstrate the use of qPCR for screening highly mycotoxin and toxigenic *Fusarium*-contaminated samples.

It is worth emphasizing its more significant potential for estimating toxigenic fungi and levels of mycotoxins when a key biosynthetic gene is used for qPCR quantification since they are directly involved in mycotoxin production. Nevertheless, qPCR assays based on gene expression could give more solid results on mycotoxin occurrence in foodstuffs, as the RNA will provide information on the overall expression of these genes. Therefore, more studies would be interesting for developing novel qPCR assays.

Credit authorship contribution statement

Letícia A. G. A. da Silva: Investigation, Validation, Writing – original draft. Liliana O. Rocha: Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Declarations of competing interest

The authors declare no competing financial interest.

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Conclusões

Os genes *TRI12* (genótipos 15-ADON e NIV), *ZEB1* e *ESYN1* foram quantificados com sucesso em grãos de cevada por meio do ensaio de qPCR proposto neste estudo. Adicionalmente, a validação do método apresentou resultados adequados e sem efeito significativo da matriz. Apenas DON e ENNs apresentaram correlações positivas com seus respectivos genes (*TRI12*/15-ADON e *ESYN1*), considerando que ZEN e NIV foram pouco detectadas. Ademais, foram encontradas correlações positivas para os genes *TRI12*/15-ADON e *ZEB1* com a ocorrência de *F. graminearum s.l.,* como também para *TRI12*/NIV e *ESYN1* com a incidência de *F. poae* nas amostras.

Estes resultados indicam o potencial no uso desta técnica como uma ferramenta mais rápida e que possibilita a avaliação de um maior número de amostras para predizer micotoxinas altamente detectadas e contaminação por espécies toxigênicas de *Fusarium* em amostras de grãos. Por fim, vale ressaltar o maior potencial da técnica quando utilizada para a quantificação de genes chave da via biosintética de micotoxinas, considerando que estes estão diretamente envolvidos em sua produção.

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