Fusarium head blight is a major concern for wheat production worldwide. The fungi that cause the disease may infect head tissues from flowering to late stages of kernel development, but a better understanding of the influence of the time of infection on grain weight reduction and mycotoxin accumulation resulting from the infection process is needed. We investigated the influence of wheat reproductive stage at the time of inoculation on disease and grain quality parameters, especially production of deoxynivalenol (DON) in mature grains. Heads of Norm wheat were spray inoculated with a macroconidial suspension of a DON-producing isolate of Fusarium graminearum at each of six reproductive stages from flowering to hard dough. Plants were incubated in a mist chamber for 48 h and then moved to the greenhouse until maturity. Norm wheat was susceptible at all stages inoculated but the highest grain weight reduction and DON accumulation occurred in plants inoculated past flowering to late milk stages. However, high incidences of kernel infection and significant levels of DON accumulation resulted from inoculations as late as the hard dough stage, even though there was no corresponding reduction in grain weight compared to non-inoculated plants. The occurrence of commercially significant levels of DON in plump, high-yielding wheat may result from infections that occur during favourable environments well after the flowering stages. Late infection and...
Influence of Growth Stage on Fusarium Head Blight and Deoxynivalenol Production in Wheat

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Abstract
Fusarium head blight is a major concern for wheat production worldwide. The fungi that cause the disease may infect head tissues from flowering to late stages of kernel development, but a better understanding of the influence of the time of infection on grain weight reduction and mycotoxin accumulation resulting from the infection process is needed. We investigated the influence of wheat reproductive stage at the time of inoculation on disease and grain quality parameters, especially production of deoxynivalenol (DON) in mature grains. Heads of Norm wheat were spray inoculated with a macroconidial suspension of a DON-producing isolate of Fusarium graminearum at each of six reproductive stages from flowering to hard dough. Plants were incubated in a mist chamber for 48 h and then moved to the greenhouse until maturity. Norm wheat was susceptible at all stages inoculated but the highest grain weight reduction and DON accumulation occurred in plants inoculated past flowering to late milk stages. However, high incidences of kernel infection and significant levels of DON accumulation resulted from inoculations as late as the hard dough stage, even though there was no corresponding reduction in grain weight compared to non-inoculated plants. The occurrence of DON is of concern for human and animal health, as this toxin is known to cause food refusal, vomiting, and depressed immune function (World Health Organization, 2001). Although progress is being made in disease reduction through chemical control, cultural control, and the development of resistant cultivars, satisfactory levels of control have yet to be attained (Bai and Shaner, 1994; Cromey et al., 2001). The fungus overwinters as a saprophyte in debris of infected cereals and grasses, including corn and wheat (Dill-Macky and Jones, 2000). Ascospores and macroconidia of the fungus are produced on overwintered residues (Khonga and Sutton, 1988) and both spore types are able to infect wheat any time from flowering through kernel filling stages if conditions are conducive (Shaner, 2003). However, there is lack of consensus about the exact size of the pheno logical window of vulnerability to infection, and especially for the accumulation of DON in the kernels. Some authors demonstrated that infection occurred principally at anthesis with the anthers as the main infection site (Atanasoff, 1920; Pearce et al., 1976). However, others have observed an extended window of infection with a peak after anthesis (Andersen, 1948; Schroeder and Christensen, 1963). DON accumulation in the kernels is influenced by many factors including host resistance, chemotype, proportions in North America and several other parts of the world in the past decades (McMullen et al., 1997). The major causal organism of this disease worldwide is Gibberella zeae (Schwein) Petch (anamorph: Fusarium graminearum Schwabe) (Schmale and Bergstrom, 2003). The disease affects wheat, barley, and other small grains both in temperate and semi-tropical areas causing reduction in grain yield and quality. Costs to farmers include increased management costs, reduced income from outright food or feed losses, and lower selling prices for commodities contaminated with mycotoxin (Jones and Mirocha, 1999). The occurrence of DON is of concern for human and animal health, as this toxin is known to cause food refusal, vomiting, and depressed immune function (World Health Organization, 2001). Although progress is being made in disease reduction through chemical control, cultural control, and the development of resistant cultivars, satisfactory levels of control have yet to be attained (Bai and Shaner, 1994; Cromey et al., 2001). The fungus overwinters as a saprophyte in debris of infected cereals and grasses, including corn and wheat (Dill-Macky and Jones, 2000). Ascospores and macroconidia of the fungus are produced on overwintered residues (Khonga and Sutton, 1988) and both spore types are able to infect wheat any time from flowering through kernel filling stages if conditions are conducive (Shaner, 2003). However, there is lack of consensus about the exact size of the phenological window of vulnerability to infection, and especially for the accumulation of DON in the kernels. Some authors demonstrated that infection occurred principally at anthesis with the anthers as the main infection site (Atanasoff, 1920; Pearce et al., 1976). However, others have observed an extended window of infection with a peak after anthesis (Andersen, 1948; Schroeder and Christensen, 1963). DON accumulation in the kernels is influenced by many factors including host resistance, chemotype,
aggressiveness of the prevalent fungal species, and environment (mainly temperature and moisture) during disease development (Hart et al., 1984; Mesterházy et al., 1991). The dynamics of toxin production in the field seem to be of a complex nature. Miller and Young (1985) reported that DON levels in heads inoculated at flowering increased for six weeks, then declined. This study was conducted to determine the influence of growth stage on disease and grain quality parameters. A brief, preliminary report of a portion of these findings was published (Del Ponte et al., 2003).

Materials and Methods

Inoculum preparation

Cultures of a single-conidium isolate of G. zeae (GZ014NY1998), with documented virulence and ability to produce DON in wheat, were grown in Petri dishes containing 1/4 strength potato-dextrose-agar (PDA) at a constant 25°C and a photoperiod of 12 h fluorescent light for 7 to 10 days. Macroconidia were harvested by washing the culture surface with sterile, distilled water. The resulting inoculum suspensions were adjusted with water to a concentration of 10^5 conidia/ml. Inoculum was used within 3 h after preparation.

Experimental design

Ten seeds of hard red spring wheat cv. Norm were sown in clay pots (2.5 l) containing a soil substrate and grown in the greenhouse at temperatures ranging from 20-23°C. By the beginning of anthesis, groups of six pots (each pot was one replicate) of homogeneous phenology were established. In each pot, 7 to 10 main tillers were maintained and secondary tillers were consistently eliminated during the experiment. Each treatment consisted of single-time spray-inoculations at one distinct growth stage, as follows, according to Zadoks scale (Zadoks et al., 1974): 65 – Anthesis half-complete; 71 – kernel watery ripe; 73 – early milk; 77 – late milk; 83 – early dough; and 87 – hard dough. Control treatment consisted of a group of wheat heads sprayed with sterile water. A hand-sprayer was used to apply water and the spore suspension; wheat heads were sprayed to saturation. After applying the treatments, plants were moved into a mist chamber for 48 h under fluorescent light for 7 to 10 days. Hence, RDS value at 6 days from inoculation to appearance of symptoms decreased from flowering until early dough stage inoculations. Typical disease symptoms were first observed for inoculations at anthesis was recorded as zero, ranging from 7 to 10 days. Hence, RDS value at 6 days for inoculations at anthesis was recorded as zero, although it increased dramatically afterwards (data not shown). Mean percentage of kernel infection (KI) was estimated by plating 10 sub-samples of 100 kernels in 10 plates with a selective medium – SNAW (Mandanhar and Cunfer, 1991). The fungal isolate inoculated previously was identified based on cultural and morphological characteristics. Three sub-samples of 100 kernels were weighed and the average used to estimate 1000-kernel weight (KW). DON levels were determined by direct competitive enzyme immunoassay RIDA-SCREEN® DON (Digen Ltd., Oxford, UK) according to manufacturer’s instructions. For each sample, 5-g grain samples were ground in a coffee mill for 12-15 s, and then stored in sealed plastic containers before analysis. One hundred millimetres of extraction buffer was added to the 5-g ground sample. Samples were shaken for 3 minutes and filtered into a collection tube. When toxin levels exceeded the test range, the test was repeated after the extract was diluted with buffer. The DON concentration was calculated accordingly.

Data analysis

Data from the two experiments were pooled due to lack of a significant difference between them (data not shown), and statistical analysis was based on all 12 combined replicates. DON analysis was an exception in that there were three replicates per experiment (total of six replicates for both experiments). Summary statistics and Pearson correlations were made in R software (R Foundation for Statistical Computing, Vienna, Austria). For the correlations of DON x KW and DON x VSK, averages for two pairs of the 12 replicates were used, in order to form six pair values of KW and VSK to correlate with six DON replicate values and to make the graphs.

Results

Norm wheat was susceptible to infection from flowering through all stages of kernel development. Disease severity could not be evaluated for heads inoculated at hard dough stage, due to natural senescence. The time from inoculation to appearance of symptoms decreased from flowering until early dough stage inoculations. Typical disease symptoms were first observed 3 to 4 days following inoculation, except in heads inoculated at flowering, in which the incubation period ranged from 7 to 10 days. Hence, RDS value at 6 days for inoculations at anthesis was recorded as zero, although it increased dramatically afterwards (data not shown). Mean values for disease and grain quality parameters evaluated are presented in Table 1. The incidence of visually scabby kernels and the degree of damage was highest for the earliest inoculations and least for inoculations at hard dough, which did not affect grain weight compared to the control. A higher incidence of visual symptoms was observed in kernels...
inoculated at early dough (72%) but the impact on grain weight was not so evident. Nearly one hundred percent of kernels from inoculations at early milk to early dough stages were colonized by the fungus.

DON levels ranged from 1.2 to 98 mg/kg with the highest toxin levels detected in kernels from inoculations at early dough or late-stage kernels. These values were around three and two times higher than toxin levels in kernels from earlier (anthesis) or later (late milk) inoculations, respectively. A high amount of DON was detected in kernels from inoculations at anthesis up to late milk stages. Inoculations at later stages resulted in 1000-kernel weights similar to the non-inoculated control. Both positive and negative correlations among the parameters were observed by analysing combined data from all six replicates in each of the six inoculation times (n = 36). A significant negative correlation (P < 0.001) was observed between kernel weight and DON (r = -0.88) and kernel weight and VSK (r = -0.77). A significant positive correlation (P < 0.001) was observed between DON and VSK (r = 0.64). Correlation between KI and DON was not significant (r = 0.09). Relationships for DON and VSK and between DON and KW are presented in Fig. 1. DON content in the kernels ranged from around 1 to 100 mg/kg. Deoxynivalenol decreased linearly with increasing kernel weight from inoculations performed at later stages of kernel development, yet a significant amount of toxin was observed in some kernels that were nearly as plump as kernels from non-inoculated plants.

**Discussion**

In the present study we demonstrated that Norm, a cultivar already known as highly susceptible to FHB, has a wide window of vulnerability to infection by *F. graminearum*. A high incidence of fungal colonization of kernels was observed following anthesis through the dough stages of kernel development. This is in agreement with Andersen (1948) who reported
Our findings also agree with a previous study by Hart et al. (1984) who observed that production of DON in wheat kernels varied according to the duration of wetness, regardless of the stage of kernel development, after they were filled. Nevertheless, we have observed a very high accumulation (49 to 98 mg/kg) of mean DON in kernels inoculated from watery ripe through milk stages and commercially significant mean toxin levels (1–13 mg/kg) in kernels inoculated at dough stages. Infections during flowering directly affect kernel development resulting in floret abortion or the development of small, shrunken and lightweight kernels that may be lost during harvest and cleaning operations (Schaaïfsma et al., 2001). In the present study, toxin-contaminated kernels resulting from inoculations at dough stages were similar in weight to non-inoculated kernels, suggesting that late infected kernels may also contribute to lower grain quality, i.e., mycotoxin contamination, even if they have a negligible effect on grain yield. Depending on the country and intended grain use, wheat may be substantially discounted or even refused by buyers at DON levels as low as 1 mg/kg, the mean level of contamination found in this study for kernels inoculated at late dough stage. Empirical evidence exists for the potential role of late infections in commercially significant toxin accumulation in mature grains. In several recent years in New York State, USA, an apparent uncoupling of DON contamination in soft winter wheat from the occurrence of FHB symptoms and grain weight reduction has been observed. That is, plump and high-yielding wheat has been contaminated with DON above 2 mg/kg (Bergstrom, unpublished). Similarly, Cowger and Sutton (2005) reported that severity of FHB in a portion of the south-eastern USA in 2003 was better correlated with weather parameters after wheat flowering than with those before or at the time of flowering, thus suggesting an important role for postflowering infections. Moreover, accuracy of prediction models for disease severity and DON increased when they included weather variables from periods past flowering (Schaaïfsma and Hooker, 2003; Del Ponte et al., 2005). A possible explanation suggested by the current findings as well as the previous findings by Hart et al. (1984) is that DON can be produced from infections late in kernel development without producing dramatic reductions in grain weight, especially if multiple infection events occur during grain filling. This biological fact complicates the already challenging task of timely prediction and control of FHB and toxin contamination. If, in fact, the results of this study and others further confirm an extended phenological window of wheat vulnerability to FHB, especially for DON accumulation, then (i) wheat breeders would need to consider screening methods for resistance to DON production resulting from late infections; (ii) farmers would need to consider management strategies aimed to protect wheat heads from infection for several weeks rather than several days around flowering; and (iii) practices which use damaged kernels as a predictor...
of DON may be less reliable in years when conditions allow for infection late in the growing season.

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