8

Novel Methods for the Quantification of Pathogenic Fungi in Crop Plants: Quantitative PCR and ELISA Accurately Determine *Fusarium* Biomass

Kurt Brunner, Andreas Farnleitner and Robert L. Mach
Vienna University of Technology, Institute of Chemical Engineering, Vienna Austria

1. Introduction

Fungi of the genus *Fusarium* are worldwide occurring plant pathogens which cause severe damages to numerous cultivable plants (Weiland et al., 2000; Mirete et al., 2004; Youssef et al., 2007; Li et al., 2008) with the highest economical losses upon infection of maize, wheat and barley (Windels et al., 2000; Nganje et al., 2004). *Fusarium* caused diseases can destroy crops within several weeks and the infection leads to quality losses in two different aspects: besides the reduced yield due to reduced kernel size, the fungus produces various toxic metabolites while colonizing the plant. These mycotoxins heavily impair the quality of the harvest (McMullen et al., 1997). The acute or chronic toxicity of *Fusarium* released compounds led to the introduction of national or international limits to regulate mycotoxin levels in food and feed (e.g. limits of the European Community since 2006).

Most *Fusarium* species are widely distributed in substrates such as soil, on subterranean and aerial plant parts, plant debris, and on dead organic matter. Many *Fusarium* species have active or passive means to disperse spores or conidia in the atmosphere. The ability to grow on a broad range of substrates combined with their efficient dispersal mechanisms enables a widespread distribution of these fungi (Burgess, 1981). *Fusarium* species are well adapted to grass hosts (Leonard & Bushnell, 2003) and can colonize on many agricultural commodities such as rice, bean and soybean. According to several studies *F. graminearum* and *F. culmorum* are among the most aggressive plant pathogenic fungi known. *F. graminearum* mainly occurs in temperate and warmer regions of the USA, China and the southern hemisphere (Osborne & Stein, 2007). It is regarded as one of the most vigorous toxin producers and has therefore become the most intensively studied plant pathogen (Goswami & Kistler, 2004). In contrast, *F. culmorum* predominates in the cooler regions including the U.K., Northern Europe and Canada (Osborne & Stein, 2007) and is also associated with the occurrence of many mycotoxins (Desjardins, 2006).

2. Toxigenicity and impacts

A worldwide problem of agricultural industry producing wheat, barley and maize is the fungal disease *Fusarium* head blight (FHB), with maize and wheat as the economically most

important host plants. The disease is associated with *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum*, *F. sporotrichoides* and *Microdochium nivale*. Each fungus contributing to FHB has particular biological and environmental requirements, which in part explains the frequency of occurrence in specific locations.

This destructive disease was initially described in 1884 in England and was considered a major threat during the early years of the twentieth century (Goswami & Kistler, 2004). Nowadays, *Fusarium* head blight is a disease of massive economic impact worldwide and has been ranked by the United States Department of Agriculture (USDA) as the worst plant disease to hit the nation (Windels, 2000). The losses due to direct and secondary economic impact of FHB on wheat and barley were estimated at $2.7 billion for the period from 1998 – 2000 in the US (Nganje et al., 2002). Epidemics of FHB are strongly influenced by various factors such as local and regional environment, the physiological state and genetic background of the host, as well as pathogen related factors including host adaptation and virulence.

Infected plant debris serves as primary substrate for common FHB pathogens, which are able to survive the winter period as saprophytic mycelium or thick walled chlamydospores. Warm, moist conditions in spring are favorable for the development and maturation of conidia and perithecia, which produce ascospores (Markell & Frankl, 2002). These sticky spores are discharged from the surface of crop debris and dispersed by wind, rain or even insects to the host plants. Infected wheat or maize presents brown, dark purple to black lesions on the exterior. The head of cereals has a characteristically bleached appearance, hence the name *Fusarium* head blight. During prolonged wet periods infected spikelets, glumes and kernels present pink to salmon-orange spore masses of the fungus. Maize infections occur at the ear apex following colonization of the kernels with white mycelium, which turns pink to red with time.

Fungal colonization is affected by numerous variables such as spike morphology, canopy density, plant height, rainfall, relative humidity, temperature and host plant resistance (Kolb et al., 2001; Rudd et al., 2001). *Fusarium* infections are increased due to the lack of completely resistant plant material and a frequent application of unsuitable cropping systems. Pathogen survival is favored in reduced tillage systems as residue burial speeds up the decomposition and reduces pathogen reproduction and survival (Khonga et al., 1988; Pereyra et al., 2004). Petcu et al., (1998) investigated the influence of crop rotations on the severity of FHB. Studies show that the economically most lucrative cultivation of alternating maize and wheat cultures turned out to be problematic. The increased production of these *Fusarium* favorable crops and the dramatic increase in their residues remaining on the soil surface provide a large increase in the amount of niche available to the pathogen. *Fusarium* non-host plants used as preceding crops or intertillage of wheat and maize are often less profitable and therefore of low interest for farmers. In North and South America erosion causes a dramatic loss of fertile topsoil. To avoid this loss, no-till systems have been established to overcome the drawback of conventional farming. However, *Fusarium* inoculum density increases in soil in reduced tillage systems compared to plough treated fields (Steinkellner & Langer, 2004).

3. Plant resistance to *Fusarium*

Plant resistance can be defined as the relative amount of heritable qualities possessed by a plant that reduces the degree of damage to the plant caused by pathogens. Schroeder and

Christensen (Schroeder & Christensen, 1963) described in their work two phenotypic measures of disease resistance; type I resistance operates against initial infection and type II resistance describes the resistance to spreading within infected tissues. A third type of *Fusarium* resistance (type III) was described by Wang and Miller (1988) as the insensitivity of wheat lines to toxins or the ability of the resistant cultivar to degrade mycotoxins.

Integrated control strategies are essential to prevent *Fusarium* diseases in modern agriculture. The use of resistant cultivars is considered necessary for numerous reasons. Firstly, modern agronomic practices such as reduced tillage and cereal and maize rich crop rotations have the tendency to increase the amount of *Fusarium* related diseases. In addition to this, chemical control measures are only partly effective as only few fungicides are sufficiently active against *Fusarium* and their application must be performed within a time frame of a few days around the flowering stage of the plants. For the protection of maize cultures no fungicides are commercially available up to now. Understanding *Fusarium* life-cycle and infection pathways is the first step in preventing disease. Nevertheless, uncontrollable factors such as weather conditions during as well as economic or ecologic interests prevent sustainable success in the reduction of these pathogens. Plant breeders have made significant progress in the development of *Fusarium* resistant maize and wheat varieties by identifying genetic regions, which are linked to the resistance of plants (Buerstmayer et al., 2003; Draeger et al., 2007). Resistance against *Fusarium* is a quantitative trait, which is governed by many independent genes distributed on several distinct genetic regions (quantitative trait loci, QTLs) in the plant genome. Therefore an effective approach to investigate FHB resistance is the identification of the QTLs and their mapping (Buerstmayer et al., 2009). The mapping of QTLs is the basis for further efforts towards a straightforward marker assisted resistance breeding strategy. Interestingly, often the best regionally adapted and highly productive crop varieties are susceptible to FHB. For this reason, breeders are faced with the difficult task to combine adaption to certain locations with high yield and *Fusarium* resistance.

The production of mycotoxins has been identified as a crucial factor for successful infection . Deoxynivalenol is considered the most common occurring mycotoxin involving *Fusarium* infection. However, DON levels in infected grain vary significantly amongst wheat cultivars (Bai et al., 2001) infected with the same *Fusarium* isolate. The evaluation of *Fusarium* resistance of either the parents in current breeding programs or the control of resistance levels of novel lines can be carried out by chemical analysis of the DON accumulation in the seeds. In general DON levels in resistant plants are much lower than those found in more susceptible cultivars. Artificial infection of wheat-ears with *Fusarium* conidia followed by determination of DON content and visual scoring of the head blight symptoms are the currently established methods for resistance assessment. Nevertheless, the determinations of disease symptoms in combination with the mycotoxin content of grain are both indirect methods to evaluate plant resistance. Furthermore, both methods observe the same effect, as visual disease symptoms are mainly caused by mycotoxin intoxication and not by the growth of mycelium. Studies have shown that the amount of fungal mycelia formed during infection not always correlates well with these parameters (Waalwijk et al, 2004; Hill et al., 2006; Brunner et al., 2009; Kulik et al. 2011). Asymptomatic kernels may contain significant amounts of mycotoxins while symptomatic kernels within the same sample may not.

4. Methods for the identification of *Fusarium* and quantification of the infection

Quantification and identification of *Fusarium* species in agriculture or plant pathology is traditionally carried out using culture based methods. Usually single Kernels are spread on petri dishes with appropriate medium and the number of infected kernels is counted after several days. For a further classification of the species a morphological investigation of the grown mycelium is necessary. This kind of species determination is time consuming and requires experience and specific expertise. Furthermore, culture based methods rely on living propagules and the obtained results not always reflects the biological situation. The pathogen detection is only reliable at late stages of the infection when a spread of the disease can no longer be controlled by fungicides (McCArtney et al., 2003). Besides these drawbacks the results are only semi-quantitative as only the number of infected kernels can be determined but not the grade of infection of each kernel. Other conventional methods include instrumental analysis such as ergosterol quantification. Ergosterol is a characteristic compound of fungal cell walls. However this approach is not specific to pathogens or a certain fungal species and the ergosterol amount relative to the fungal mycelia varies within *Fusarium* species (Pasanen et al., 1999). Finally, the analytical determination of ergosterol is not easier than that of DON, resulting in an alternative method, which is not commercially applicable to determine the *Fusarium* resistance of plants.

Recent studies aim at developing more direct techniques to quantify *Fusarium* related diseases which can i) reduce the number of analyses to accurately assess *Fusarium* infection, ii) eliminate pleiotropy between disease symptomology and mycotoxin production, iii) reduce the error associated with environmental effects and iv) reduce errors related with asymptomatic expression of FHB (Hill et al., 2008). In general, two distinct approaches have become accepted within the last two decades: immunoassays and (quantitative) PCR. The enzyme linked immunosorbent assay (ELISA) has been developed as a direct measure of *Fusarium* spp. biomass in infested grain samples or plant tissue. ELISA provides at least genus specificity through specific fungal antigens and its ease of sample preparation and low costs make it an interesting novel method. Another approach to overcome the drawbacks of conventional identifications is the development of screening techniques based on DNA identification which are nowadays well established for *Fusarium* species and a broad range of available literature guarantees to find a suitable PCR assay for many applications. Unlike conventional detection methods, samples can be tested directly with ELISA or PCR – no elaborate isolation and cultivation steps are necessary for a suitable detection or quantification.

4.1 Enzyme linked immunosorbent assays (ELISAs)

Enzyme linked immunosorbent assays are based on the specific recognition capabilities of antibodies. These antibodies are usually derived from the immunization of animals (usually rabbits, mice, chicken or goat) with certain immunogens such as culture filtrates or mycelial compounds. After repeated injection of the immunogen blood samples are taken and the serum is used either as a whole or it is applied after certain clean-up steps for the ELISA tests. For the production of the well-defined monoclonal antibodies lymphocytes are isolated from immunized mice and are fused to myeloma cells. The resultant clonal hybridoma cells can be maintained in culture. Soon after the development of the ELISA method in the early 1970s

scientists working on agricultural and plant pathology related topics recognized the potential of this analytical tool. Within only a few years the number of annual ELISA based publications in the AGRICOLA database rose from zero to more than 150 and numerous antibodies were developed to detect plant pathogens belonging to different genera.

4.1.1 ELISAs based methods for the detection and quantification of *Fusarium*

The first immuno assay for *Fusarium* was developed at the University of Göttingen in 1989 by J.G. Unger and published in his PhD thesis. The author succeeded in the recognition of *F. culmorum* exoantigens (compounds secreted by *Fusarium* during growth) by an antibody. However, after this first success of the ELISA in *Fusarium* detection almost 15 years passed by until this *F. culmorum* ELISA was applied to practical applications (Chala et al., 2003). During these field tests the authors found out that the developed antibody is not specific to *F. culmorum* but binds to antigens from various *Fusarium* species. Over the years numerous studies on the development of anti-*Fusarium* antibodies have been published. Although most authors used *F. graminearum* and/or *F. culmorum* for immunization, no group succeeded in the production of highly specific antibodies. Gan et al., (1997) immunized chicken with soluble exoantigens and with soluble frations from homogenized mycelia of F. graminarum, *F. poae* and *F. sporotrichioides*. The *F. graminearum* and *F. sporotrichioides* exoantigen antibodies showed cross-reactivities with almost all common *Fusarium* species. The antibody against *F. poae* exoantigens showed absolutely no cross-reactivity against other *Fusarium* species or against other filamentous fungi. Up to now this is the only species-specific antibody for a *Fusarium* spp. Hill et al., (2006) probably produced the best-characterized antibody so far by isolating monoclonal antibodies produced by IF8 cell lines for the detection of *Fusarium*. These antibodies detected species from different phylogenetic clades and the authors also demonstrated that no cross-reaction – even with closely related ascomycetes – could be observed. Unfortunately, no publication describes the essential analytical parameters like limit of detection, limit of quantification or a linear range of the developed tests.

In general, antibodies detect many different *Fusarium* species but the specificity of PCR based assays has not yet been obtained by immunoassays. Neither the detection of particular species – except one example for *F. poae* – nor the detection of a particular toxigenic group (e.g. trichothecene producers or fumonisin producers) is possible with ELISA methods. Furthermore, most antibodies were raised against unknown compounds secreted by *Fusarium* during growth in liquid medium (exoantigens). As usually the secretion is not a constant attribute but is subjected to complex regulation, the amount of exoantigens secreted during the infection of grains might not always be constant. For this reason, the amount of *Fusarium* antigen detected in a sample may not always correlate with the fungal biomass.

4.1.2 Applications of *Fusarium* ELISAs for agriculture and plant pathology

Many of the developed PCR based tests for the determination of *Fusarium* focus on the screening of harvested grain and frequently the correlation of *Fusarium* DNA content and toxin accumulation is the central topic. Only few (references) of the many *Fusarium* PCR publications deal with plant pathology related questions whereas all developed ELISA methods were extensively used in field applications to monitor the efficacy of fungicide

application (Chala et al., 2003) or to assess the *Fusarium* resistance of crop lines (Miedaner et al., 1994, Miedaner et al., 2004, Hill et al., 2006, Slikova 2009, Rohde & Rabenstein 2005).

4.1.2.1 Evaluation of fungicide efficacy with a *Fusarium* ELISA

Chala et al., (2003) presented a comprehensive study on the evaluation of fungicide application to reduce *Fusarium* infection and DON content in wheat. Winter wheat was planted in four field replications and artificially infected with *F. culmorum* conidia at flowering stage by spray inoculation. Five different fungicides were tested at different application times alone and in various combinations. The efficiency of the treatment was determined by visual scoring of disease symptoms, *Fusarium* ELISA, yield a 1000-grain weight. The combination of these different methods allows the evaluation of the results obtained by the immunoassay. The results of the field trials reveal clearly that only one fungicide showed a high efficiency in the reduction of fungal biomass and DON content. All applied methods led to the same conclusion. Interestingly, the application of some fungicides at a later growth stage after flowering did not affect the fungal biomass but slightly increased the production of DON. In general the study demonstrates clearly the potential of the developed *Fusarium* ELISA to obtain a deeper insight into biomass formation. The main advantage of the ELISA based determination of infection is the enhanced failure-safety.

4.1.2.2 Evaluation of *Fusarium* resistance with ELISA tests

The comparison of the resistance of different plant varieties is often a challenge for breeders. The increase in *Fusarium* resistance of novel crop lines is just marginal and for some plants like durum or triticale disease symptoms are usually not obvious. Furthermore, other fungal diseases like *Microdochium nivale* or *Septoria nodurum* also lead to similar symptoms as *Fusarium* head blight and the result of the visual assessment might be misinterpreted. Besides the PCR based methods described above the ELISA tests represent the only available tool to determine fungal biomass and so the application for resistance tests is self-evident.

The first field test for *Fusarium* resistance based on ELISA analysis was performed in the early 1990s (Miedaner et al., 1994). Winter rye was infected with *F. culmorum* colonized wheat flour in November when the plants have reached the three-leaf status. The colonization of the host plant started early after inoculation and increased continuously till full maturity. Interestingly, different genotypes showed the highest variance in *Fusarium* protein content during medium growth states. In adult states almost no difference in *Fusarium* infection between resistant and susceptible plants was found. The disappearance of the discrepancy results in saprophytic growth of *F. culmorum* on rye during ripening and therefore the medium growth stages are the optimal date to discriminate resistant from susceptible varieties. The same group also investigated the *F. culmorum* resistance of wheat and triticale cultivars in six different environments and demonstrated the performance of ELISA biomass determination for two more cereal crops (Miedaner et al., 2004). However, the correlation of the ELISA based *Fusarium* protein content to the DON content in a sample was not better than the correlation between DON and visual disease symptom assessment. Another ELISA test based on monoclonal antibodies was optimized for the quantification of *Fusarium* in barley (Hill et al., 2006) and was used to study the influence of the applied analytical method on the results of resistance tests (Hill et al., 2008). A mapping population was grown in two environments and breeding lines were grown at four different locations

and disease data were collected by visual disease assessment, determination of the DON accumulation and the *Fusarium* ELISA. The obtained data were subjected to statistical analyses and the authors calculated a model for the prediction of different replications and different environments necessary to certainly identify differences in *Fusarium* resistances. Interestingly, the determination of DON was identified as the factor that is least feasible for resistance studies. The quantification of the *Fusarium* biomass by ELISA required less different locations and fewer field replicates per location to classify barley varieties according to their *Fusarium* resistance than visual disease assessment or toxin measurements.

Often the correlation between visual disease scoring and DON content is better than between ELISA results and DON content. This fact has also been demonstrated in a Slovakian study (Slikova et al., 2009) for the infection of winter wheat with *F. culmorum*. As visual assessment just records the damage caused by the fungal toxins the correlation between symptoms and the DON content is obvious. The fact that visual symptoms and DON content are not independent features makes the combined use of only these two factors for resistance determination questionable. Therefore, Slikova et al. (2009) recommend the application of the *Fusarium* ELSIA to get more reliable results for plant resistance than with the two other methods.

4.2 Detection and quantification of *Fusarium* by PCR methods

The extensive application of DNA based identification technologies has increased the knowledge on suitable diagnostic DNA fragments of *Fusarium* species such as ITS (internal transcribes spacer) or IGS (intergenic spacer) sequences (Gagkaeva & Yli-Mattila, 2004, Jurado et al., 2006, Konstantinova & Yli-Mattila, 2004, Kulik 2008, Yli-Mattila, 2004), mitochondrial DNA (Laday et al., 2004), the β-tubulin encoding gene (Yli-Mattila, 2004, Mach et al., 2004, Reischer et al., 2004), the translation elongation factor gene (Knutsen et al., 2004) and the calmodulin gene (Mule et al., 2004) which were sequenced from numerous *Fusarium* spp. As a result highly specific PCR primers could be developed for *Fusarium* detection. In order to distinguish between producers and non-producers of certain toxins the genes from biosynthesis pathways for mycotoxins were accurately studied. The sequence information gained throughout numerous molecular taxonomic investigations allows not only qualitative applications to detect particular species but also quantitative measurement of fungal biomass. Different techniques like DGGE, RFLP and AFLP can be applied for the identification of adequate PCR targets for a review see Brunner & Mach, (2010), for quantitative detection methods however, only the real-time PCR has been shown to be applicable. Molecular diagnosis of plant pathogenic fungi has been proven to be highly specific, very sensitive and fast and relatively insensitive to microbial backgrounds and non-target organisms (McCartney et al., 2003).

Within the last decade numerous PCR based assays have been published for the detection of most agriculturally important *Fusarium* species. An intensive literature study reveals that all such publications can be separated into two distinct groups. Either the authors focus on highly specific test systems to allow the differentiation of *Fusarium* species or the focus is the comprehensive detection of a whole group sharing a common feature. The latter assays usually use a key-gene for a biosynthetic pathway – e.g. for mycotoxin synthesis – as a target for PCR. This system allows the detection or even the quantification of all isolates, which are able to produce a certain class of toxins even if they belong to different species or to different genera.

4.2.1 Species-specific PCR assays

Species-specific assays allow the determination of one particular *Fusarium* species. This method focuses on high selectivity in order to quantify a target species even in a background of highly similar isolates. Several TaqMan based PCR assays have been developed for the quantification of some of the predominant species associated with head blight in Europe including *F. graminearum*, *F. poae*, *F. culmorum*, F. sporotrichioides, F. verticillioides or *F. avenaceum* . The authors clearly demonstrated the advantage of quantitative real time PCR systems combined with a high-throughput DNA extraction protocol over morphologic based methods. Unlike conventional agar-plating techniques, the quantification of different fungal species is possible directly from infested material (grain or plant tissue) and the entire procedure is less time consuming than microbiological methods (for a review see Brunner & Mach, 2010).

4.2.2 Group specific assays

Group specific assays were designed for the simultaneous quantification of all strains, which produce certain toxins such as trichothecenes, fumonisins or enniatin, based on key-genes involved in the mycotoxin biosynthesis. In contrast to the species-specific assays, a whole group of *Fusarium* spp., regardless of taxonomic origin, that is able to produce a certain class of mycotoxins can be quantified in a single run. A correlation between qPCR determined fungal biomass and DON, fumonisin or enniatin content in cereals could be shown using these group specific assays.

Detectable species	PCR target	Type of assay	Reference
F. graminearum, F. culmorum, F. avenaceum	RAPD derived	qualitative	Schilling et al., 1996
trichothecene producers	*tri5*	qualitative	Niessen et al., 1998
F. graminearum, F. culmorum	RAPD derived	qualitative	Nicholson et al., 1998
trichothecene producers	*tri5*	quantitative	Schnerr et al., 2001
trichothecene producers	*tri5*	qualitative	Edwards et al., 2001
F. graminearum, F. poae	IGS	quantitative	Yli-Mattila et al., 2008
fumonisin producers	fum1	quantitative	Bluhm et al., 2004
F. graminearum	*tub1*	quantitative	Reischer et al., 2004
F. graminearum, F. culmorum, F. avenaceum	RAPD derived	quantitative	Waalwijk et al. 2004
F. solani	rRNA, small subunit	quantitative	Li et al., 2008
fumonisin producers	*fum1*	quantitative	Waalwijk et al. 2008
enniatin producers	*esyn1*	quantitative	Kulik et al. 2011

Table 1. Overview of frequently applied PCR assays. This table cites the original method development papers (qualitative and quantitative) but not potentially following publications demonstrating the practical application of these assays.

4.2.3 Applications of the *Fusarium* PCR in agriculture and plant pathology

In contrast to the practical applications of the developed ELISA tests, the PCR assays are only rarely used to address typical questions related to plant pathology.

Some methods were developed to identify the chemotype of different *Fusarium* strains on a molecular basis. These assays are designed to differentiate between nivalenol, 3- and 15-acetyl-deoxynivalenol chemotypes of *F. graminearum*, *F. culmorum*, and F. cerealis. The biosynthetic pathway of trichothecene production is highly conserved between different *Fusarium* species and the genes within the trichothecene gene cluster are well investigated. Based on this knowledge three genes were chosen in all studies to identify the chemotypes: tri3, tri5, tri7 and tri13 or regions between these genes. The developed assays were applied to *Fusarium* monitoring programs in numerous countries of Europe and Nortehrn America (Chandler et al., 2003; Jennings et al. 2004; Lia et al., 2005; Quarta et al., 2006; Stepien et al., 2008;).

A tri5 based assay was used to evaluate the efficiency of fungicides against *F. graminearum* and *F. culmorum* (Edwards et al., 2001. Three chemical fungicides were applied alone in various concentrations and in combinations. Interestingly, the authors could demonstrate that different pathogenic fungi require different fungicides for the optimal control of the disease. The study revealed a good correlation between the DON content of samples and the *Fusarium* DNA. Furthermore, the ration between DON and the fungal DNA was not altered by the application of fungicides. This fact is of particular interest as previous in vitro studies postulated the enhanced toxin production of *Fusarium* after the treatment with antifungal compounds.

Most of the published studies focus on correlations between accumulated toxins and the PCR determined fungal DNA concentration in harvested grain and the PCR is postulated as a screening method for applications in food and feed safety. For some of these studies species specific assays were used to assess the biomass of various *Fusarium* species (Schnerr et al., 2000; Waalwijk et al., 2004; Brandfass & Karlovsky, 2006; Yli-Mattila et al. 2008) or group specific assays were used to identify of quantify certain toxin producers (Schnerr et al. 2001, 2002; Bluhm et al. 2004; Waalwijk et al., 2008; Kulik et al., 2011).

Although the sensitive PCR methods represent an optimal tool to monitor even minor amounts of *Fusarium* during colonialization of a host these applications are rare. Reischer et al. (2004) developed a TaqMan based quantitative PCR assay to quantify *F. graminearum* directly from infected plant material. A central spikelet of wheat heads was artificially infected with conidia and the increase of fungal biomass was monitored by real-time PCR and the disease symptoms were also assessed visually. Due to the superior sensitivity of the PCR assay the disease could be detected and quantified the first day after inoculation, whereas the first visual symptoms became obvious five days later. Nicholson et al. (1998) used PCR tests for *F. culmorum* and *F. graminearum* to address the fungal spread in wheat. The authors found out that the severity of *Fusarium* stem rot caused by *F. culmorum* correlates not only with the inoculums load but also with the time point the inoculums is applied. The earlier in the season the conidia are applied the more fungal DNA could be measured in the stem. The influence of trichothecene production on successful infection was tested in the same study with toxigenic and atoxigenic *F. graminearum* isolates. The toxin producers colonized grain better than non-producers, supporting the theory of trichothecens

as a virulence factor. However, non-producing strains could also infect the wheat heads but the amount of detected fungal DNA was only 1 to 10% of the DNA amount found in plants infected with toxin producing strains. The infected wheat heads were also subjected to visual assessment of disease symptoms. Although the conservative method led to the same results the difference between toxigenic and atoxigenis strains was less pronounced. The above described assay for *F. culmorum* was also used to monitor natural stem rot in wheat in the U.K. (Nicholson et a., 2002), The authors found out that usually other pathogens than *F. culmorum* were the causal agent of this disease as almost no quantifiable DNA of *Fusarium* was present in the samples. The same *F. culmorum* assay was again used in combination with a *F. poae* assay to evaluate fungicide efficiency for *Fusarium* head blight control in a greenhouse experiment (Doohan et al., 1999). Under all tested conditions the applied fungicides reduced the disease severity between 20% and 80% and in general the results could be confirmed by visual monitoring of the disease symptoms.

A Canadian *Fusarium* monitoring program integrated PCR tests for *F. avenaceum*, *F. culmorum*, F. crookwellense, *F. poae*, *F. sporotrichioides*, *F. equiseti*, *F. pseudograminearum*, and *F. graminearum* to compare results with the traditional agar plating method (Demeke et al., 2005). For 83% of the tested grain samples the two methods led to the same results. However, the agar plate method not always gave positive results when DNA was found in samples. This leads to the assumption that PCR based methods provide enhanced sensitivity. Additionally, *F. graminearum*, *F. pseudograminearum* and *F. crookwellense* could not be distinguished by morphological analysis whereas PCR could clearly differentiate these species. This comprehensive study clearly demonstrated the power of DNA based methods if integrated into *Fusarium* monitoring programs. PCR allows the quantification (if applied as real-time PCR) and classification of numerous strains within a few hours of analysis. Classical methods need some days to weeks to give the same results and quantification is difficult as only the number of infected kernels can be determined.

An LNA-TaqMan based assays was used to investigate the differences in *Fusarium* resistance of twenty novel wheat lines ranging from highly susceptible to highly resistant (Brunner et al., 2009). The wheat-ears were inoculated with *F. graminearum* and *F. culmorum* in two consecutive years and the formation of mycotoxins, the accumulation of *Fusarium* DNA and the visual disease symptoms were recorded. This study demonstrated a certain discrepancy between visual scoring and quantitative PCR results. In accordance to other studies the visible symptoms matched perfectly the DNA content in medium to low resistant lines. In contrast, highly resistant lines with low *Fusarium* biomass – but nevertheless high amount of toxins – did sometimes not show any disease symptoms. The authors addressed this effect to the detoxification mechanism of high resistant plants as some toxins can be "masked" by the plant. So they are converted to less plant-toxic metabololites which do not damage the wheat-ears.

5. Conclusions

Visual assessment of disease caused by *Fusarium* species is frequently insufficient to identify the causal agent or to quantify the plant pathogen. In natural systems pathogenic fungi often occur in a combination of species, which can induce similar symptoms and a visual discrimination might be impossible. Other methods like agar plate assays rely on the fact that viable propagules are present in a sample. Especially in harvested grain this is not

always the case as long dry periods during ripening in combination with intense UV radiation can reduce the survival of fungal mycelium or even of conidia. Furthermore, the morphologic discrimination of grown mycelium is difficult and experienced personal is indispensible. Novel methods for the detection and quantification of fungal biomass based on marker molecules (e.g. antigens or DNA) have the potential to revolutionize the field of pathogen monitoring in plant pathology. Within the last twenty years immunoassays and PCR methods found their way into this scientific field. ELISA test for different *Fusarium* species have been developed intensely in the 1990s and were applied in numerous field studies. However, the specificity of the produced antibodies leaded a lot to desire. Although particular species were used for the immunization of animals the resulting antibodies did detect many different *Fusarium* species. Only one group succeeded in the production of polyclonal antibodies which were specific to a single species, namely to *F. poae* . Furthermore, the re-production of antibodies is difficult as immunization is a complex procedure. The resulting immunoglobulins can vary in their features and therefore a careful characterization must be performed each production cycle. Interestingly with the availability of affordable real-time PCR cyclers on the market published studies using *Fusarium* ELISA almost disappeared. Most probably this tendency can be ascribed to two crucial advantages of PCR methods: i) these methods can be performed in high throughput and are easily transferred to other laboratories. Primers are synthesized commercially, are cheap and they are available within a few days. ii) PCR assays are highly specific and can be used to detect either genera or species and even distinctive isolates can be differentiated. Although quantitative PCR provides numerous advantages to study *Fusarium* in its environment only a few studies have been published to address biological problems. This might be due to the fact that nowadays food safety related questions are of high relevance and so the comparison between fungal DNA and mycotoxins is estimated as more important than other applications. On the other hand, the barrier for traditional plant pathologist might still be high to enter a technical field like quantitative PCR.

An investigation of the NCBI pubmed database reveals that the published studies applying *Fusarium* PCR methods in field-trials increase continuously. Although less novel assays are developed, more and more previously published methods are included in current studies, which might indicate the changeover from PCR based method development to PCR applications.

6. Acknowledgements

The authors thank the Federal Country Lower Austria and the European Regional Development Fund (ERDF) of the European Union for financial support.

7. References

Bai, G.H., Plattner, R., Desjardins, A. & Kolb, F. (2001). Resistance to *Fusarium* head blight and deoxynivalenol accumulation in wheat. *Plant Breeding*, 120(1), pp 1-6.

Baird, R., Abbas, H.K., Windham, G., Williams, P., Baird, S., Ma , P., Kelley, R., Hawkins L. & Scruggs M. (2008). Identification of select fumonisin forming *Fusarium* species using PCR applications of the polyketide synthase gene and its relationship to fumonisin production in vitro. *International Journal of Molecular Sciences*, 9: 554-570.

Bluhm, B.H., Cousin, M.A. & Woloshuk, C.P. (2004). Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. *Journal of Food Protection*, 67(3): 536-543.

Brandfass, C. & Karlovsky, P. (2006). Simultaneous detection of *F. culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. *BMC Microbiology*, 6:4

Brunner, K., Kovalsky Paris, M.P., Paolino, G., Bürstmayr, H., Lemmens, M., Berthiller, F., Schuhmacher, R., Krska, R. & Mach R.L. (2009). A reference-gene-based quantitative PCR method as a tool to determine *Fusarium* resistance in wheat. *Analytical and Bioanalytical Chemistry*, 395(5), pp 1385 - 1394.

Brunner, K. & Mach R.L. (2010). Quantitative Detection of Fungi by Molecular Methods: A Case Study on *Fusarium*. In: *Molecular Identification of Fungi*. Gherbawy, Y., Springer-Verlag, Berlin Heidelberg, pp 93-105.

Buerstmayr, H., Steiner, B., Hartl, L., Griesser, M., Angerer, N., Lengauer, D., Miedaner, T., Schneider, B. & Lemmens, M. (2003). Molecular mapping of QTLs for *Fusarium* head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. *Theoretical and Applied Genetics*, 107(3), pp 503-508.

Buerstmayr, H., Ban, T. & Anderson, J.A. (2009). QTL mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. *Plant Breeding*, 128(1), pp 1-26.

Burgess, L.W. (1981). General ecology of the Fusaria. P. E. Nelson, T. A. Toussoun, & R. J. Cook, In: *Fusarium: diseases, biology, and taxonomy*. Pennsylvania State University Press, University Park, 1981: p. 225-235.

Chala, A., Weinert J., Wolf G.A. (2003). An Integrated Approach to the Evaluation of the Efficacy of Fungicides Against *Fusarium culmorum*, the Cause of Head Blight of Wheat. *Journal of Phytopathology*, 151, 673-678.

Chandler, E.A., Simpson, D.R., Thomsett,M.A. & Nicholson, P. (2003). Development of PCR assays to Tri7 and Tri13 trichothecene biosynthetic genes, and characterisation of chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis*. *Physiological and Molecular Plant Pathology* , 62 (2003) 355–367.

Demeke, T., Clear, R.M., Patrick, S.K. & Gaba, D. (2005). Species-specific PCR-based assays for the detection of *Fusarium* species and comparison with the whole seed agar plate method and trichothecene analysis. *International Journal of Food Microbiology*, 103, pp 271-284.

Desjardins, A.E. (2006). *Fusarium* mycotoxins. Chemistry, Genetics, and Biology. APS Press, St. Paul, MN, 2006.

Doohan, F.M., Parry, D.W. & Nicholson, P. (1999). *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. *Plant Pathology*, 48, pp 209-217

Draeger, R., Gosman, N., Steed, A., Chandler, E., Thomsett, M., Srinivasachary, J. Schondelmaier, H., Buerstmayr, H., Lemmens, M. & Schmolke, M.. (2007). Identification of QTLs for resistance to *Fusarium* head blight, DON accumulation and associated traits in the winter wheat variety Arina. *Theoretical and Applied Genetics*, 115(5), pp 617-625.

Edwards, S.G., Progozliev, S.R., Hare, M.C. & Jenkinson, P. (2001). Quantification of trichothecene producing *Fusarium* species in harvested grain by competitive PCR to determine efficiacies of fungicides against *Fusarium* head blight of winter wheat. *Applied and Environmental Microbiology*, 67(4): 1575-1580.

Gagkaeva, T.Y. & Yli-Mattila, T. (2004). Genetic diversity of *Fusarium* graminearum in Europe and Asia. *European Journal of Plant Pathology*, 110: 551-562.

Gan, Z., Marquardt, R.R., Abramson, D. & Clear R.M. (1997). The characterization of chicken antibodies raised against *Fusarium* spp. by enzyme-linked immunosorbent assay and immunoblotting. *International Journal of Food Microbiology*, 16, 38(2-3), pp 191-200.

Goswami, R.S. & Kistler, H.C. 2004). Heading for disaster: *Fusarium* graminearum on cereal crops. *Molecular Plant Pathology*, 2004. 5(6): p. 515-525.

Hill, N.S., Hiatt, E.E. & Chanh, T.C. (2006). ELISA analysis for *Fusarium* in barley. *Crop Science*, 46: p. 2636 - 2642.

Hill, N.S., Neate, S.M., Cooper, B., Horsley, R., Schwarz, P., Dahleen, L.S., Smith, K.P., O'Donnell, K. & Reeves, J. (2008). Comparison of ELISA for *Fusarium*, Visual Screening, and Deoxynivalenol Analysis of *Fusarium* Head Blight for Barley Field Nurseries. *Crop Science*, 48, pp 1389-1398.

Jennings, P., Coates, M.E., Turner, J.A., Chandler, E.A. & Nicholson P. (2004). Determination of deoxynivalenol and nivalenol chemotypes of *Fusarium* culmorum isolates from England and Wales by PCR assay. *Plant Pathology,* 53, pp 182–190.

Jurado, M., Vázquez, C., Sanchis, V. & González-Jaén, M.T. (2006). PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in maize. *Systematic and Applied Microbiology*, 29: 681-689.

Khonga, E.B. & Sutton, J.C. (1988). Inoculum production and survival of Gibberella zeae in maize and wheat residues. Canadian Journal of Plant Pathology 10: p. 232-240.

Knutsen, A.K. & Holst-Jensen, A. (2004). Phylogenetic analyses of the *Fusarium* poae, *Fusarium* sporotrichioides and *Fusarium* langsethiae species complex passed on partial sequences of the translation elongation factor-1 alpha gene. *International Journal of Food Protection*, 95: 287-295.

Kolb, F.L., Baib, G-H., Muehlbauer, G.J., Andersonc, J.A., Smithc K.P. & Fedak G. (2001). Host Plant Resistance Genes for *Fusarium* Head Blight: Mapping and Manipulation with Molecular Markers. *Crop Science*, 41(3), pp 611-619.

Konstantinova, P. & Yli-Mattila, T. (2004). IGS-RFLPanalysis and development of molecular markers for identification of *Fusarium* poae, *Fusarium* langsethiae, *Fusarium* sporotrichioides and *Fusarium* kyushuense. *International Journal of Food Microbiology*, 95: 321-331.

Kulik, T. (2008). Detection of *Fusarium* tricinctum from cereal grain using PCR assay. *Journal of Applied Genetics*, 49(3): 305–311.

Kulik, T., Jestoi, M. & Okorski, A. (2011). Development of TaqMan assays fort he quantitative detection of *Fusarium* avenaceum/*Fusarium* tritinctum and *Fusarium* poae esyn1 genotypes from cereal grain. *FEMS Microbiology Letters*, 314, pp49-56.

Laday, M., Mule, G., Moretti, A., Hamari, Z., Juhasz, A., Szecsi, A., Logrieco, A., (2004). Mitochondrial DNA variability in *Fusarium* proliferatum (Gibberella intermedia). *European Journal of Plant Pathology*, 110, pp 563–571

Leonard, K.J. & Bushnell, W. (2003). *Fusarium* head blight of wheat and barley. St.Paul, Minn, USA: APS Press, 2003.

Li, S., Hartman, G. L., Domier L.L. & Boykin, D. (2008). Quantification of *Fusarium solani* f. sp. glycines isolates in soybean roots by colony-forming unit assays and real-time quantitative PCR. *Theoretical and Applied Genetics*, 117: 343-352.

Lia, H., Wua, A., Zhaoa, C., Scholten, O., Löffler H., & Yu-Cai Liaoa (2005).Development of a generic PCR detection of deoxynivalenol- and nivalenol-chemotypes of *Fusarium* graminearum. *FEMS Microbiology Letters*, 243(2), pp 505-511

Mach, R.L., Kullnig-Gradinger, C.M., Farnleitner, A.H., Reischer, G. Adler, A. & Kubicek, C.P. (2004). Specific detection of *Fusarium* langsethiae and related species by DGGE and ARMS-PCR of a β-tubulin (*tub1*) gene fragment. *International Journal of Food Protection*, 95, pp 333-339.

Markell, S.G. & Francl, L.J. (2003). *Fusarium* head blight inoculum: species prevalence and *Gibberella zeae* spore type. *Plant Disease*, 87, pp 814-820.

McCartney, H.A., Foster, S.J., Fraaije, B.A. & Ward E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Sciences*, 59, pp129-142

McMullen, M., Jones, R., & Gallenberg, D. (1997). Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. *Plant Disease* 81(12): 1340-1348.

Miedaner, T., Beyer, W., Höxter, H. & Geiger, H.H. (1994). Growth Stage Specific Resistance of Winter Rye to *Microdochium nivale* and *Fusarium* spp. In the Field Assessed by Immunological Methods. *Phytopathology*, 85, pp 416-421.

Miedaner, T., Heinrich, N., Schneider, B., Oettler, G., Rohde, S. & Rabenstein, F. (2004). Estimation of deoxynivalenol (DON) grain content by symptom rating and exoantigen content for resistance selection in wheat and triticale. *Euphytica*, 139(2), pp 123-132

Mirete, S., Vázquez, C., Mulè, G., Jurado M. & González-Jaén M.T. (2004). Differentiation of *Fusarium verticillioides* from Banana Fruits by IGS and EF-1α Sequence Analyses. *European Journal of Plant Pathology*, 110(5-6), pp 515-523.

Mulè, G., Susca, A. Stea, G. & Moretti, A. (2004). A species specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. roliferatum* and *F. subglutinans*. *European Journal of Plant Pathology*, 110: 495-502.

Mulfinger, S., Niessen, L. & Vogel, R. (2000). PCR based quality control of toxigenic *Fusarium* spp.in brewing malt using ultrasonication for rapid sample preparation. *Advances in Food Science*, 22(1/2): p. 38 - 46.

Nganje, W.A., Bangsund, D.A., Leistritz, F.L., Wilson,W.W., & Tiapo, N.M. (2004). Regional Economic Impacts of *Fusarium* Head Blight in Wheat and Barley. *Review of Agricultural Economics*, 26(3), pp 332-337.

Nicholson, P. Simpson, D.R., Wilson, A.H., Chandler, E. & Thomsett A. (2004). Detection and differentiation of trichothecene and enniatin-producing *Fusarium* species on small-grain cereals. *European Journal of Plant Pathology*. 110: 503-514.

Nicholson, P., Simpson, D.R., Weston, G., Rezanoor, H.N., Lees, A.K., Parry, D.W., & Joyce, D. (1998). *Physiological and Molecular Plant Pathology*, 53: 17-37

Osborne, L.E. and Stein, J.M. (2007). Epidemiology of *Fusarium* head blight on small-grain cereals. *International Journal of Food Microbiology*, 119(1-2): p. 103-8.

Pasanen A.L., Yli-Pietilä, K., Pasanen, P., Kalliokoski, P. & Tarhanen J. (1999). Ergosterol Content in Various Fungal Species and Biocontaminated Building Materials. *Applied and Environmental Microbiology*, 65(1), pp 138-142.

Pereyra, S.A., Dill-Macky, R. & A.L. (2004). Sims, Survival and inoculum production of *Gibberella zeae* in wheat residue. *Plant Disease*, 88, pp 724-730.

Petcu, G. & Ioniþã, S. (1998). Influence of crop rotation on weed infestion and *Fusarium* spp. attack, yield and quality of winter wheat. Romanian Agricultural Research, 9-10: 83-91.

Quarta, A., Mita, G., Haidukowski, M., Logrieco, A., Mulè, G., & Visconti, A. (2006). Multiplex PCR assay for the identification of nivalenol, 3- and 15-acetyl-deoxynivalenol chemotypes in *Fusarium*. *FEMS Microbiology Letters*, 259(1), pp 7-13

Reischer G.H., Lemmens, M., Farnleitner, A.H., Adler, A. & Mach, R.L. (2004). Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan probe. *Journal of Microbiology Methods*, 59, pp 141-146.

Rhode, S. & Rabenstein, F. (2005). Standardization of an indirect PTA-ELISA for detection of *Fusarium* spp. In infected grains. *Mycotoxin Research,* 21(2), pp 100-104.

Rudd, J.C., Horsley, R.D., McKendry, A.L. & Elias, E.M.. (2001). Host Plant Resistance Genes for *Fusarium* Head Blight: Sources, Mechanisms, and Utility in Conventional Breeding Systems. *Crop Science*, 41(3), pp 620-627.

Schilling, A.G., Möller, E.M. & Geiger H.H. (1996). Polymerase Chain Reaction-Based Assay for Species-Specific Detection of *F. culmorum*, *F. graminearum* , and *F. avenaceum*. *Molecular Plant Pathololog*y, 86(5):515-522

Schnerr, H., Niessen, L. & Vogel, R.F. (2001). Real-time detection of the tri5 gene in *Fusarium* by LightCycler-PCR using SYBR Green I for continous fluorescence monitoring. Int. *Journal of Food Microbiology*, 71, pp 53-61.

Šliková, S., Šudyová, V., Martinek, P., Polišenská, I., Gregová, E. & Mihálik D. (2009). Assessment of infection in wheat by *Fusarium* protein equivalent levels. *European Journal of Plant Patholohy*, 124, pp 163-170.

Schroeder, H.W. & Christensen, J.J. (1963). Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. *Phyto*pathology 53: pp 831-839.

Steinkellner, S. & Langer, I. (2004). Impact of tillage on the incidence of *Fusarium* spp. in soil. *Plant and Soil*, 267(1-2): pp 13-22.

Stepień, L., Popiel, D., Koczyk, G. & Chełkowski, J. (2008). Wheat-infecting *Fusarium* species in Poland--their chemotypes and frequencies revealed by PCR assay. *Journal of Applied Genetics*, 49(4), pp 433-441

Waalwijk, C., Koch, S.H., Ncube, E., Allwood, J., Flett, B., de Vries, I. & Kema, G.H.J. (2008). Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal*, 1(1), pp 39-47.

Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Corainville, G.C., Häusler-Hahn, I., Kastelein, P., Köhl, J., Lonnet, P., Demarquet, T. & Kema, G.H.J. (2004). Quantitative detection of *Fusarium* in wheat using TaqMan. *European Journal of Plant Pathology*. 110: 481-494.

Wang, Y.Z. & Miller, J.D. (1988). Effects of *Fusarium* graminearum metabolites on wheat tissue in relation to *Fusarium* head blight resistance. *Journal of Phytopathology*, 122, pp 118-125.

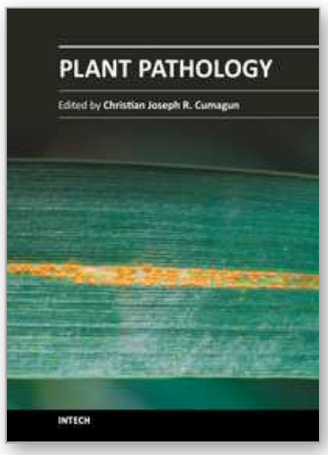
Weiland, J.J. & Sundsbak, J.L. (2000). Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region oft he rRNA gene. *Plant Disease*, 84(4), pp 475-482.

Windels, C.E. (2000). Economic and Social Impacts of *Fusarium* Head Blight: Changing Farms and Rural Communities in the Northern Great Plains. *Phytopathology*, 90(1), pp 17-21.

Yli-Mattila, T., Mach, R.L., Alekhina, I.A., Bulat, S.A., Koskinen, S., Kullnig-Gradinger, C.M., Kubicek, C.P. & Klemsdal, S.S. (2004). Phylogenetic relationship of *Fusarium* langsethiae to *Fusarium* poae and *Fusarium* sporotrichioides as inferred by IGS, ITS, β-tubulin sequences and UP-PCR analysis. *International Journal of Food Microbiology*, 95, pp 267-285.

Yli-Mattila, T., Paavanen-Huhtala, S., Jestoi, M., Parikka, P., Hietanieme, V., Gagkaeva, T., Sarlin, T., Haikara, A., Laaksonen, S. & Rizzo, A. (2008). Real-time PCR detection and quantification of *Fusarium poae*, *F. graminearum*, *F. sporotrichioides* and *F. langsethiae* in cereal grains in Finland and Russia. Archives of Phytopathology and Plant Protection, 41(4), pp 243-260.

Youssef, S.A., Maymon, M., Zveibil, A., Klein-Gueta, D., Sztejnberg, A., Shalaby, A.A. & Freeman S. (2007). Epidemiological aspects of mango malformation disease caused by *Fusarium mangiferae* and source of infection in seedlings cultivated in orchards in Egypt. *Plant Pathology*, 56, pp 257–263.



Plant Pathology

Edited by Dr. Christian Joseph Cumagun

ISBN 978-953-51-0489-6
Hard cover, 362 pages
Publisher InTech
Published online 04, April, 2012
Published in print edition April, 2012

Plant pathology is an applied science that deals with the nature, causes and control of plant diseases in agriculture and forestry. The vital role of plant pathology in attaining food security and food safety for the world cannot be overemphasized.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Kurt Brunner, Andreas Farnleitner and Robert L. Mach (2012). Novel Methods for the Quantification of Pathogenic Fungi in Crop Plants: Quantitative PCR and ELISA Accurately Determine Fusarium Biomass, Plant Pathology, Dr. Christian Joseph Cumagun (Ed.), ISBN: 978-953-51-0489-6, InTech, Available from: http://www.intechopen.com/books/plant-pathology/novel-methods-for-the-quantification-of-pathogenic-fungi-in-crop-plants-quantitative-pcr-and-elisa-a

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen