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Importance of cell wall degrading enzymes produced by *Fusarium graminearum* during infection of wheat heads

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Abstract

Cytological studies were carried out to elucidate the importance of cell wall degrading enzymes (CWDE) during infection of wheat spikes by *Fusarium graminearum.* Scanning electron micrographs revealed that at 6–24 hours after inoculation (hai) of single spikelets with macroconidia of *F. graminearum,* the fungus germinated by forming several germ tubes and developed a dense hyphal network in the cavity of the spikelet. At 24–36 hai, the fungus formed infection hyphae which invaded the ovary and inner surface of the lemma and palea. Transmission electron microscopical studies revealed that the fungus extended inter- and intracellularly in the ovary, lemma and rachis and caused considerable damage and alterations to the host cell walls. In different tissues of healthy and *F. graminearum*-infected wheat spikes the cell wall components cellulose, xylan and pectin were localized by means of enzyme-gold and immuno-gold labelling techniques. Localization of cellulose, xylan and pectin showed that host cell walls which were in direct contact with the pathogen surface had reduced gold labelling compared to considerable higher labelling densities of walls distant from the pathogen–host interface or in non-colonized tissues. The reduced gold labelling densities in the infected host cell walls indicate that these polysaccharide degrading enzymes might be important pathogenicity factors of *F. graminearum* during infection of wheat spikes. The results revealed that, infection and colonization of wheat spikes by *F. graminearum* and reactions of infected host tissue were similar to those reported for *F. culmorum*.

Introduction

Fusarium head blight (FHB), also known as scab or ear blight, is a seed and head disease characterized by bleaching of the wheat head, shriveled kernels and accumulation of mycotoxins in grain (Parry et al., 1995; McMullen et al., 1997). In North America and in southern Europe, *F. graminearum* (Schwabe) (Teleomorph *Gibberella zeae* (Schw) Petch) predominates, while *F. culmorum* (Smith) Sacc. predominates in cooler climates in Europe (Parry et al., 1995; McMullen et al., 1997). Most cultivars of wheat are susceptible to these pathogens and resistance to scab is horizontal (i.e. race non-specific). These two pathogens are closely related, and plant resistance to *F. culmorum* is correlated with resistance to *F. graminearum* (Mesterhazy et al., 1999).

In general, cell wall degrading enzymes (CWDE) produced by plant pathogenic fungi are considered important pathogenicity factors. However, although significant amounts of work have been published on the role of CWDE in fungal pathogenicity, it has not resulted in any definitive conclusions on the importance of CWDE for fungal pathogenicity (Annis and Goodwin, 1997). Using molecular DNA techniques, mutants deficient in expression of a specific CWDE did not necessarily have reduced pathogenicity (Bowen et al., 1995; Wu et al., 1997). This is because the

disruption of a single enzyme may be complemented by the production and secretion of other isoforms of the enzyme exhibiting similar activities (Hammer and Holden, 1997).

Using immuno-gold and enzyme-gold labelling techniques, Kang and Buchenauer (2000a) demonstrated the production of CWDE such as pectinase, cellulase and xylanase during infection and colonization of infected wheat spikes by *F. culmorum*. Since *F. culmorum* and *F. graminearum* are closely related, we suspected that extracellular CWDE are potentially involved in pathogenesis of *F. graminearum* on wheat.

In this article, we present cytochemical evidence that CWDE are involved in infection and colonization of different tissues of wheat spikes by *F. graminearum*.

Materials and methods

Plant material and source of inoculum

The susceptible wheat cv. agent was used. Seeds were planted in November 1998 and 1999, respectively, in 12 litre pots filled with loamy organic soil. After growth stage (GS) 25 (Zadoks, 1974), plants were fertilized weekly with 100 ml per pot of 1% (v/v) solution of the fertilizer Wuxal (N–P–K: 8–8–6; Aglukon, Germany). At GS 39, all side tillers were eliminated and the number of plants was thinned to 15 main tillers per pot. For inoculum production, autoclaved oat seeds were inoculated with a spore suspension of *F. graminearum* isolate 18.7 (Institute of Phytomedicine, University of Hohenheim) (Kang and Buchenauer, 2000a). Conidial suspensions were prepared by washing the colonized oat kernels with distilled water and after filtering through a double layer of cheese cloth, the spore suspensions were adjusted to 1×10^5 conidia ml^{-1}.

Inoculation of wheat spikes

A 10-µl conidia suspension of *F. graminearum* was pipetted into the cavity between the lemma and palea of every third spikelet on one side of the spike at mid anthesis (GS 65; Zadoks, 1974). After inoculation, the heads were covered with a wet plastic bag tied to the stalk to uphold a high relative humidity for 24 h.

Tissue processing for electron microscopy

For transmission electron microscopy, florets from infected and uninfected spikes were taken and the ovaries, lemmas and rachis excised and fixed in 3% (w/v) glutaraldehyde in 50 mM phosphate buffer (pH 6.8) for 3–6 h at 4 °C. The same buffer was used to thoroughly rinse the samples which were later post-fixed with 1% (w/v) osmium tetroxide in the same phosphate buffer for 2 h at 4 °C. However, for immuno-gold labelling, the samples were fixed with 1% (w/v) glutaraldehyde and 4% (w/v) formaldehyde in 100 mM sodium cacodylate buffer (pH 7.2) for 3 h. Subsequently, all samples were dehydrated in a graded ethanol series, embedded in LR white (TAAB Laboratories, Munich, Germany) and polymerized at 50 °C for 2 days. Ultrathin sections of the samples were cut with a diamond knife and collected on 200-mesh nickel grids for cytochemical labelling. Three replicate experiments were performed. For scanning electron microscopy, the infected and healthy wheat spikes were taken and processed according to Kang and Bucheneaur (2000b).

Preparation of enzyme-gold probes

The method of Frens (1973) was used for preparing a colloidal gold solution with particles of 15 nm average diameter. For the cellulase-gold probe, the method of Berg (1990) was used. Colloidal gold solution (10 ml, pH 5.4) were added to 1 mg cellulase of *Trichoderma reesei* (Worthington Biochemical Corporation, Freehold, NJ, USA), supplied as a chromatographically purified enzyme complex containing cellobiohydrolase I, cellobiohydrolase II and endoglucanase. The mixture was centrifuged for 30 min, the pellet resuspended in 10 ml of 50 mM citrate buffer (pH 5.4), again centrifuged and the residue suspended in 1 ml of citrate buffer containing 0.02% (w/v) of polyethylene glycol (20 000 molecular weight). For xylanase-gold probe, the method of Giesbert et al. (1998) was followed whereby 800 µg of xylanase (EC 3.2.1.8) purified from *T. viride* were conjugated with 10 ml of the gold solution, pH 5.5. The gold solution was further stabilized with 1% w/v BSA. After centrifugation at 17 000 *g* for 30 min, the pellet was resuspended in double distilled water, centrifuged again and recovered in 50 mM acetate buffer, pH 5.0.

Cytochemical labelling

For localization of cellulose and xylan, ultrathin sections were quenched with buffer containing 1% BSA

and 0.5% gelatine (Merk, Germany) for 20 min and then incubated with the respective enzyme-gold solution (1 : 10 diluted) for 20 min. For immuno-gold detection of pectin, the monoclonal antibody JIM7, which binds specifically to methyl-esterified pectin, was used (Knox et al., 1990; Kang and Buchenauer, 2000a). Briefly, ultrathin sections were first blocked with 1% (w/v) of BSA in Tris-buffered saline (TBS, 10 mM Tris–HCl, 150 mM NaCl, pH 7.4) for 20 min, then incubated with monoclonal antibody (JIM7) diluted 1 : 1 with TBS for 14 h at 4 °C. After rinsing with TBS, the sections were incubated for 2 h with goat anti-rat immunoglobulin linked to 15 nm colloidal gold particles (GAT–gold antibody, EM GATIg G15, Biocell, Cardiff, UK) diluted 1 : 30 in TBS. Ultrathin sections were contrasted with uranyl acetate and lead citrate before examination with a Zeiss Model EM10 electron microscope at 80 kV. For the controls, sections were incubated with uncoated colloidal gold solution or goat anti-rat immunoglobulin solution alone, or incubated with cellulase-gold, xylanase-gold or with the monoclonal antibody JIM7, to which carboxymethylcellulose, xylan or pectin from citrus was previously added, respectively (Kang and Buchenauer, 2000a).

Results

Infection of wheat spikes by F. graminearum

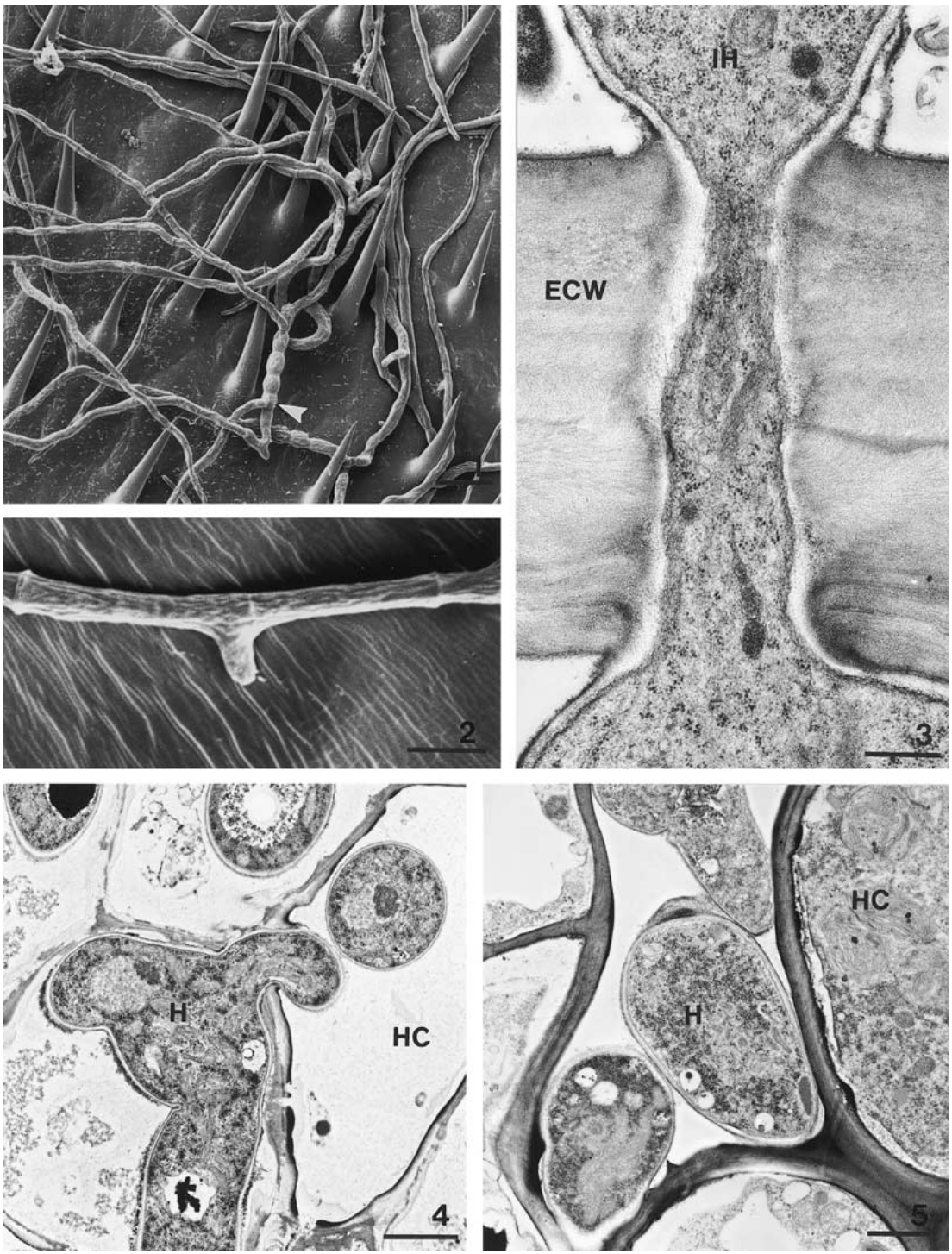
Spikelets of 6–12 hours after inoculation (hai) with macroconidia of *F. graminearum,* the fungus germinated by forming several germ tubes and up to 24 hai it developed a dense hyphal network in the cavity of the spikelet (Figure 1) especially between the pollen grains on the top of the ovary. At 24–36 hai, the fungus formed infection hyphae (Figure 2) which produced penetration pegs and invaded the ovary and inner surface of the lemma and palea. The hyphae grew either within the epidermal cell walls or penetrated the epidermal cell walls (Figure 3). Three days after inoculation (dai), hyphae developed inter- and intracellulary in the pericarp parenchyma cells of the ovary (Figure 4). In the lemma tissue, the fungus grew predominantly intercellularly (Figure 5). At 5 dai, the hyphae reached the rachis from the infected ovary, lemma and palea. The fungus extended inter- and intracellularly in the cortical tissue and vascular bundles of the rachis (Figure 6). Colonization of the host tissues by the hyphae of *F. graminearum* caused considerable alterations of the cell walls, degeneration of host cytoplasm, disintegration of host organelles such as chloroplasts and collapse of some parenchyma cells.

Localization of cellulose, xylan and pectin in healthy and infected wheat spikes

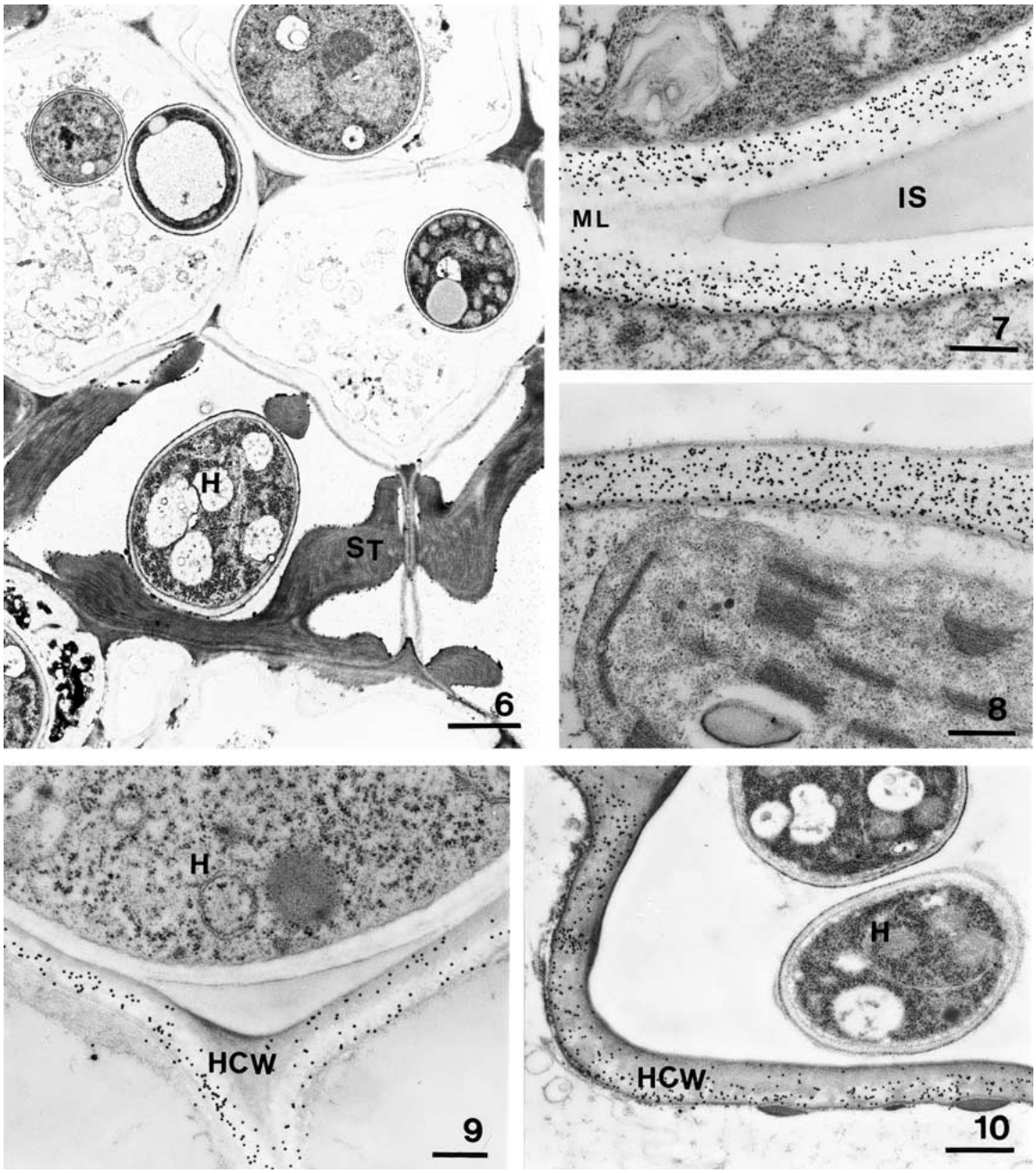
After incubation of ultrathin sections of non-inoculated healthy tissues with the cellulase-gold probe, the cell walls of host tissues were intensely and regularly labelled. In the ovary, labelling densities of the cell wall layers varied considerably across the cell wall; while the inner wall layer facing the cytoplasm in the parenchyma cell walls was densely labelled, the outer layer facing the intercellular spaces and the middle lamella was scantly labelled (Figure 7). In the lemma, the parenchyma cell walls, as well as the thick-walled collenchyma cell walls, were densely and evenly labelled (Figure 8). In the rachis, the xylem vessels, sieve tubes and secondary wall of cortical parenchyma cells showed dense cellulase-gold labelling while the middle lamella was weakly labelled. Control sections incubated with either colloidal gold solution alone or with cellulase-gold probe to which carboxymethylcellulose had been added previously exhibited little or no labelling. Incubation of ultrathin sections of infected host tissues with cellulase-gold probe revealed alterations in the pattern of gold particles in the cell walls as compared to sections of the corresponding healthy tissues. At 3 or 5 dai, host cell walls of the parenchyma cells either in the ovary or lemma, which were in direct contact with the pathogen surface showed a reduced cellulase-gold labelling compared to the considerably higher labelling density of walls distant from the pathogen–host interface or in non-colonized tissue areas (Figures 9 and 10). This reduced cellulase-gold labelling indicates that there was enzymatic degradation of cellulose in the polysaccharide network of the host cell wall at the vicinity of the pathogen.

Xylan

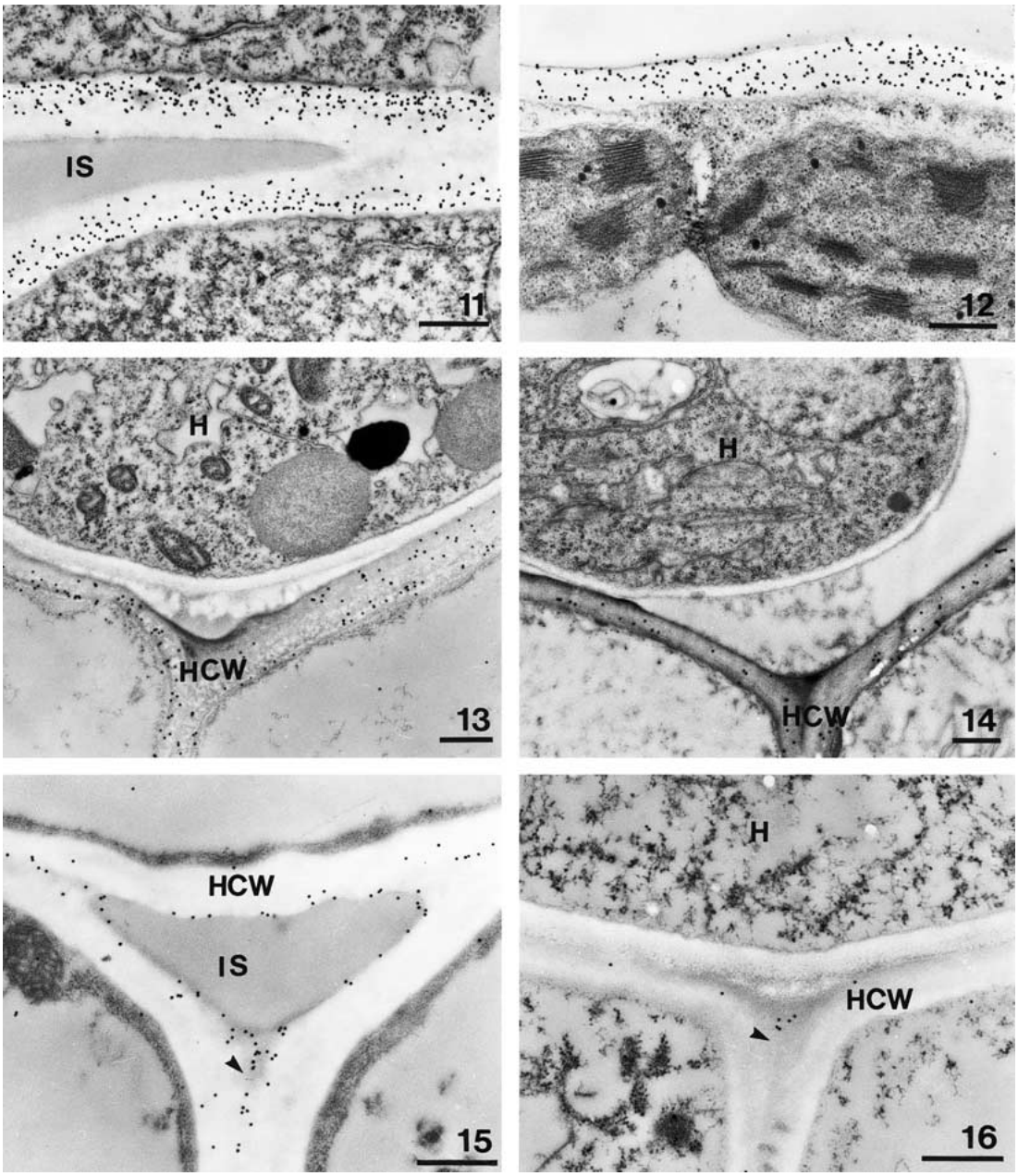
Incubation of ultrathin sections of non-inoculated healthy host tissues with the xylanase-gold probe resulted in uniform labelling patterns. In the ovary, the labelling density increased from the outer to the inner wall layer of the epidermal and parenchyma cells. The outer layer facing intercellular spaces and middle lamella had little or no gold labelling (Figure 11). In the lemma, the cell walls of parenchyma cells showed an even distribution of gold particles (Figure 12). In the



Figures 1–5. Scanning (Figures 1 and 2) and transmission (Figures 3–5) electron micrographs of colonization of wheat spikes after inoculation with *F. graminearum* macroconidia. (1) *F. graminearum* macroconidia (arrowhead) germinated with several germ tubes which had formed dense hyphal mycelium on the inner surface of the lemma, 24 hai (Bar = 20 μm). (2) Infection hypha developing from the hypha adhering to the inner surface of lemma, 36 hai (Bar = 5 μm). (3) A penetration peg, arising from an infection hypha and penetrated through the epidermal cell wall on the inner surface of lemma, 2 dai (Bar = 0.5 μm). (4) The inter- and intracellular growth of the fungus in the pericarp of parenchyma cells of the ovary, 3 dai (Bar = 2 μm). (5) Hyphae growing intercellularly between host cells in the parenchyma cells of the lemma, 3 dai (Bar = 2 μm). ECW = epidermal cell wall; IH = infection hypha; H = hyphal cell; HC = host cell.



Figures 6–10. Transmission electron micrograph of rachis (Figure 6) and enzyme-gold localization of cellulose by a cellulase-gold probe over the cell walls in different tissues of healthy wheat spikes (Figures 7 and 8) and *F. graminearum* inoculated wheat spikes (Figures 9 and 10). (6) Hyphae growing intracellularly in the xylem vessels in the infected rachis, 5 dai. (Bar = 2 μm). (7) Parenchyma cells in the ovary. Inner wall layers facing the cytoplasm were densely labelled while the outer layer facing the intercellular spaces and the middle lamella were scantly labelled (Bar = 0.5 μm). (8) Densely and evenly labelled parenchyma cell wall in the lemma (Bar = 0.5 μm). (9) Parenchyma cells in the pericarp of the infected ovary, 3 dai. Walls in direct contact with the pathogen surface show a reduced cellulase-gold labelling (Bar = 0.5 μm). (10) Damaged parenchyma cells in the infected lemma, 5 dai. The labelling density of cellulose over the cell walls adjacent to the hyphal cell was markedly reduced (Bar = 0.5 μm). ST = secondary thickening; H = hyphal cell; HCW = host cell wall; IS = intercellular space; ML = middle lamella.



Figures 11–16. Cytological labelling for xylan by a xylanase-gold probe over the cell walls of healthy wheat spikes (Figures 11 and 12) and in *F. graminearum* inoculated wheat spikes (Figures 13 and 14) and immuno-gold labelling for pectin by monoclonal antibody, JIM7 (Figures 15 and 16). (11) Parenchyma cells in the ovary. The labelling density for xylan increased from the outer to the inner wall layer. The outer layer facing intercellular spaces and middle lamella show little or no gold labelling (Bar = 1 μm). (12) Uniform labelling of xylan on cell wall of the parenchyma cell in the lemma (Bar = 1 μm). (13) Parenchyma cells in the pericarp of the infected ovary, 3 dai. The host cell walls show a reduced xylanase-gold labelling (Bar = 1 μm). (14) Parenchyma cells in the infected lemma, 5 dai. The labelling density for xylan over the cell wall was markedly reduced (Bar = 0.5 μm). (15) The parenchyma cells in the lemma of a healthy spike, incubated with monoclonal antibody, JIM7. The cell wall and the middle lamella (arrowhead) were labelled with gold particles (Bar = 0.5 μm). (16) Parenchyma cells in the lemma of the infected spike incubated with monoclonal antibody, JIM7, 5 dai. Few gold particles were found over the cell walls and the middle lamella (arrowhead) (Bar = 0.5 μm). IS = intercellular space; H = *F. graminearum* hyphal cell; HCW = host cell wall.

rachis, labelling was found evenly distributed on the cortical cell walls, sieve tube walls and the primary and secondary thickenings of xylem vessels. There was no labelling in the control sections incubated either with colloidal gold solution alone or xylanase-gold to which xylan had been added previously. When ultrathin sections of *F. graminearum*-infected host tissues were incubated with xylanase-gold probe considerable alterations of density and pattern of gold particles in the cell walls were observed as compared to the sections of the corresponding healthy tissues. Figures 13 and 14 show that, when the hyphae were attached or close to the cell walls of the parenchyma cells either in the ovary or lemma tissues, the xylanase-gold labelling decreased tremendously. The same was true for the secondary thickening in the rachis.

Pectin

When healthy host tissues were incubated with the JIM7 monoclonal antibody directed against methyl esterified pectin and goat anti-rat gold solution, there was specific gold labelling over the cell walls of host tissue with differing labelling patterns. As expected, gold labelling was concentrated in the middle lamella and the outermost wall layer of parenchyma cells (Figure 15) and in junction areas between cells of the ovary or lemma. The cuticle and inner layers of these parenchyma cells showed no labelling. In the rachis, gold particles were observed in the middle lamella of xylem vessels and sieve tubes. Incubation of ultrathin sections of *F. graminearum*-infected tissue with JIM7 and goat anti-rat gold solution, resulted in reduced and irregular labelling of the primary wall and the middle lamella (Figure 16) as compared to the results of the healthy host tissues (Figure 15).

Discussion

The primary penetration sites of *F. graminearum* were mainly the ovary and the inner surfaces of the lemma and palea following single spikelet inoculations. After penetration of the plant cuticle, the fungal hyphae grew either inside the outer epidermal cell walls or penetrated the epidermis cells. *F. graminearum* hyphae developed both intercellularly and intracellularly in the tissues of wheat spikelets. These findings indicate similar behaviour of the two FHB pathogens *F. culmorum* and *F. graminearum* regarding penetration and spreading in the spike tissue (Kang and Buchenauer, 2000b).

When healthy host tissues were incubated with the monoclonal antibody (JIM7) and goat anti-rat gold solution, gold labelling was found mainly concentrated in the middle lamella and in primary cell walls, confirming that these regions are rich in pectin. Findings of Kang and Buchenauer (2000a) working with *F. culmorum* indicated that these junction regions may be more preferable sites for the pathogen entry. The same could be true for *F. graminearum* since, a very close relationship was found between these two *Fusarium* head blight pathogens with respect to penetration and spreading. Incubation of infected tissue with JIM7 and goat anti-rat gold solution, resulted in reduced and irregular labelling in comparison with the labelling patterns of sections in healthy host tissues. The reduced immuno-gold labelling indicates the potential role of pectolytic enzymes synthesized and secreted by *F. graminearum* during infection.

During inter- and intracellular growth of *F. graminearum* in the spike tissues, fungal hyphae penetrated host cell walls which contain a high proportion of cellulose according to the molecular architecture of grass cell walls (Carpita and Gibeaut, 1993). Cellulases are regulated by substrate induction and their optimum is usually at pH 5.0. The endo-glucanases are composed of a catalytic core and a terminal tail which are separated by an *O*-glycosylated linker region. The tail is involved in binding of the enzyme to polymeric cellulose (Knowles et al., 1987). Incubation of ultrathin sections of infected host tissues with cellulase-gold probe revealed alterations of density and pattern of gold particles as compared to the results of the corresponding healthy tissues. Host cell walls of the parenchyma cells either in the ovary or lemma, which were in direct contact with the hyphae of the pathogen showed a reduced cellulase-gold labelling compared to the considerably higher labelling density of walls distant from the pathogen–host interface or in non-colonized tissue areas. The reduced cellulase-gold labelling indicates that there was an enzymatic cellulose degradation of the polysaccharide network of the host cell wall at the vicinity of the pathogen. These results imply a potential role of cellulases in pathogenicity of *F. graminearum*.

Production of xylanases has been found predominantly with grass pathogens and synthesis of xylanases is induced by xylan and plant cell walls (Cooper et al., 1988; Degefu et al., 1995). The pH optimum of xylanases is around 5.0 and they hydrolyze β-1,4-glycosidic bonds and show catalytic similarities to cellulases. Results from incubation of ultrathin

sections of non-inoculated healthy host tissues with xylanase-gold probe resulted in regular and different labelling patterns in the ovary, lemma and the rachis. When the hyphae were attached or close to the cell walls, the gold labelling decreased tremendously, demonstrating effective enzymatic degradation of xylan by *F. graminearum*. This indicates that xylan degrading enzymes are important for the colonization of the host tissue.

In conclusion, the results reveal that germination of macroconidia and hyphal growth patterns in the host tissue were similar with those reported for *F. culmorum*. The studies of gold labelling of the cell wall polymers cellulose, xylan and pectin in infected tissues indicated that *F. graminearum* produces cellulase, xylanase and pectinase during penetration and colonization of the wheat spike tissues. The production and secretion of these CWDE aid infection by *F. graminearum* and its spread in the host tissues, as well as satisfying its nutritional requirements.

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