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Scanning Electron Microscopy of *Fusarium*-Infected Kernels of Winter Triticale (x *Triticosecale* Wittmack)

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ABSTRACT

Infection tests involved kernels of four winter triticale (x *Triticosecale* Wittmack) cultivars, which showed different infection severity following artificial inoculation of triticale heads with an aqueous suspension of *Fusarium culmorum* (W.G.Smith) Sacc. spores applied at mid-anthesis. After threshing, the concentration of ergosterol, an indicator of fungal biomass, was determined in kernels. Kernels, which differed in their severity of infection, were examined using scanning electron microscopy (SEM). We confirmed the presence of hyphae both on the surface and in the tissues of triticale kernels. Observations of the endosperm of severely infected kernels, classified as FDKs (*Fusarium* damaged kernels), proved the presence of hyphae in the endosperm and changes in its structure such as damage of its cellular structure, loose arrangement of starch granules, disappearance of small starch granules and damage to large starch granules. The microscopic image of the starch endosperm of triticale FDKs resembles that of wheat FDKs.

Keywords: *Fusarium culmorum*, kernel, scanning electron microscopy, triticale
Abbreviations: ERG, ergosterol; **FDK**, *Fusarium* damaged kernels; **FHB**, *Fusarium* head blight; **SEM**, scanning electron microscopy

INTRODUCTION

Triticale (x *Triticosecale* Wittmack), a wheat and rye hybrid, is a unique cereal crop in that it has been artificially created by man. Triticale is among those plant breeding achievements that have been most promptly implemented in agriculture. Triticale is recommendable for commercial cultivation owing to its tolerance to poor soil conditions, high yield potential and high fodder quality of grain. Triticale has become a specialty of plant breeding in Poland. It has been quickly accepted by Polish farmers and gained much popularity – in 2006 it was grown on 1.2 million ha in Poland (3.6 m ha worldwide) (FAOSTAT 2008). Most triticale cultivars are universal. The high value of Polish triticale varieties has been confirmed by their registration in many European countries as well as outside our continent. Triticale grain, which is an excellent source of protein and starch, is mainly used as animal fodder, but the quality assets of this cereal are far from being fully appreciated. Triticale grain is used, although on a small scale, to bake different types of bread, oriental noodles and soft-wheat products. It has also found some use in the malt and brewery industries or in the production of starch and bioethanol (Peña 2004; Glatthar *et al*. 2005; Kučerová 2007).

With time, some diseases of triticale plants have become more intense. This cereal is quite commonly infected by *Puccinia triticina* Eriks. (brown rust), *Septoria tritici* Roberge (septoria leaf) in Desmaz and *Stagonospora nodorum* (Berk.) E. Castell. & E.G. Germano (glume blotch) and various *Fusarium* spp. fungi. *Fusarium* head blight (FHB, scab) is a severe fungal disease of small grain plants caused by several species of the genus *Fusarium* (Perkowski and Wiwart 2002). It is a preharvest disease although *Fusarium* species can also grow after harvest if wet grain is not dried thoroughly and quickly. *Fusarium graminearum* Schwabe and *F. culmorum* (W.G. Smith) Sacc are the predominant *Fusarium* species which infect cereals. *F. graminearum* prevails in warmer, more humid parts of the world, such as the USA, whereas *F. culmorum* has been identified as the predominant species in cooler areas, for example north, central and west Europe. Due to their parasitic (facultative parasites) and saprophytic (competitive parasites) mode of life, *Fusaria* are present in the natural soil environment, but can also be transmitted with seed material. Predominance of a particular species is mainly conditioned by the climate, especially temperature and water demand (Parry *et al*. 1995; McMullen *et al*. 1997; Wagacha and Muthomi 2007). Triticale is less susceptible to head blight than wheat, although infection of this cereal by *Fusarium* fungi brings about similar results: depression of yield structure components and accumulation of a certain profile of fusarial mycotoxins in grain (Arseniuk *et al*. 1999; Perkowski and Kaczmarek 2002). Severity of infection depends on the virulence of a pathogen whereas differences in the plant's resistance as well as environmental conditions during the flowering and grain maturing phases are responsible for a specific response of a triticale cultivar to infection (Arseniuk *et al*. 1999; Chełkowski *et al*. 2000). The growth of mycelia in tissues of kernels as well as the presence of fungal metabolites in infected grain cause changes in the chemical composition and microstructure of kernels, which deteriorates their quality and technological parameters. Changes in the microstructure of wheat and barley grains infected by *F. graminearum* and *F. culmorum* revealed by scanning electron microscopy (SEM) assays have been well documented (Meyer *et al*. 1986; Šrobárová 1996; Nightingale *et al*. 1999; Schwarz 2003; Jackowiak 2005). Analogous studies on other cereals are fragmentary. The purpose of the present study was to examine using SEM winter triticale kernels, which were infected to various degrees after artificial inoculation of heads with *F. culmorum* under field conditions and examine the extent of damage caused by the pathogen.

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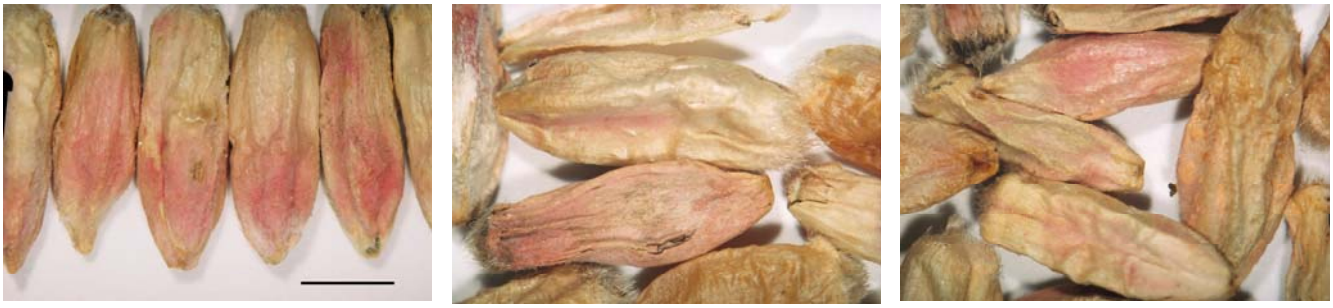

Fig. 1 Triticale kernels infected by *Fusarium culmorum* (W.G. Smith) Sacc (Nikon 102 stereoscopic microscope). Scale bar = 3 mm.

MATERIALS AND METHODS

Kernels of four winter triticale (x *Triticosecale* Wittm.) cultivars: 'Malno', 'Ugo', 'Prado' and 'Mundo,' were tested. Kernels were obtained from a trial set up to test resistance of several winter triticale cultivars and lines to *Fusarium* head blight (FHB) under field conditions, following artificial inoculation of heads with a mixture of isolates of *Fusarium culmorum*. These isolates were obtained from naturally infected wheat kernels and identified from pigmentation and morphological characters on PDA and SNA (Burgess *et al.* 1994). Isolates are deposited in collection of *Fusarium* isolates in the Department of Plant Pathology, Plant Breeding and Acclimatization Institute, Radzików, Poland. Isolates were incubated with autoclaved wheat grain in glass flasks for about 4 weeks and next exposed to permanent UV for 4 to 7 days at 18°C. The mycelium-colonized grain were dried and stored in refrigerator at 4°C until usage. At the day of inoculation grain was suspended in tap water and filtered to obtain conidial suspension. The suspensions from each of the 5 isolates were adjusted to 5×10^5 spores/ml with the aid of a hemocytometer and equal volumes were combined. Heads of triticale plants were inoculated by spraying with a suspension of spores. Inoculations were performed individually on each plot at the mid-anthesis phase (Feekes scale 10.52).

Symptoms of the disease were first assessed about 14 days after the last inoculation. In total, three such evaluations were performed at 7-day intervals. FHB severity was scored according to the mean percentage of blighted spikelets per each infected head and the percentage of infected heads per plot. At the maturity phase (Feekes scale 11.4), heads from infected plants were harvested manually and threshed with laboratory thresher (HEGE 16, Wintersteiger-Hege Maschinen, Austria) at a low wind speed to prevent loss of low-weight infected kernels. Triticale grain was analyzed by mean GC/MS for deoxynivalenol (DON), a trichothecene mycotoxin. The results of the study on FHB resistance of winter triticale cultivars and lines as well as their ability to accumulate DON in kernels were the subject of a separate report (Góral *et al*. 2002). In the present paper, we focused on the microstructure of triticale kernels characterized by various infection severity rates, which were scored according to the concentration of ergosterol.

For the microscopic observations, dry samples of kernels were fixed with 1% osmium tetroxide (for electron microscopy, Serva Electrophoresis GmbH, Germany) in 0.1 M cacodylate buffer (buffer substance for biochemistry, Merck, Germany) (pH 7.2) vapors for 12 h and then air-dried. Specimens were mounted on aluminum stubs using double-sided adhesive carbon tape and coated with gold (7 nm). The preparations were examined by means of a ZEISS 435 VP (Germany) SEM at high vacuum at 10–15 kV accelerating voltage (Jackowiak *et al*. 2005). Three kernels of each triticale cultivar derived from the inoculated combinations were examined. The surface of kernels, both on the dorsal and ventral side, was examined as well as their inner structure, paying particular attention to the endosperm.

The concentration of ergosterol in infected kernels was determined according to the method described by Perkowski *et al*. (2007). Briefly, 10 g sample of grain was grounded by mean of WŻ-1 laboratory grinder (Research Institute of Baking Industry Ltd., Bydgoszcz, Poland) and than 100 mg samples were placed into 17 ml culture tubes, suspended in 1 ml of methanol (HPLC grade, Sigma-Aldrich, Steinheim, Germany), treated with 0.1 ml of 2 M aqueous NaOH (Chempur, Poland), and sealed tightly. Then the culture tubes were placed within 250 ml plastic bottles, sealed tightly, and placed inside a microwave oven (Whirlpool model AVM 401/WH) operating at 2450 MHz and 900 W maximum output. Samples were irradiated (370 W) for 20 s, after *c*. 5 min, for an additional 20 s and extracted with pentane (HPLC grade, Sigma-Aldrich, Steinheim, Germany) (3 × 4 ml) within the culture tubes. The combined pentane extracts were evaporated to dryness in a gentle stream of a high purity nitrogen. Prior to analysis samples were dissolved in 4 ml of methanol, filtered through 13 mm syringe filters with a 0.5 μm pore diameter (Fluoropore Membrane Filters), evaporated to dryness by a nitrogen stream and dissolved in 1 ml of methanol. Prepared samples were analyzed by HPLC. Separation was achieved on a 150 mm length x 3.9 mm diameter Nova Pak C-18, 4 μm particle size column and eluted with methanol/acetonitrile (90:10) at a flow rate of 0.6 ml/min. ERG was detected with a Waters 486 Tunable Absorbance Detector set at 282 nm. Quantification of ERG was made by a comparison of peak areas with those of external standards of ERG (> 95%, Aldrich, Milwaukee, USA). Confirmation of ERG was achieved by a comparison of retention times with the external standard and by co-injection every tenth sample with an ERG standard.

Values of CV was 5.4%, recovery (± SD) was 96.7 ± 3.64% (RSD 3.76%). Limit of detection was 0.02 mg/kg and limit of quantitation was 0.06 mg/kg.

RESULTS AND DISCUSSION

Morphology of infected kernels and ERG content

Cereal kernels infected by *Fusarium* spp. fungi were thin and shrunken and show typical symptoms such as poor seed fill, changed colour and presence of mycelium, white, white and pink, salmon pink or bright red in colour, on the surface of a kernel (**Fig. 1**). Kernels of two winter triticale cultivars, 'Mundo' and 'Malno', which were slightly or very slightly infected, showed very low levels of ERG, which is considered to be an indicator of fungal biomass (Müller and Lehn 1988). In cv. 'Mundo' kernels, the concentration of ergosterol was less than 3 mg/kg, i.e. below the ergosterol threshold level accepted for good mycological quality grains (Schnürer and Jonsson 1992). Kernels produced by cultivars 'Mundo' and 'Malno' were relatively well filled and the hyphae present on their surface were more numerous in the crease on the ventral side (**Fig. 2**). Kernels of the other two cultivars, 'Ugo' and 'Prado', which showed very severe infection symptoms such as irregular shape of kernels, damage to the seed cover and abundant mycelium on the surface, contained high amounts of ergosterol (**Table 1,**

Table 1 Content of ergosterol in kernels of four winter triticale cultivars characterized by different severity of *Fusarium culmorum* infection.

Cultivar (cv.)	Ergosterol (mg/kg)
Mundo	2.10
Malno	9.70
Ugo	84.10
Prado	97.90

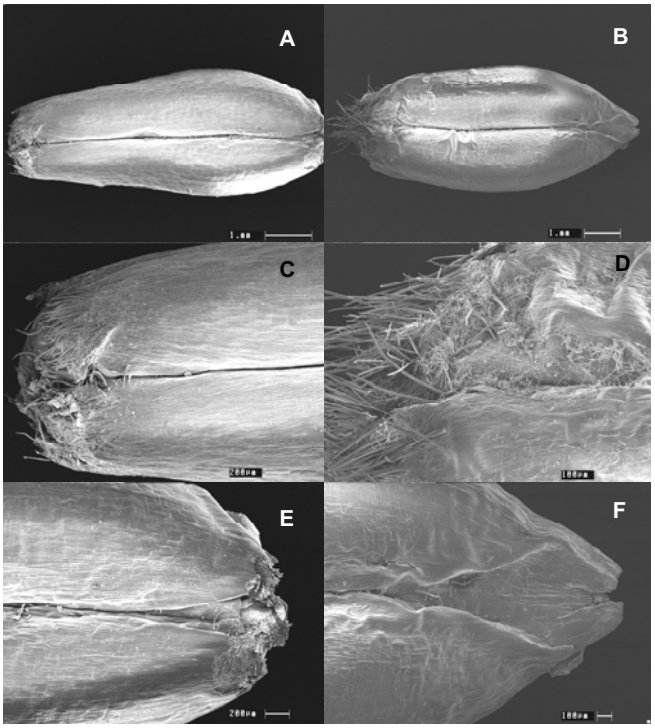

Fig. 2 Morphology of winter triticale kernels low in ergosterol. (**A, B**) Whole kernels, viewed from the ventral side. (**C, D**) Enlarged fragments of a kernel on the side of the brush, visible hyphae among hairs of the brush. (**E, F**) Enlarged fragments of a kernel on the side of the embryo, visible damage to the pericarp. A, C, E: cv. 'Mundo'; B, D, F: cv. 'Malno'.

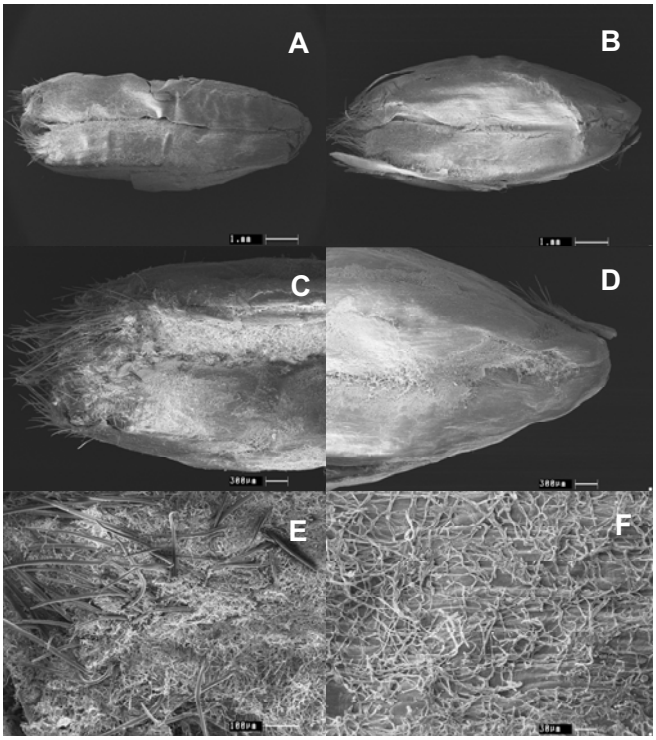

Fig. 3 Morphology of severely infected triticale kernels high in ergosterol. (**A, B**) Whole kernels with damaged pericarp and abundant mycelium on the surface, seen on the ventral side. (**C, E**) Enlarged fragments of a kernel on the side of the brush, with abundant mycelium on the surface and between hairs of the brush. (**D**) Enlarged fragment of a kernel on the side of the embryo with abundant mycelium on the surface. (**E**) Mycelium of the dorsal side of an infected kernel. **A, C, E**: cv. 'Ugo'; **B, D, F**: cv. 'Prado'.

Fig. 3). In all the triticale cultivars examined, higher levels of ergosterol corresponded to higher DON concentrations, which reached 7.5, 32.5, 96.3 and 118.8 mg/kg for cvs. 'Mundo', 'Malno', 'Ugo' and 'Prado', respectively (Góral *et al*. 2002).

Internal structure of kernels

The inside of healthy-looking kernels was completely free from hyphae. These kernels were regular in shape at the cross-section (**Fig. 4A**). In contrast, badly infected kernels were deformed, their pericarp was damaged and hyphae were found both on their surface and inside tissues (**Fig. 4B-F**). Areas where hyphae were observed inside triticale kernels are identical to those observed in spring wheat kernels (Jackowiak *et al*. 2005).

One possible measure of fungal disease severity is the extent to which the endosperm structure has been changed. Starch in the endosperm of triticale is deposited in two types of starch granules: large (type A) and small (type B) ones, which is similar to wheat, rye and barley grains (Lorenz *et al*. 1978; Hoseney 1994; Jane *et al*. 1994; Perez 2004). Triticale starch is bimodal, with round pancake granules 22-36 μm thick and 6-10 μm in diameter for large granules or up to 5 μm for small ones (Jane *et al*. 2004). The endosperm of kernels, which were slightly infected on the surface only (without penetration into the endosperm), was completely free from hyphae. The inside of the endosperm cells was densely packed with large and small starch granules immersed in the protein matrix (**Fig. 5**). Analysis of FDKs revealed the presence of hyphae in the starch endosperm, among starch granules, as well as typical changes in the morphology of starch granules (**Fig. 6**). The structural changes in the endosperm which we noticed included: disappearance of the cellular structure of the endosperm and loose arrangement of starch granules; disappearance of small starch granules; damage to large starch granules.

The microscopic image of the starch endosperm of FDKs of winter triticale resembles damages that of FDKs of common wheat (Meyer *et al*. 1986; Nightingale *et al*. 1999; Jackowiak *et al*. 2005) and barley (Schwarz 2003). A similar type of damage to starch granules was observed to

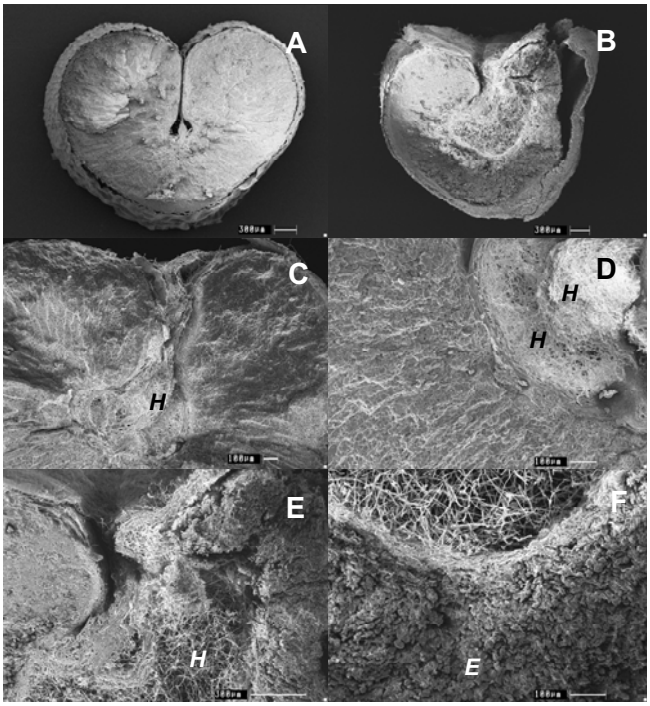

Fig. 4 Transverse sections of triticale kernels characterised by different infection severity. (**A**) Transverse section across a regularly shaped and very weakly infected kernel. (**B**) Transverse section across a severely infected kernel. (**C, D**) Hyphae (*H*) in the crease and air spaces of the pericarp at the base of the crease. (**E**) A cavern with hyphae (*H*) inside the starch endosperm of a deformed kernel. (**F**) Changed (loose) structure of the endosperm (*E*) at the base of the crease filled with hyphae (*H*). **A**: cv. 'Malno'; **B, D, E, F**: cv. 'Prado'; **C**: cv. 'Ugo'.

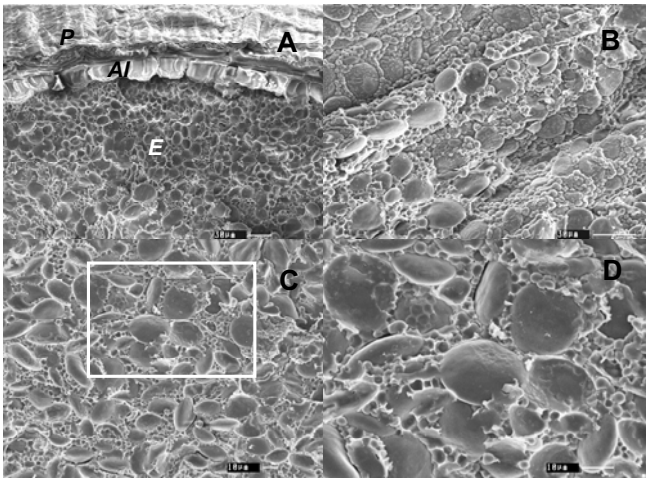

Fig. 5 Structure of the endosperm in surface infected triticale kernels. (**A**) Fragment of a transverse section comprising the pericarp (*P*), aleurone cells (*Al*) and the starchy endosperm (*E*) without hyphae. (**B, C**) Transverse section across the endosperm with visible large and small starch granules immersed in the protein matrix. (**D**) Enlarged fragment of the endosperm in (**C**). **A, B**: cv. 'Malno'; **C, D**: cv. 'Mundo'.

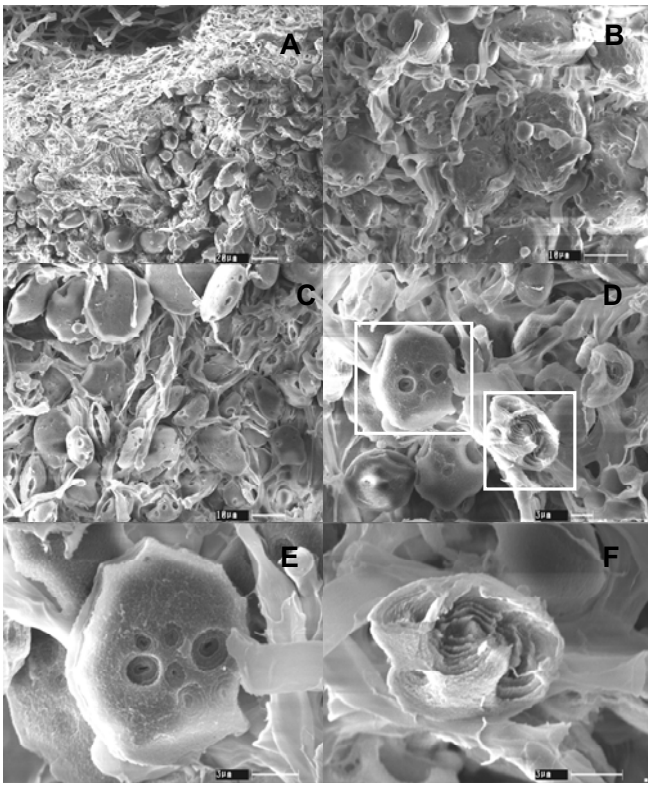

Fig. 6 Structure of the endosperm in severely infected triticale kernels. (**A**) Loose arrangement of starch granules in the endosperm at the base of the crease. (**B**) Damaged, porous structure of large starch granules, fewer small starch granules, lack of the protein matrix, visible hyphae of *F. culmorum* between damaged starch granules. (**C, D**) Very badly damaged large starch granules, lack of small granules or the protein matrix, visible hyphae between damaged starch granules. (**E**) A large 'hamburger-like' starch granule with holes along the equatorial groove and funnel-shaped craters on the lateral side (enlarged fragment of (**D**)). (**F**) Very badly damaged large starch granule with a visible concentric structure of particular layers which constitute a starch granule (enlarged fragments of Fig. (**D**)). **A, C, D**: cv. 'Prado'; **B**: cv. 'Ugo'.

have been more pronounced at the edge of the kernel along the equatorial groove than on the lateral sides (**Fig. 6C-E**). Very severe damage revealed the concentric structure of starch granules (**Fig. 6F**). By analogy to wheat kernels, the pattern of damage caused by penetration of fungal hydrolytic enzymes towards the inside of starch granules was random. Possible correlations between the degree of starch endosperm damage in FHB infected kernels versus the hydrolytic activity of *Fusarium* spp., which have been discussed in detail in our previous paper (Jackowiak *et al*. 2005), hold true for the *F. culmorum* and winter triticale pathosystem. Atomic force microscopic (NC-AFM) studies on triticale starch granules have demonstrated that the surface of triticale starch granules contains holes or pores as well as minor surface structures, which may have been produced by more intense activity of endogenous amylolytic enzymes present in the endosperm of triticale (Juszczak 2003). Obtained results suggest that *Fusarium* infection can enhance the activity of endogenous amylolytic and/or proteolytic enzymes in the triticale endosperm and result in accelerated hydrolysis of major storage materials, such as proteins and starch.

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