Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 73 (2008) 33–40
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for down-stream real-time PCR quantification and correlation to mycotoxin levels

Elisabeth Fredlund [a,*], Ann Gidlund [a], Monica Olsen [a], Thomas Börjesson [b], Niels Henrik Hytte Spliid [c], Magnus Simonsson [a]

[a] *National Food Administration, P.O Box 622, SE-751 26 Uppsala, Sweden*
[b] *Lantmännen Business development, Grain, SE-531 87 Lidköping, Sweden*
[c] *Institute of Integrated Pest Management, Forsøgsvej 1, DK-4200 Slagelse, Denmark*

Received 30 October 2007; received in revised form 17 January 2008; accepted 17 January 2008
Available online 26 January 2008

Abstract

Identification of *Fusarium* species by traditional methods requires specific skill and experience and there is an increased interest for new molecular methods for identification and quantification of *Fusarium* from food and feed samples. Real-time PCR with probe technology (Taqman®) can be used for the identification and quantification of several species of *Fusarium* from cereal grain samples. There are several critical steps that need to be considered when establishing a real-time PCR-based method for DNA quantification, including extraction of DNA from the samples. In this study, several DNA extraction methods were evaluated, including the DNeasy® Plant Mini Spin Columns (Qiagen), the Bio robot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen), and the Fast-DNA® Spin Kit for Soil (Qbiogene). Parameters such as DNA quality and stability, PCR inhibitors, and PCR efficiency were investigated. Our results showed that all methods gave good PCR efficiency (above 90%) and DNA stability whereas the DNeasy® Plant Mini Spin Columns in combination with sonication gave the best results with respect to *Fusarium* DNA yield. The modified DNeasy® Plant Mini Spin protocol was used to analyse 31 wheat samples for the presence of *F. graminearum* and *F. culmorum*. The DNA level of *F. graminearum* could be correlated to the level of DON ($r^2 = 0.9$) and ZEN ($r^2 = 0.6$) whereas no correlation was found between *F. culmorum* and DON/ZEA. This shows that *F. graminearum* and not *F. culmorum*, was the main producer of DON in Swedish wheat during 2006.

Keywords: Absolute quantification; Deoxynivalenol; *Gibberella zeae*; TaqMan; qPCR; Zearalenone

1. Introduction

Fusarium species are found in cereals and other cultivated crops in Europe and in other parts of the world (Bahtnager et al., 2002; Miller, 1994; Parry et al., 1995; Placinta et al., 1999; Tanaka et al., 1988). Most of them are able to produce one or more mycotoxins with varying degree of toxicity (Bottalico and Perrone, 2002; Langseth et al., 1999). The trichothecenes constitute the largest group of *Fusarium* toxins found in cereals but also zearalenone (ZEN) and fumonisins are detected (Bahtnager et al., 2002; Hussein and Brasel, 2001; Tanaka et al., 1988). The *Fusarium* toxins have been evaluated by the European Commission (Scientific Committee on Food, 1999, 2000a,b,c, 2001) and by JECFA (WHO, 2001). Deoxynivalenol (DON), T2, and HT-2 were identified as the most critical mycotoxins based on their occurrence in cereals being close to the Tolerable Daily Intake (TDI) (Scientific Committee on Food, 1999, 2001). In 2006, the European Union decided on a uniform legislation to protect the health of the consumers and set limit values for DON, ZEN, Fum B1 and B2 in unprocessed and processed foods including cereals (EC 1881/2006). Limit values will also be determined for T2 and HT-2 (EC 1881/2006). *F. graminearum* (sexual stage = *Gibberella zeae*), *F. culmorum*, *F. avenaceum, F. poae*, and *F. sporotrichioides* are common in grain and are

* Corresponding author. National Food Administration, P.O Box 622, SE-751 26 Uppsala, Sweden. Tel.: +46 18 175788; fax: +46 18 105848.
E-mail address: Elisabeth.Fredlund@slv.se (E. Fredlund).

0167-7012/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2008.01.007

causative agents of Fusarium head blight disease (FHB), which causes great yield loss worldwide (Parry et al., 1995). Each *Fusarium* species produce a more or less characteristic set of toxins but strain variations within a species also occurs (O'Donnell et al., 2000). *F. graminearum* and *F. culmorum* are the main producers of DON and ZEN in wheat (WHO, 2001) and consumption of these toxins can cause nausea, diarrhoea, vomiting, skin irritation, and feed refusal (WHO, 2001). *F. graminearum* is common worldwide and dominates in warmer climates whereas *F. culmorum* has been limited to cooler regions (Miller, 1994; Parry et al., 1995). However, there are reports showing that *F. graminearum* is spreading also in the cooler regions of Northern Europe (Bottalico and Perrone, 2002; Waalwijk et al., 2003). The increased cultivation of maize, which is an important host for *F. graminearum*, changes in tillage practice, as well as climatic changes, has been suggested to explain this (Birzele et al., 2002; Dill-Mackay and Jones, 2000).

Quantification and identification of *Fusarium* species have traditionally relied on culture methods and morphological classification that require specific expertise and experience. Culturing methods take time and are dependent on living propagules, which may not be related to toxin levels. In recent years, several PCR-based techniques have been developed to overcome this problem, for review see Edwards et al. (2002). PCR-based identification has several applications such as to study the dynamics of different *Fusarium* species over time and between geographical regions in cereals or other environments or to study disease development in the field. It can also be an important tool in the risk assessment of grain as a screening method to identify samples with potentially high mycotoxin content to reduce costs for chemical analyses.

Real-time PCR methods have been described for several *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* and correlation between DNA from single or groups of *Fusarium* species and their corresponding mycotoxins has been evaluated (Leisová et al., 2006; Sarlin et al., 2006; Schnerr et al., 2002; Waalwijk et al., 2004; Yli-Mattila et al., in press). In wheat from the Netherlands, correlation between DON and DNA was found for both *F. graminearum* and *F. culmorum* (Waalwijk et al., 2004) whereas in Finnish barley only *F. culmorum* could be correlated to DON production (Sarlin et al., 2006). Schnerr et al. (2002) used the *tri5* as the target gene and showed that DNA from the group of trichothecene-producing species correlated to DON in wheat. However, when using the *tri5* gene as target, no information is obtained on the species contribution to the toxin production. Different PCR-based methods employ different DNA extraction methods as well as different primer and probe systems. The degree of correlation between DNA and mycotoxin in grain has also shown to vary substantially. As a first step to a more standardized approach, this paper presents an evaluation of several DNA extraction methods from fungal mycelia in wheat and an optimized extraction method for the external standard used for absolute quantification of *Fusarium* DNA in grain. We also investigated the correlation between DNA levels of *F. graminearum* and *F. culmorum* and their mycotoxins DON and ZEN in 31 Swedish wheat samples.

2. Materials and methods

2.1. Fungal strains and mycelium production

Fusarium graminearum, strain IBT 1958, and *Fusarium culmorum*, strain IBT 2303, were used to produce DNA standards for the absolute quantification of *Fusarium* in wheat. Both strains are deposited at the DTU culture collection in Lyngby, Denmark. To produce fungal mycelia, the isolates were grown in MEA-broth (LP0039, Oxoid Ltd, Hampshire, UK) in a water bath (100rpm) at 25 ± 0.5°C for 5days. The mycelium was washed twice in sterile tap water, centrifuged for 10min at 4000×*g*, and then freeze-dried (Edwards Modulyo freeze dryer). The freeze-dried mycelia were stored in eppendorf tubes at − 20°C until DNA extraction.

2.2. Wheat samples

Thirty-one wheat samples were selected from field trials from the harvest of 2006 in the middle and south parts of Sweden and collected within the project "Survey of fusarioses and fusariumtoxins in winter wheat" sponsored by Swedish Farmers' Foundation for Agricultural Research.

The wheat samples (100g) were milled to fine powder on a Waring Blender (Torrington, Connecticut, USA) and used for both chemical toxin analysis and DNA extraction. The samples were freeze-dried (see above) and stored at − 20°C until DNA extraction.

2.3. DNA isolations from fungal mycelia

Genomic DNA of *F. graminearum* and *F. culmorum* was extracted from 10mg of freeze-dried mycelia using four different methods (A, B, C, and D). All extractions were performed in duplicates. Method A was based on the commercial kit DNeasy® Plant Mini Spin Columns (Qiagen, Solna, Sweden) but modified in the cell lysis and protein removal steps. Fungal mycelium (10mg), 200µl S3 lysis buffer [66mM Tris, 3.3% Triton-X, 1.65M guanidinium–HCL, 0.825M NaCl, and ddH_2O, pH 7.9; (Mulfinger et al., 2000)], 4µl RNAse A (100mg ml^{-1}, Qiagen), 5µl proteinase K (20mg ml^{-1}, Sigma-Aldrich), and 200µl AP1 lysis buffer (provided by the DNeasy® Plant Mini Spin Column kit) were added to Lysis Matrix A tubes (BIO101 Systems, Qbiogene). The tubes were run in a FastPrep® Cell Disrupter, model FP120 (BIO101 Savant, Qbiogene) for 20s at speed level 5.0. The lysate was incubated in a heating block at 65°C for 1h and thereafter treated according to the protocol supplied with the DNeasy® Mini Spin Column kit (Qiagen). An additional phenol extraction step [once with one volume phenol:chloroform: isoamylalcohol (25:24:1) and twice with one volume chloroform:isoamylalcohol (24:1)] was added after the sample had passed through the QIAshredder column supplied by the DNeasy® Mini Spin Column kit.

Method B used the Bio robot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen). Freeze dried mycelia was lysed in 350µl G2 buffer (supplied with the kit) and 250U of lyticase (Sigma-Aldrich, MO, USA) as suggested in the user-