European Journal of Plant Pathology **108:** 653–660, 2002.

Studies on the infection process of *Fusarium culmorum* in wheat spikes: Degradation of host cell wall components and localization of trichothecene toxins in infected tissue

Z. Kang and H. Buchenauer
Institute of Phytomedicine, University of Hohenheim, D-70593 Stuttgart, Germany
(Fax: +494592408; E-mail: hbuchen@uni-hohenheim.de)

Key words: Fusarium culmorum, *Triticum aestivum*, infection process, deoxynivalenol, cell wall degrading enzymes

Abstract

After single spikelet inoculation, the infection process of *Fusarium culmorum* and spread of fungal hyphae in the spike tissues were studied by scanning and transmission electron microscopy. While hyphal growth on outer surfaces of the spike was scanty and no successful penetration was observed, the fungus developed a dense mycelium on the inner surfaces and effectively invaded the lemma, glume, palea and ovary by penetration pegs. During the inter- and intracellular spreading of the fungus, marked alterations in the host tissues were observed, including degeneration of cytoplasm, cell organelles, and depositions of electron dense material between cell wall and plasmalemma. Ultrastructural studies revealed that host cell walls in proximity of the penetration peg and in contact with hyphae were less dense or transparent which suggested that cell wall degrading enzymes were involved in colonisation of host tissues by fungal hyphae. Enzyme- and immunogold-labelling investigations confirmed involvement of extracellular enzymes, that is cellulases, xylanases and pectinases, in degradation of cell wall components. Localization studies of trichothecenes indicated that toxins could be detected in host tissues at an early stage of infection.

Introduction

Fusarium head blight or scab of wheat (*Triticum aestivum*) is one of the most destructive diseases in humid and semi-humid areas. The disease is caused by a number of *Fusarium* species, mainly *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum* and *F. avenaceum* (teleomorph *G. avenacea*) (Bai and Shaner, 1994; Parry et al., 1995). The disease causes substantial yield losses as a result of a diminished number of grains per spike and reduced grain weight. The infected grains are of low quality with damaged starch granules and storage proteins (Bechtel et al., 1985).

Moreover, *Fusarium* species produce highly toxic metabolites, for example trichothecene mycotoxins, such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (Wang and Miller, 1988; Snijders, 1990). These toxins are detrimental to livestock and human health (Trenholm et al., 1983; Marasas et al., 1984). In addition, some mycotoxins, for example DON, exert phytotoxic effects and are involved, as virulence factors, in the pathogenicity of *F. culmorum* and *F. graminearum* (Miller, 1989; Snijders and Krechting, 1992).

Wheat heads are most susceptible to the infection of the pathogen at the flowering stage (Pugh et al., 1933; Andersen, 1948; Strange and Smith, 1971). Detailed studies on the infection process of the pathogen on wheat heads, especially during the early stages, are limited and contradictory. In this study, the infection course of *F. culmorum* in wheat spikes was investigated by scanning and transmission electron microscopy. Also, the degradation of host cell wall components in *F. culmorum*-infected wheat spikes was demonstrated by means of enzyme-gold and immunogold-labelling.

Finally, the distribution of *Fusarium* toxins in wheat spikes at early infection stages was revealed by immunogold-labelling.

Materials and methods

Wheat plants of the cultivar Agent, which is susceptible to *Fusarium* head blight, were grown in 12 litre pots under outdoor conditions and inoculated at mid-flowering (GS 65) with a spore suspension of *F. culmorum* (isolate no. 46; Institute of Phytomedicine) by spraying the whole wheat spikes with a macroconidial suspension (5×10^5 spores ml^{-1}) or by injecting a conidial suspension (10^5 spores ml^{-1}) into the floral cavity between the lemma and palea of the first floret on spikelets using a pipette. Control plants were treated with distilled water. Immediately after inoculation, spikes were covered with plastic bags and plants were transferred into a growth chamber (16 h fluorescent light at 23 °C and 8 h darkness at 18 °C). After two days, the plastic bags were removed and plants were returned to outdoor conditions.

Whole single spikelets, including glumes, lemmas, paleas, ovaries and rachis from inoculated wheat spikes were sampled 6 and 12 h and 1, 2, 3, 4, 6 and 8 days after inoculation. The samples were fixed in glutaraldehyde (3–4%, v/v) in 50 mM phosphate buffer for 3–8 h at 4 °C. The samples were processed for scanning electron microscopy and light and transmission electron microscopy (Kang and Buchenauer, 2000a). The mycotoxins deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON) produced by *F. culmorum* were localized in host and fungal cells by immunogold-labelling (Kang and Buchenauer, 1999). The cell wall components cellulose, xylan and pectin were detected in healthy and *F. culmorum*-infected wheat spikes using enzyme-gold and immunogold-labelling methods (Kang and Buchenauer, 2000b).

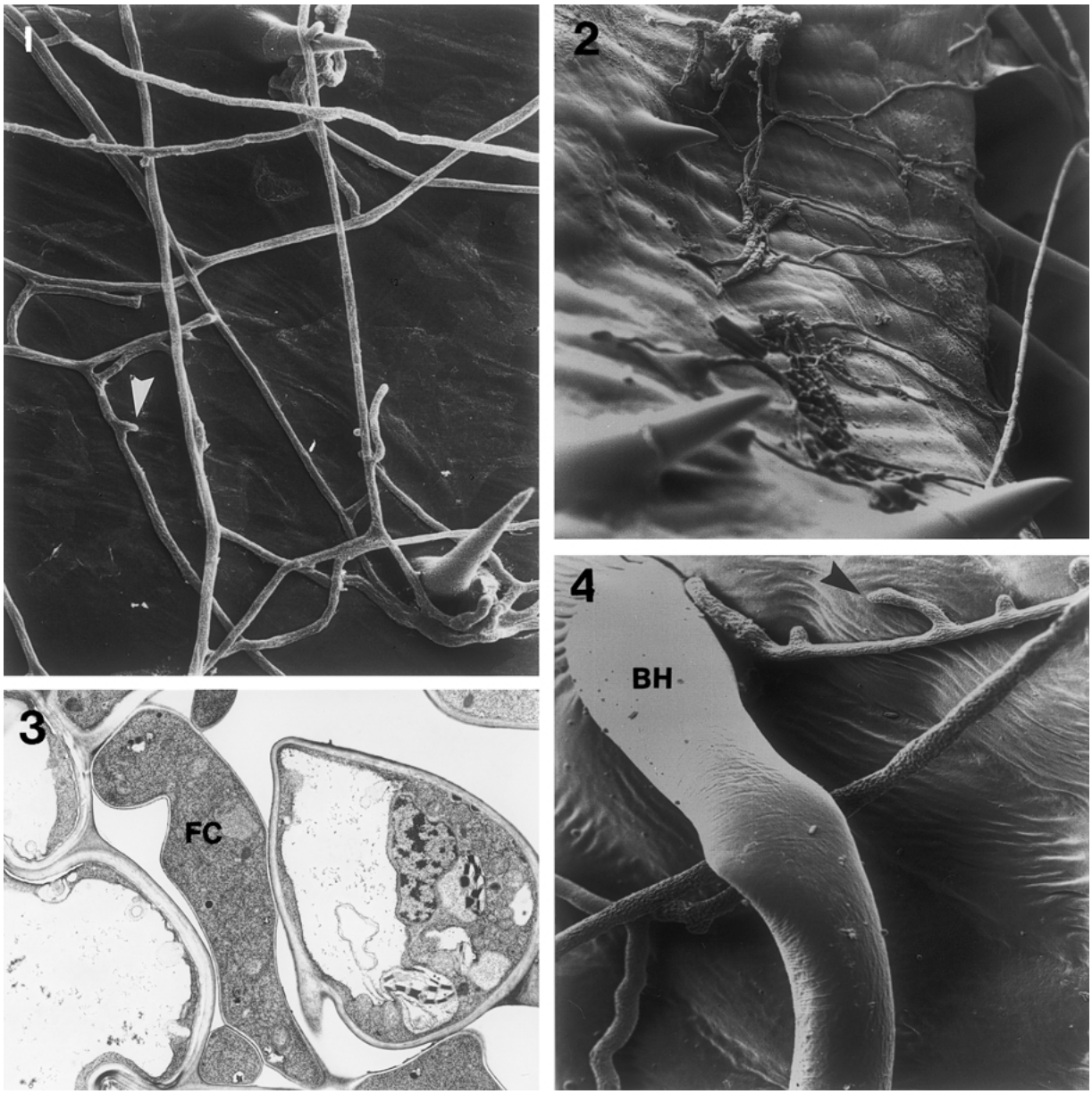
Results

F. culmorum macroconidia germinated with several germ tubes within 6–12 h after inoculation. Further development of the germ tubes differed markedly on the outer and inner surfaces of the floret tissues.

Growth of the hyphae on the outer surfaces was severely retarded. Two days after inoculation, the germ tubes on the convex middle part of the glume were short and possessed morphological alterations. Four days after inoculation, hyphal growth was very limited and no hyphal network developed. Hyphae near the edges of the lemma and glume exhibited more intensive growth, tending to extend to the edges in order to reach the inner surfaces of the glume, lemma or palea (Figure 2). Two days after inoculation, the fungus had developed a dense hyphal network on the inner surfaces of the lemma (Figure 1), glume, palea and top of the ovary. A dense mycelium developed on the anthers and pollen grains which were often present between the brush hair on the top of the ovary. With extended incubation time, hyphal growth on the inner surfaces of the spikelet became more dense and 3–4 days after inoculation the hyphae had reached the bases of the lemma, glume and palea. *F. culmorum* did not penetrate the inner surfaces of the host tissue immediately after germination of the macroconidia, but infections were observed after development of the dense hyphal network.

By 36 h after inoculation, hyphae in contact with the inner surfaces of the spikelet tissues formed short branches, the infection hyphae. The hyphae and the infection hyphae in touch with the host surfaces often produced cutinases and the cuticular layer beneath the hyphae was digested. The infection hyphae did not form appressoria but penetrated the epidermal cell wall directly by developing a constricted penetration peg. A septum was usually formed between the hyphal cell and the penetration peg. The fungus did not always penetrate the epidermal cell wall completely, but sometimes hyphae developed between the cuticule and the cell wall or within the cell wall layers. After penetration, during the first phase of tissue colonization, hyphae predominantly extended intercellularly between parenchyma cells in the lemma (Figure 3) and palea. During growth in the intercellular spaces, the fungus induced alterations in the host cells which were in contact or in advance of the hyphae. Small appositions were detected between the secondary cell wall and the plasmalemma. In the vacuoles, electron dense material was found and chloroplasts became swollen. Subsequently, the pathogen penetrated the affected host cells and colonized the tissues of the lemma, palea and ovary by both inter- and intracellular hyphal growth.

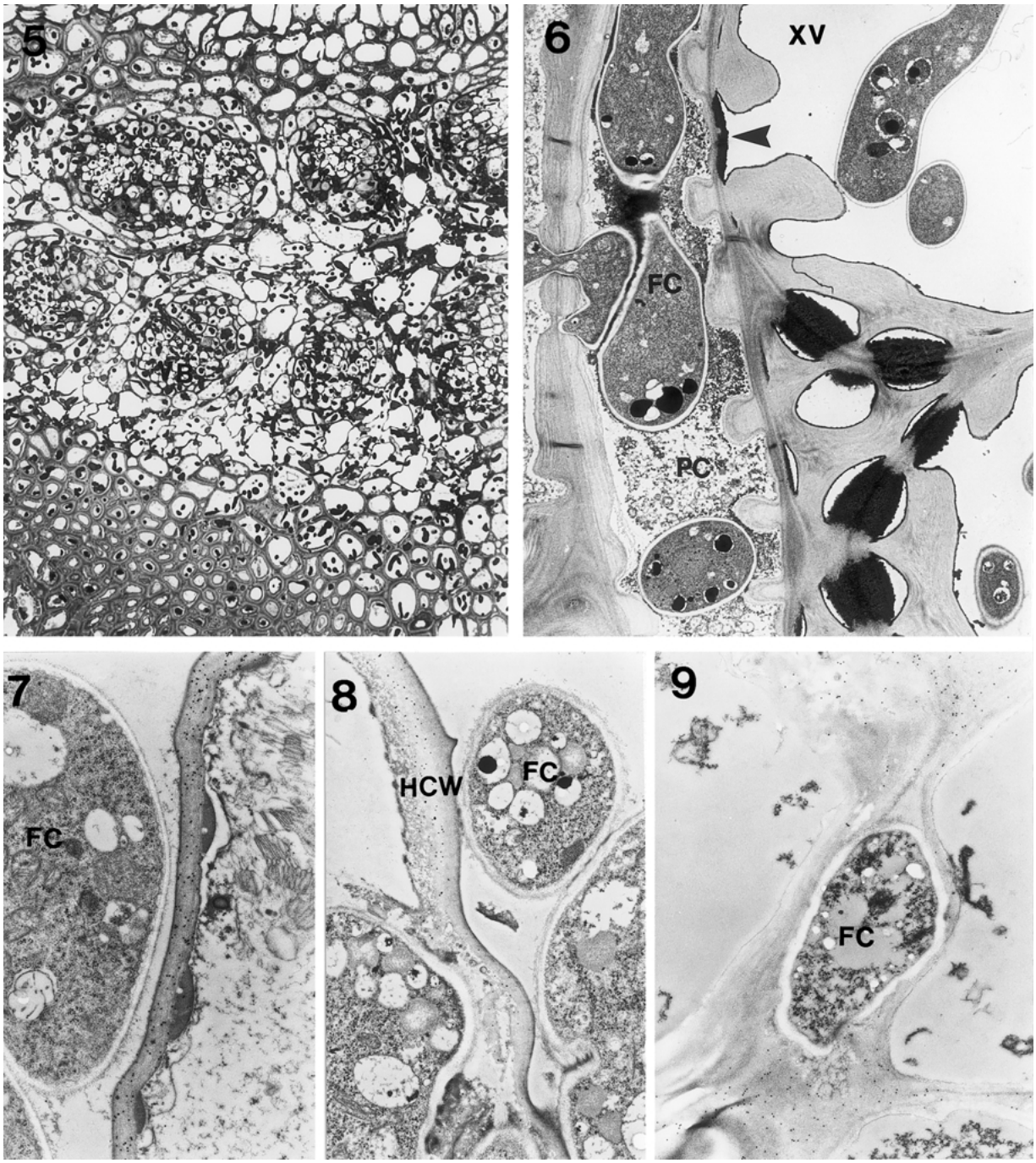
Three to four days after inoculation, the hyphae of *F. culmorum* had reached the bases of the lemma and palea by growing on the inner surfaces of the lemma and palea. At that time, the fungus had spread inter- and intracellularly to the base of the lemma and palea as well as horizontally from the inner surfaces of the lemma and palea to their outer surfaces. Typical



Figures 1–4. Scanning and transmission electron micrographs of colonization of wheat spike by *Fusarium culmorum*. **1**: Hyphal network on the inner surface of the lemma, 2 days after inoculation (dai). Infection hyphae (arrow) were already formed from hyphae adhering to the surface of the lemma. **2**: Hyphae on the outer surface near the top edge of the glume, 3 dai. The hyphae grew upwards and over the edge of the glume. **3**: Hyphae in the intercellular spaces between parenchyma cells in the lemma, 2 dai. Appositions appeared between host cell wall and plasmalemma. **4**: Hyphae with infection hyphae (arrow) on the top of the ovary, 2 dai. FC: fungal cell; BH: brush hair.

symptoms of brown water-soaked spots appeared on the infected tissues.

The cells of the colonized host tissue exhibited severe damage, such as disintegration of the cytoplasm and cell organelles, as well as collapse of some parenchyma cells. Host cell walls in close contact with hyphae or around the penetration pegs were, in general, less dense or transparent, suggesting that the pathogen produced cell-wall degrading enzymes during colonization of the host tissue.



Figures 5–9. Light and transmission electron micrographs of colonization of wheat spike by *Fusarium culmorum*. **5**: A cross-section of the rachis node, 4 dai. Hyphae are distributed in the vascular bundles, the parenchyma tissue outside the vascular bundle, and also in the thick-walled tissue around the vascular tissue. **6**: A cross-section of the rachis, 6 dai. Hyphae spreading in the xylem vessels and the paratracheal parenchyma cells. The primary wall of vessels became much more dense and there was a dense coating on the inner surfaces of the vessel walls (arrow). **7**: Cytochemical labelling for cellulose by a cellulase-gold probe over the cell walls in the *Fusarium culmorum*-infected lemma, 3 dai. The density of gold particles over the host cell wall decreased, particularly adjacent to the hypha. No labelling was seen over the appositions between the host cell wall and plasmalemma. The fungal cell wall was free of gold particles.

Fungal hyphae growing on the upper surface of the ovary also developed infection hyphae about 36 h after inoculation (Figure 4) and usually penetrated the tissue through the junctions between the epidermis or between the epidermis and the brush hair. The hyphae either extended between the epidermal wall layers or penetrated the cell wall. The fungus also infected the stigma and grew downwards to the upper pericarp cells. Hyphae extended very rapidly by inter- and intracellular growth from the upper part of the pericarp to the base of the ovary. The pericarp cells invaded by the fungus or in proximity to the intercellular hyphae were degenerated and host cell walls had disintegrated.

The hyphae extending either on the inner surface of the lemma, palea and the outer surface of the ovary or spreading within the host tissues reached the base of the lemma, palea or ovary about 3–4 days after inoculation and entered the rachilla, which is characterized by a central core of very thick-walled cells. After colonization of the rachilla, hyphae extended into the rachis node (Figure 5) and then grew into the rachis, which was reached 4–5 days after inoculation. In the rachis, hyphae of *F. culmorum* spread in the xylem vessels, phloem sieves and paratracheal parenchyma cells and colonized the rachis by inter- and intracellular hyphal extension (Figure 6). *F. culmorum* started to produce conidiophores and macroconidia when the outer surface of the lemma and palea had been reached, 5–6 days after inoculation.

The cell wall components cellulose, xylan and pectin were localized in different tissues of the non-inoculated, healthy and *F. culmorum*-infected wheat spikes by means of enzyme-gold and immuno-gold labelling (Kang and Buchenauer, 2000b). The intra- and intercellular colonization of the lemma, palea, ovary and rachis of the wheat spike by hyphae of *F. culmorum* caused pronounced alterations of the matrices of the host cell wall and middle lamella, and also resulted in marked modifications of the host cell wall components such as cellulose, xylan and pectin (Figures 7–9). The significant reduced binding sites of the cell wall macromolecules revealed by enzyme gold and immunogold-labelling of the infected tissue, as compared to the healthy non-infected cell walls, indicated excretion of cellulases, xylanases and pectinases by the hyphae of the pathogen during penetration and spreading. From the results obtained, it may be deduced that during the initial infection and spreading process of the pathogen, pectinases activity is higher than the activities of the cellulases and xylanases.

An immunogold-labelling method was also developed to study the subcellular localization of the trichothecene mycotoxins deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON) (Kang and Buchenauer, 1999). The toxins were produced by the hyphae before penetration, when they were growing in close contact on the inner surface of the lemma and palea or on the surface of the ovary. Thirty-six hours after inoculation, toxins were detected in the walls and cells of the epidermis. During penetration, toxins were found in the host cell wall around the infection peg and in the cytoplasm of the host cells (Figure 10). During spreading of the pathogen, higher intensities of toxin labelling were present in host cells in proximity to the hyphae.

In plant cells, toxins were localized in the cytoplasm, chloroplasts, plasmalemma and sometimes associated with endoplasmic reticulum and ribosomes (Figure 11). Figure 12 shows a parenchyma cell of the lemma with disorganized cytoplasm, 3 dai. Toxins were present over the host cell wall, cytoplasm and vacuole.

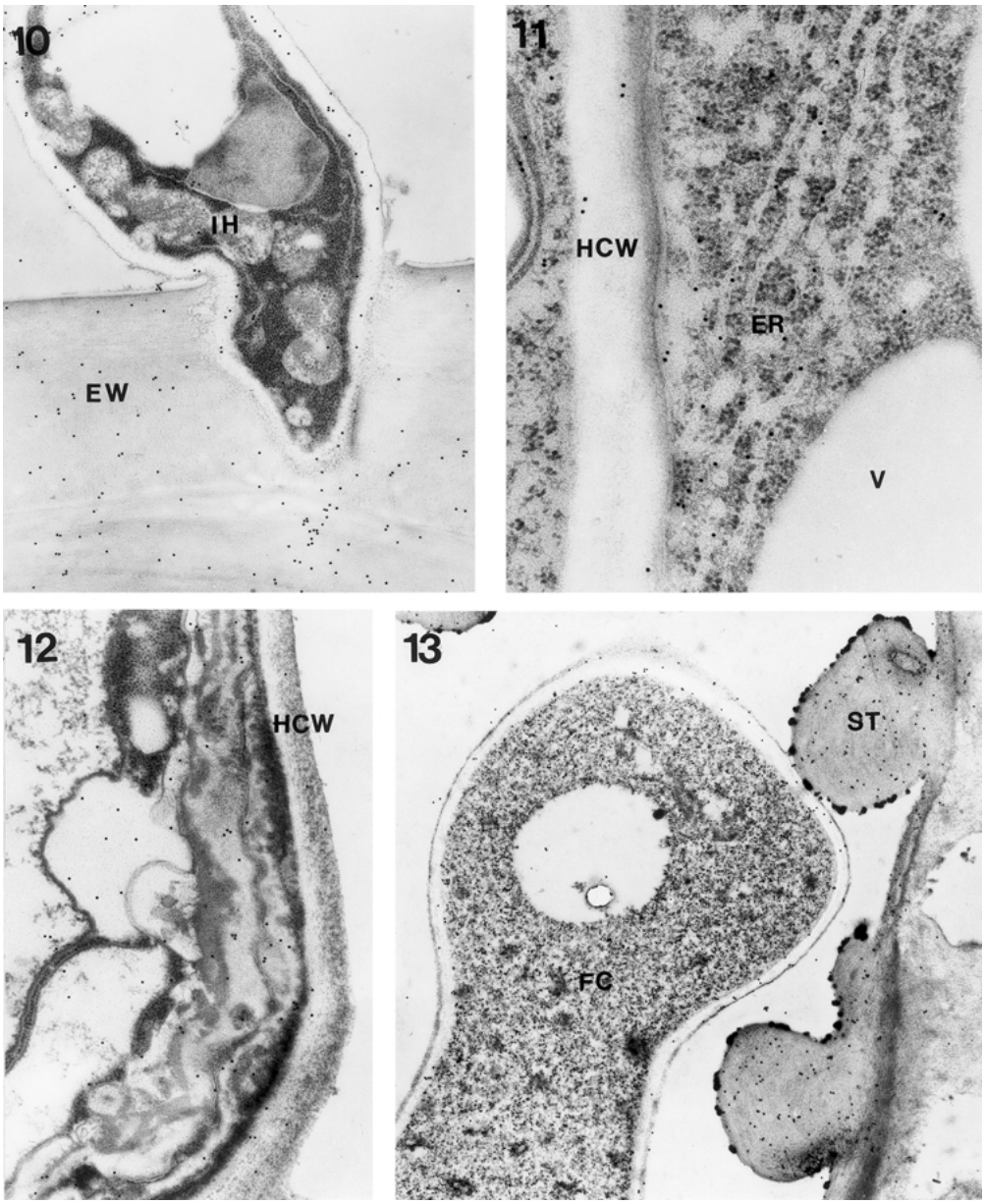
In infected wheat kernels, toxins were detected at different concentrations in the pericarp tissues, pigment strand, aleurone cells and in the starch endosperm. In the rachis colonized by the fungus, toxins were found in xylem vessels (Figure 13) and phloem sieve tubes and parenchyma cells outside the vascular bundles.

The studies also revealed that toxins were translocated upwards in the xylem and phloem sieve tubes and downwards in the phloem sieve tubes to areas of the spike which were not colonized by the fungus.

Discussion

The study revealed rapid and effective colonization of wheat spikes by *F. culmorum*. The hyphae of the pathogen extended inter- and intracellularly in the parenchyma and vascular tissues of the lemma, glume,

8: Cytochemical localization of xylan with a xylanase-gold probe in cell walls of the *Fusarium culmorum*-infected ovary, 3 dai. The cytoplasm of the cells was destroyed and labelling decreased over the host cell wall in contact with the hyphae. **9**: Immunogold labelling for pectin by monoclonal antibody, JIM7, in the cell walls of the *Fusarium culmorum*-infected sieve tubes in the rachis, 5 dai. The growth of the hypha in the middle lamella of the sieve tube resulted in cell wall displacement and reduction of gold particles in the wall. FC: fungal cell; HCW: host cell wall; PC: paratracheal parenchyma cell; XV: xylem vessel.



Figures 10–13. Immunogold localization of Fusarium toxins in infected host tissue by *Fusarium culmorum*. **10**: The epidermal cell wall of the lemma, 36 h after inoculation, was incubated with DON-antiserum and goat anti-rabbit immunoglobulin-linked colloidal gold particles (GAR-gold antibody). Gold particles were localized over the host cell wall and the hyphal cell wall. Note that dense accumulation of gold particles was found over the host cell wall in advance of the penetration peg. **11**: The epidermal cell in the ovary, 2 dai, incubated with DON-antiserum and GAR-gold antibody. The gold particles were found over the cell wall, cytoplasm, endoplasmic reticulum and ribosomes. **12**: The parenchyma cell with disorganized cytoplasm in the lemma, 3 dai, incubated with DON-antiserum and GAR-gold antibody. Gold labelling was found over the host cell wall, cytoplasm and the vacuole. **13**: The infected xylem vessel in the rachis, 6 dai, incubated with DON-antiserum and GAR-gold antibody. The vessel was labelled with numerous gold particles on the secondary thickenings and with fewer gold particles on the primary wall. The hyphal cell in the vessel was also labelled. ER: endoplasmic reticulum; EW: epidermal cell wall; FC: fungal cell; HCW: host cell wall; IH: infection hypha; ST: secondary thickening; V: vacuole.

ovary and rachis, and caused severe damage to the host tissues.

Scanning microscopical studies revealed striking differences regarding hyphal growth of *F. culmorum* on the outer surfaces of the glume, lemma and rachis and the inner surfaces of the lemma, glume and palea. While the fungus developed a dense mycelium on the inner surfaces and effectively invaded these tissues, hyphal growth on outer surfaces of the spike was scanty and no successful penetration was observed.

The unfavourable conditions for hyphal growth on the outer surfaces of the wheat spikes may be associated with physical and chemical as well as environmental properties. The pronounced wax layer covered with wax crystals may present a water-repellent surface and also might contain antifungal components. As a result of these inhospitable conditions, almost no infections by *F. culmorum* occurred on the outer surfaces. These observations are in agreement with studies of Strange and Smith (1971) which showed that no infections occurred after placing droplets of spore suspensions on the outer surface of the glume.

Ultrastructural analysis showed that the host cell walls in contact with the hyphae or in proximity to the penetration pegs were less dense or transparent suggesting that cell-wall degrading enzymes are involved in the spread of the fungus in the host tissues. Cytochemical labelling studies confirmed degradation of the cell wall components cellulose, xylan and pectin in *F. culmorum*-infected wheat spikes. The study also showed that during the early stage of infection (e.g. 3 dpi) pectin degradation in the epidermal cell walls of the wheat ovary was more pronounced than that of cellulose and xylan. This finding suggests that *F. culmorum* may secrete pectinases earlier or in higher activities than cellulases or xylanases. Following infection, the pathogen invaded host cells only when they exhibited signs of disintegration or necrosis.

Numerous studies have shown that *F. culmorum* produces the trichothecene mycotoxins DON, 3-ADON, 15-ADON and nivalenol (Wang and Miller, 1988; Wong et al., 1995). It has been demonstrated that these toxins also display detrimental effects to plant cells such as inhibition of protein synthesis, electrolyte losses, cytoplasm convolution and disintegration of organelles (Miller and Ewen, 1997; Pavlovkin et al., 1986).

The studies indicated, that as early as 36 h after inoculation, hyphae of *F. culmorum* growing on the surfaces of the lemma and ovary had already secreted trichothecene toxins which were present in the host tissues before the pathogen had invaded the host cells. During penetration and growth the hyphae continued to produce toxins which were detected in host cells adjacent to and in advance of the hyphae. In the host cells, *Fusarium* toxins were associated with the plasmalemma and in the cytoplasm sometimes with ribosomes and endoplasmic reticulum. These localization studies suggest that the trichothecene toxins interfere with the wheat spike/*F. culmorum*-interaction by impairing host defence reactions. Their association with the plasmalemma might result in alterations of membrane permeability, and because of the affinity to the ribosomes, it is assumed that the toxins interfere in protein synthesis which might result in severe impairment of post-infection defence mechanisms. The delayed or inhibited post-infection responses also became evident from the ultrastructural studies. It was difficult to detect defence structures such as callose or papilla formations in the infected tissues.

Wheat kernels colonized by *F. culmorum* were also contaminated with the toxins (Snijders and Perkowski, 1990; Sinha and Savard, 1997). The labelling density in the different tissues not only correlated with the hyphal density; toxins were also detected in advance of the hyphae in the starchy endosperm. It may be concluded that, in addition to the production of cell-wall degrading enzymes, the secretion of trichothecene mycotoxins may also constitute an important factor in head blight development of wheat by *F. culmorum*.

Acknowledgements

The authors thank Mrs H. Brandl and Mrs M. Harfold for excellent technical assistance, Mrs G. Moll for preparing the photographs and Mrs M. Sarkar for typing the manuscript. This study has been supported by the Deutsche Forschungsgemeinschaft (DFG; Forschergruppe 'Fusarium-Toxine').

References

Anderson AL (1948) The development of *Gibberella zeae* head blight of wheat. Phytopathology 38: 595–611

Bai G and Shaner G (1994) Scab of wheat: Prospects for control. Plant Disease 78: 760–766

Bechtel DB, Kaleikan LA, Gaines RL and Seitz LM (1985) The effects of *Fusarium graminearum* infection on wheat kernels. Cereal Chemistry 62: 191–197

Kang Z and Buchenauer H (1999) Immunocytochemical localization of Fusarium toxins in infected wheat spikes by *Fusarium culmorum*. Physiological and Molecular Plant Pathology 55: 275–288

Kang Z and Buchenauer H (2000a) Cytology and ultrastructure of the infection of wheat spikes by *Fusarium culmorum*. Mycological Research 104: 1083–1093

Kang Z and Buchenauer H (2000b) Ultrastructural and cytochemical studies on cellulose, xylan and pectin degradation in wheat spikes infected by *Fusarium culmorum*. Journal of Phytopathology 148: 263–275

Marasas WFO, Nelson PE and Toussoun TA (1984) Toxigenic *Fusarium* species: Identity and mycotoxicology. The Pennsylvania State University Press, University Park, PA

Miller JD (1989) Effects of *Fusarium graminearum* metabolites on wheat cells. In: Graniti A, Durbin RD and Ballio A (eds) Phytotoxins and Plant Pathogenesis. NATO ASI Ser. Ser. H, Cell Biology 27: 449–452

Miller JD and Ewen MA (1997) Toxic effects of deoxynivalenol on ribosomes and tissues of the spring wheat cultivars Frontana and Casavant. Natural Toxins 5: 234–237

Parry DW, Jenkinson P and Mc Leod L (1995) *Fusarium* ear blight (scab) in small grain cereals – a review. Plant Pathology 44: 207–238

Pavlovkin J, Mistrik I, Zajcenko AM and Mraz J (1986) Effects of mycotoxins on cell membrane of higher plant roots. Biologia (Bratislava) 41: 681–686

Pugh GW, Johann H and Dickson JG (1933) Factors affecting infection of wheat heads by *Gibberella sambinetii*. Journal of Agricultural Research 46: 771–791

Sinha RC and Savard ME (1997) Concentration of deoxynivalenol in single kernels and various tissue of wheat heads. Canadian Journal of Plant Pathology 19: 8–12

Snijders CHA and Krechting CF (1992) Inhibition of deoxynivalenol translocation in *Fusarium* head blight resistant wheat. Canadian Journal of Botany 70: 1570–1576

Snijders CHA and Perkowski J (1990) Effects of head blight caused by *Fusarium culmorum* on toxin content and weight of wheat kernels. Phytopathology 80: 566–570

Snijders CHA (1990) *Fusarium* head blight and mycotoxin of wheat, a review. Netherlands Journal of Plant Pathology 96: 187–198

Strange RN and Smith H (1971) A fungal growth stimulant in anthers which predisposes wheat to attack by *Fusarium graminearum*. Physiological Plant Pathology 1: 141–150

Trenholm HL, CochraneWP, Cohen H, Elliot JI, Farnworth ER, Friend DW, Hamilton RMG, Standish JF and Thompson BK (1983) Survey of vomitoxin contamination of the Ontario 1980 white winter wheat crop: Results of survey and feeding trials. Journal of the Association of Official Analytical Chemistry 66: 92–97

Wang YZ and Miller JD (1988) Effects of *Fusarium graminearum* metabolites on wheat tissue in relation to Fusarium head blight resistance. Journal of Phytopathology 122: 118–125

Wong LSL, Abramson D, Tekauz A, Leisle D and McKenzie RIH (1995) Pathogenicity and mycotoxin production of *Fusarium* species causing head blight in wheat cultivars varying in resistance. Canadian Journal of Plant Sciences 75: 261–267