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Antifungal Properties of Wheat Histones (H1−H4) and Purified Wheat Histone H1

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ABSTRACT: Wheat (*Triticum* spp.) histones H1, H2, H3, and H4 were extracted, and H1 was further purified. The effect of these histones on specific fungi that may or may not be pathogenic to wheat was determined. These fungi included *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Fusarium solani*, *Fusarium graminearum*, *Penicillium digitatum*, *Penicillium italicum*, and *Greeneria uvicola*. Non-germinated and germinating conidia of these fungi were bioassayed separately. The non-germinated and germinating conidia of all *Fusarium* species were highly susceptible to the mixture (H1−H4) as well as pure H1, with viability losses of 99−100% found to be significant ($p < 0.001$) at ≤10 μM or less for the histone mixture and pure H1. *F. graminearum* was the most sensitive to histone activity. The histones were inactive against all of the non-germinated *Penicillium* spp. conidia. However, they significantly reduced the viability of the germinating conidia of the *Penicillium* spp. conidia, with 95% loss at 2.5 μM. Non-germinated and germinating conidia viability of the *Aspergillus* spp. and *G. uvicola* were unaffected when exposed to histones up to 10 μM. Results indicate that *Fusarium* spp. pathogenic to wheat are susceptible to wheat histones, indicating that these proteins may be a resistance mechanism in wheat against fungal infection.

KEYWORDS: Wheat, histones, *Fusarium*, fungi, antifungal

INTRODUCTION

Wheat (*Triticum* sp.) is a grass that has been cultivated domestically for over 10 000 years. Worldwide, it is the third most important food grain after maize and rice. *Fusarium* species cause leaf blotch, head blight, root rot, foot rot, and crown rot in wheat. This genus is one of three fungal genera that cause black point in wheat.[1] *Fusarium graminearum* is the most common causal agent of *Fusarium* head blight (FHB), a destructive disease of cereal grain crops with worldwide economic impact.[2] Economic losses because of this disease alone are huge. In wheat and barley, such losses because of FHB were estimated at $3 billion between 1990 and 2002.[3] Direct and secondary economic losses because of FHB for all crops in only the northern great plains and central United States were approximately $2.7 billion from 1998 to 2000.[2] It is a major limiting factor on wheat production in certain sections of the world.[4]

F. graminearum produces a number of potent mycotoxins that render contaminated wheat unusable. These include the trichothecenes and zearalenone, an estrogenic toxin. Deoxynivalenol, a trichothecene, inhibits protein biosynthesis. In animals, this toxin causes feed refusal, diarrhea, emesis alimentary hemorrhaging, and contact dermatitis.[5] Trichothecenes are also linked to the potentially lethal alimentary toxic aleukia and Akakabi toxicosis.[2]

There are six highly conserved histone types (H1, H2A, H2B, H3, H4, and H5). The "core" histones (H2A, H2B, H3, and H4) form an octameric complex that constitutes the nucleosome, which consists of two of each of the four core histone families.[6] Initially, it was thought that histones merely serve as a spindle upon which DNA is wound. However, more recent experiments have shown that these proteins are also involved in transcription regulation, DNA replication, repair, and condensation.[7] Core histones are crucial to the condensed packing of DNA.[8] H1 and H5 are "linker" histones that seal the loops of DNA extending from nucleosomes and keep the nucleosome structure condensed and compact.[9]

Histones are highly conserved throughout nature. However, differences do exist in the histone structures not only between the plant and animal kingdoms[10] but also between different plants.[11,12] Variations exist among the linker H1 histones of higher eukaryotes where the central globular domain is conserved but the N-terminal and C-terminal tails are less conserved.[11] There are differences in histones of the same plant family. For example, differences exist among the histone H1 of legume species and H2A in wheat.[12,13] Histones are known to possess potent antimicrobial activity, as either intact proteins or cleaved peptides.[14] Antimicrobial histones are present in a wide range of organisms from shrimp to humans.[14]

The antimicrobial nature of histones is not a factor of the combined group but resides in certain histones, which varies according to the host.[15,16] Minor changes in the structure of the antimicrobial histones can significantly affect antimicrobial properties of peptides derived from the antimicrobial portion of histones. For example, parasin I is a histone H2A-derived antimicrobial peptide. A loss of a single lysine from this molecule causes the loss of antimicrobial properties.[17] In other cases, proline is crucial to the antimicrobial properties of peptides obtained from histone H1 or H2A regions.[18]

Although some reports have been published on the antibacterial properties of various histones, little is known of histone

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antifungal activity. The purpose of this study was to determine the *in vitro* antifungal properties of mixed wheat histones (H1−H4) as well as purified H1 against several genera of filamentous fungi found in the environment. These included several *Fusarium* species as well as species of genera (*Aspergillus*, *Penicillium*, and *Greeneria*) not known to be wheat pathogens.

MATERIALS AND METHODS

Wheat Histone Mixture Extraction. Histone extraction from wheat seeds was performed by acid extraction according to a modification of an earlier protocol.[19] Sodium bisulfate (50 mM) was added as a protease inhibitor to all buffers used in the preparation of chromatin. Wheat germ (100*g*) was homogenized at full speed for 1 min in ice with coarse knives in 400 mL of TBT buffer [0.05 M Tris·HCl (pH 8.1), 15 mM β-mercaptoethanol, and 0.5% (w/v) Triton X-100] and 100 μL of 1-butanol (to reduce foaming). The slurry was poured into a beaker, and 100 mL of TBT was added and stirred in ice for 10 min using a magnetic stirrer. The homogenized material was passed through a series of nylon fabrics with defined mesh (2, 1, 0.5, and finally, 0.3 mm).

Ammonium sulfate solution (3 M) was added dropwise to a final concentration of 50 mM to aggregate the chromatin. This mixture was gently stirred for 10 min at 4 °C, and the chromatin recovered by centrifugation at 10000*g* at 4 °C for 10 min. The pelletted chromatin was resuspended in 200 mL of TBT−0.5 mM ammonium sulfate in a hand homogenizer with a Teflon pestle. The homogenized chromatin was centrifuged (10000*g* at 4 °C for 10 min). The supernatant was removed, and the chromatin was homogenized and washed a total of 4 times in TBM buffer. Finally, the pelleted chromatin was resuspended in 0.1× SSC−5 mM sodium bisulfite to a final volume of 100 mL and stored in ice overnight at 4 °C.

To the recovered chromatin (100 mL) was added dropwise 25 mL of 0.5 M sulfuric acid followed by stirring at 4 °C for 4 h. Subsequently, the suspension was centrifuged at 10000*g* for 10 min at 4 °C, and the supernatant was harvested.

The chromatin pellet was re-extracted with 50 mL of 0.1 M sulfuric acid and stirred for 4 h at 4 °C, followed by centrifugation (10000*g* at 4 °C for 10 min). The supernatant was collected and combined with the previous supernatant.

The combined supernatants were transferred to dialysis tubing [molecular weight cut-off (MWCO) = 3500], covered with polyethylene glycol (PEG) 6000 on a tray, and stored overnight at 4 °C to concentrate the material. Next, the dialysis tubing was rinsed with tap water to remove excess PEG, then dialyzed against 0.1% acetic acid (3 × 4000 mL) at 4 °C, and finally, clarified by centrifugation at 10000*g* for 15 min at 4 °C. The clarified material containing the histone mixture was stored at −20 °C until further use.

Analysis of Wheat Germ Histones. To confirm the presence and purity of the histone mixture, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), spectrophotometric analysis, and protein determination were performed. SDS−PAGE was performed (with T = 15% and C = 3.3%) by running the samples at 120 V in a Mini-Protean system (BioRad, Hercules, CA), in which the Laemli discontinuous buffer system was employed. An absorption spectrum of acid-extracted histones in 0.1% acetic acid was performed in a NanoDrop spectrophotometer (Saveen and Werner AB, Limhamn, Sweden). Finally, the protein concentrations of the histone preparations were performed using bicinchoninic acid (BCA) and with bovine serum albumin as the protein standard.

Hydroxyapatite Chromatography. Wheat germ histones were dialyzed against 100 mM potassium phosphate buffer at pH 6.7 and 300 mM NaCl and applied to a 2.5 × 17 cm Bio-Gel HT hydroxyapatite (Bio-Rad) column equilibrated with the same buffer. After the column was washed with the same buffer, histone proteins were eluted using a 300 mM−2 M NaCl gradient in 100 mM potassium phosphate buffer at pH 6.7. The recovered material was finally dialyzed against 0.1% acetic acid and stored at −20 °C.

Bioassays. Filamentous fungi used in bioassays included *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioides*, *F. graminearum*, *Penicillium digitatum*, *Penicillium italicum*, and *Greeneria uvicola*. Assays to determine the fungicidal properties of the histones against the non-germinated and germinating conidia were determined separately as described previously.[20] Because the histones (H1−H4) in a mixture have various molecular weights, an average molecular weight of 19 081 was used to determine final test concentrations. A molecular-weight average of 25 900 was used for pure H1 in the respective bioassays.

The fungi were grown on potato dextrose agar (PDA) (Difco, Detroit, MI) slants at 30 °C for 1 week, followed by storage at 4 °C. Prior to testing, 3 mL of 1% potato dextrose broth (PDB; Difco) was added to the slant culture and the conidia were suspended by gentle agitation with the pipet. Conidial suspensions (3×10^4/mL) were prepared using a hemocytometer to calculate the conidial concentration. One set of conidia was used immediately in the non-germinated conidial assays. Serial dilutions of the histone mixture were prepared in 1% PDB with final histone concentrations of 0−8.7 μM. To the histone mixture solution was added 25 μL of the conidial suspension with a final volume of 250 μL. The test suspensions were incubated at 30 °C for 30 min. Aliquots (50 μL) from each sample were spread on each of four PDA plates that were incubated at 30 °C for 48 h, after which viable colonies were enumerated. Bioassays were performed on three separate occasions (n = 12).

A time-course study using the H1−H4 mixture was performed with the non-germinated and germinating conidia of *F. graminearum*. The protocol used was the same as above, except for the incubation times (0, 10, 20, 30 min).

Pure H1 histone was tested as described above, except with a concentration range of 0−11.8 μM. Because of the small amount of H1 histone available, only members of the *Aspergillus* and *Fusarium* genera were tested in the bioassays.

Statistical Analysis and Graph Preparation. Statistical analysis was performed using SigmaStat, which determined mean, standard error, and significance ($p < 0.001$). These data were used to prepare the graphs showing viability loss or lack of the same for each fungal species. To obtain the graph points, the mean of the viable count for the respective histone concentration treatments was divided by the mean of the viable count for the respective control. The result was then multiplied by 100 to obtain the percent viability mean for the respective histone concentration. Error bar values were determined by dividing the mean of the standard error for the respective histone concentration treatment by the mean of the respective control and then multiplying the resulting value by 100.

Scanning Electron Microscopic Studies. The effect of wheat histones (H1−H4) on *F. graminearum* was studied by scanning electron microscopy (SEM). Suspensions of *F. graminearum* non-germinated and germinating conidia were prepared as described above. Four 2 mL mixtures of non-germinated conidia and 1% PDB (200 μL of conidia and 1800 μL of 1% PDB) were prepared as controls. The same number of test samples containing non-germinated conidia (200 μL), 1% PDB, and wheat histone mixture (H1−H4) to achieve a final concentration of 2.5 μM was prepared. A similar number of control and test samples of germinating conidia was prepared. After the samples were vortexed, both sets of conidia were incubated for 30 min at 30 °C.

After incubation, the samples were centrifuged in an Eppendorf microcentrifuge model 5417C (Hamburg, Germany) in a swinging bucket rotor at 11700*g* relative centrifugal force (RCF) for 2 min. The supernatant was removed, and 500 μL of a 3% solution of glutaraldehyde was added. After mixing, the samples were placed at room temperature for 48 h.

The samples were then centrifuged, and the supernatant was removed. The samples were then dehydrated in successive single treatments (500 μL) of 20, 40, 60, and 80% ethanol and three treatments with 100% ethanol. Each ethanol concentration was kept quiescently for 2 h, after which the samples were centrifuged as before and the supernatant was removed. The third aliquot of 100% ethanol was not removed from the sample.

The dehydrated samples were then filtered to remove the alcohol using a 13 mm Nucleopore filter (2 μM) that was held by a Swinnex filter holder. Next, a 5.5 cm no. 52 filter paper (Whatman, Maidstone, U.K.) was folded in half with the edges folded and stapled and was placed in a 5.0 cm Petri dish containing about 10 mL of 100% ethanol. The Nucleopore filter was quickly removed from the Swinnex holder and placed inside the folded filter paper. The open end of the filter paper was stapled shut and returned to the 100% ethanol. Lastly, the sample in the folded "filter paper sandwich" was critical-point-dried from liquid carbon dioxide by a standard protocol in a model 28 000 Ladd critical point dryer (Ladd Research Industries, Williston, VT).

After critical point drying, the Nucleopore filters and/or filter paper were mounted on standard Cambridge SEM stubs using double-stick Avery photo tabs. The SEM mounts were coated with 60:40 gold/palladium using a Hummer II sputter coater to a thickness of 200 nm. The specimens were examined in an environmental scanning microscope (FEI, Hillsboro, OR) at an accelerating voltage from 15 to 20 kV under high vacuum conditions.

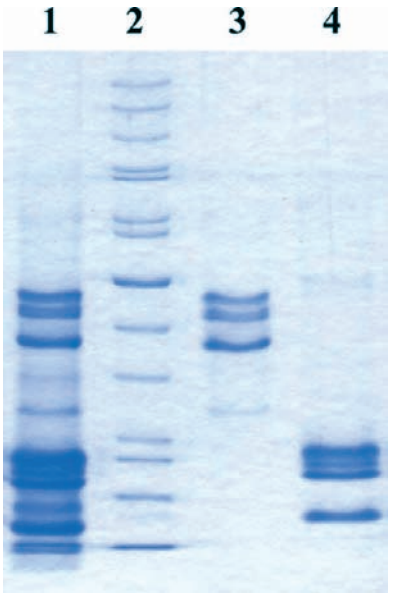

Figure 1. SDS−PAGE analysis of wheat germ histones: lane 1, total protein from acid extraction of wheat chromatin; lane 2, molecular-weight standard (Fermentas PageRuler no. SM0661); lane 3, histone H1 from hydroxyapatite chromatography (molecular weights of 17 000−28 000); and lane 4, histones from hydroxyapatite chromatography, from the top, histone H3 (15 300 Da), H2A (13 900 Da), H2B (13 800 Da), and H4 (11 300 Da).

RESULTS

Histone Extraction and Analyses. About 800 mg of acid-soluble proteins were recovered from 100*g* of wheat germ. SDS−PAGE showed that mainly histones were present in the material from acid-extracted chromatin (Figure 1). The molecular weights varied among the four histones. Pure wheat histone H1 (Figure 1) was determined to have a molecular weight of 24 300−27 500 Da. The molecular weights (Figure 1) of the remaining histones were determined to be as follows: H3 (17 475 Da), H2A (15 586 Da), H2B (16 433 Da), and H4 (13 197 Da). The aberrant mobility of the histones in SDS−PAGE may be the result of the high content of positive charged amino acids.[21] The size heterogeneity of H1 is likely due to post-translational modifications.[21] The protein patterns are the same as reported by others for histones.[12,22]

The extracted proteins were further purified by hydroxyapatite chromatography, and two peaks were obtained (Figure 2). Pure H1 was harvested from "peak A" (Figure 2), while "peak B" contained the remaining histones (Figure 2).

Figure 3 shows a spectrum for total histones in 0.1% acetic acid in the region of 215−300 nm. Although they are proteins, the absorbance of histones at 280 nm is low because the content of aromatic amino acids is very low. However, histones do absorb at a wavelength around 230 nm.[23]

Fungicidal Properties. The fungicidal properties of the histone mix for the tested *Fusarium* species are shown in panels A and B of Figure 4. The histones were highly lethal for both the non-germinated and germinating conidia of these fungi. A loss of viability (100%; $p < 0.001$) was observed for the non-germinated and germinating conidia of *F. oxysporum* and *F. solani* between 4.4 and 8.7 μM (Figure 4A). A total viability loss ($p < 0.001$) was

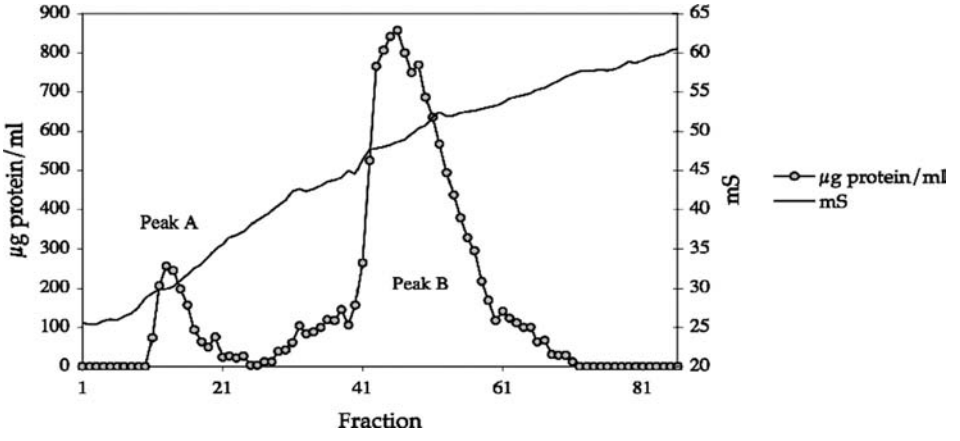

Figure 2. Hydroxyapatite chromatogram of acid-extracted wheat germ histones. Fractions corresponding to "peak A" and "peak B" were pooled separately and dialyzed against 0.1% acetic acid.

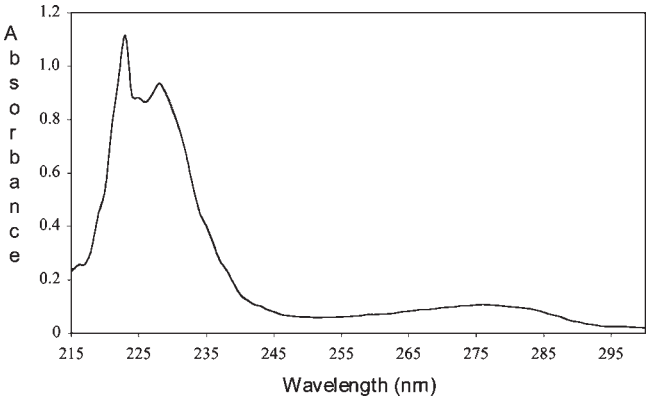

Figure 3. Total wheat germ histone mixture was in 0.1% acetic acid analyzed by spectrophotometry in a NanoDrop photometer.

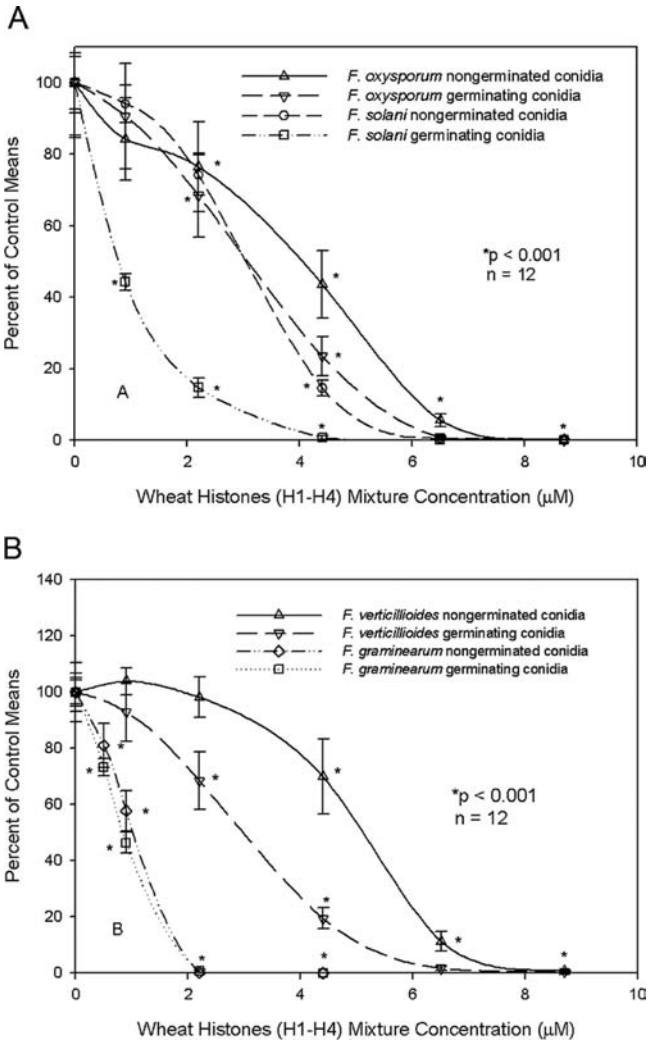

Figure 4. (A) Wheat histones (H1−H4) *in vitro* activity against *F. solani* and *F. oxysporum*. (B) Wheat histones (H1−H4) *in vitro* activity against *F. verticillioides* and *F. graminearum*.

observed for the non-germinated and germinating conidia of *F. graminearum* and *F. verticillioides* at 2.2 and 8.7 μM, respectively (Figure 4B).

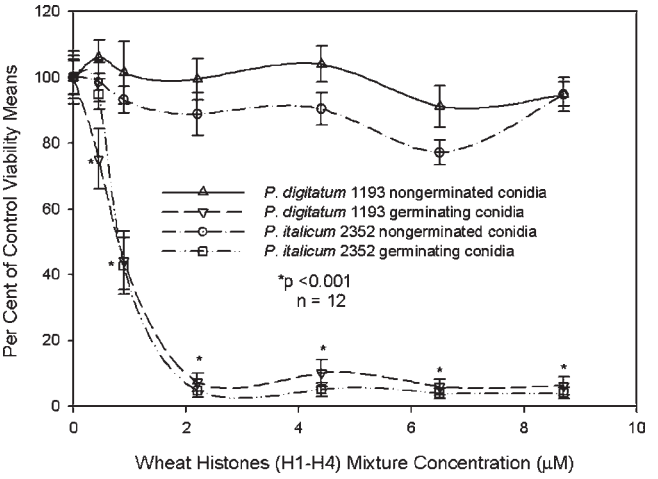

Figure 5. Wheat histones (H1−H4) *in vitro* activity against *P. digitatum* and *P. italicum*.

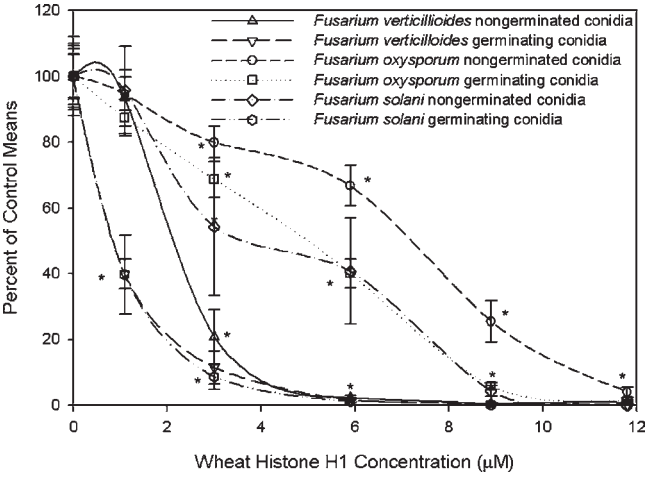

Figure 6. Purified wheat histone (H1) *in vitro* activity for *F. verticillioides*, *F. oxysporum*, and *F. solani*.

Non-germinated conidial viability of *P. digitatum* and *P. italicum* were not reduced by the mixed histones (Figure 5). However, the viability of the germinating conidia of these two species was reduced by 90−95% beginning at 2.2 μM. In contrast, no viability reduction was observed for the *Aspergillus* species tested or for *G. uvicola* (data not shown). Pure H1 histone only showed minor activity against *A. niger* non-germinated conidia and no activity against the non-germinated conidia of *A. flavus* and *A. fumigatus* or the germinating conidia of all three species (data not shown).

In contrast, pure wheat histone H1 displayed significant activity against the non-germinated and germinating conidia of the three tested *Fusarium* species beginning at 3.5 μM. Pure H1 (Figure 6) was more active against the non-germinated and germinating conidia of *F. verticillioides* than the H1−H4 mixture (Figure 4B). Viability losses for *F. solani* and *F. oxysporum* were very similar with the same concentrations of pure H1 and the H1−H4 mixture.

Results of the time-course study are shown in panels A (0 and 10 min incubation) and B (20 and 30 min incubation) of Figure 7. The data show significant viability reduction for both

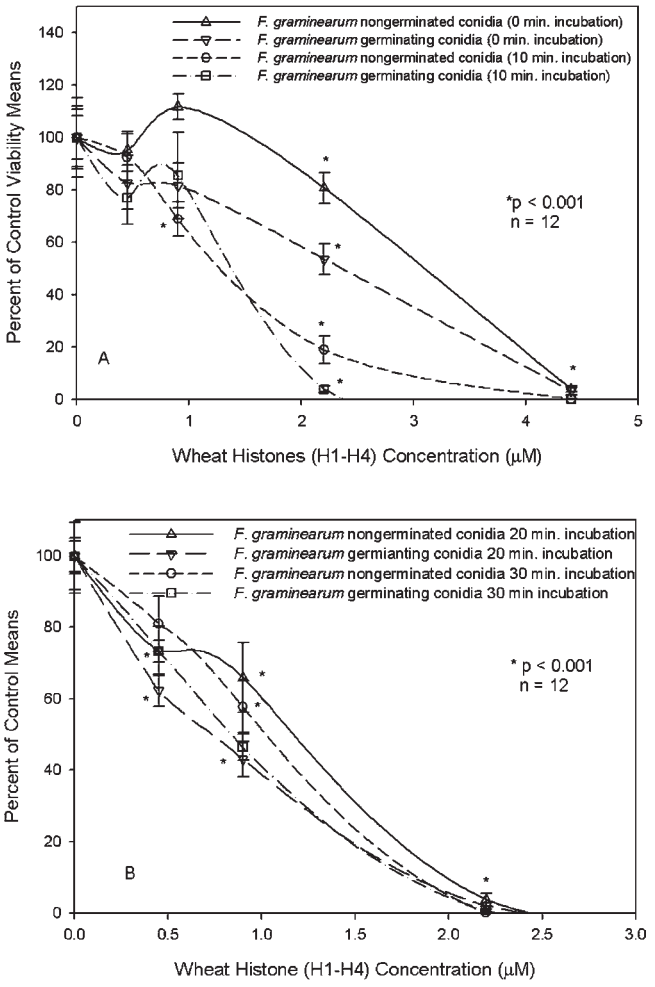

Figure 7. (A) *In vitro* time-course study of wheat histones (H1−H4): 0 and 10 min incubation with *F. graminearum*. (B) *In vitro* time-course study of wheat histones (H1−H4): 20 and 30 min incubation with *F. graminearum*.

the non-germinated and germinating conidia (19 and 46% loss, respectively) of *F. graminearum* at 2.2 μM at 0 min of incubation (Figure 7A). After 10 min of incubation, the viability loss of 46 and 97% at 2.2 μM was more pronounced for the non-germinated and germinating conidia, respectively. The viability reduction graphs produced by 20 and 30 min incubation periods were essentially the same (Figure 7B). For both incubation periods, approximately 100% viability reduction was achieved with the histones at 2.2 μM for the non-germinated and germinating conidia.

SEM Results. Figure 8A shows the *F. graminearum* non-germinated conidia control sample, while Figure 8B displays *F. graminearum* non-germinated conidia after incubation for 30 min with the wheat histone mixture. The conidia in Figure 8B appear to be covered by a matrix that is possibly comprised of histones and which is not present in Figure 8A. No pore formation is evident in the conidial wall of the conidia (Figure 8B).

Figure 8C displays *F. graminearum* germinating conidial control sample, which shows a normal conidium with developing hyphae. A germinating conidium displaying early growth hyphae that was incubated with histones is shown in Figure 8D. Pore formation is not visible; however, the hyphae appear to be covered by a mass of histones. The mass could also be many "bleb"-like structures, which would indicate a weakening of the developing hyphae or fungal wall because of histone interaction with possible binding sites.

DISCUSSION

Histones are cationic proteins whose antimicrobial properties reside in the basic residue of the N terminus, which is also important for the antimicrobial activity of histone-derived peptides.[18,19] For example, parasin 1 is a 19 amino acid histone H2A-derived antimicrobial peptide. Parasin 1 antimicrobial activity is lost when polar, neutral, or acidic residues are substituted for basic residues, but activity is regained when a basic amino acid is reintroduced.[18]

The cationic wheat histones investigated in the present study displayed rapid and significant fungicidal properties in less than 1 min. The cationic nature of some peptides cause permeabilization and pore formation of the cytoplasmic membrane, leading to rapid cell death. It is possible that antimicrobial peptides and wheat histones kill microorganisms via a mode of action similar to the "carpet model" of the peptide interaction with microbial membranes.[24]

However, the SEM images do not suggest pore formation after incubation of either the non-germinated or germinating conidia of *F. graminearum*. Research has indicated that membrane permeability is needed for the peptides to affect their primary, internal, anionic targets, namely, protein and DNA synthesis.[25] For example, buforin II, is a H2A-derived antimicrobial peptide whose analogues were shown to penetrate but not permeabilize bacterial membranes and accumulate in the cytoplasm, where it is believed to interact with nucleic acids.[26]

The ability of cationic wheat histones to significantly reduce the non-germinated conidial viability of the tested *Fusarium* species is similar to that observed with cationic peptides, such as dermaseptin (*Phyllomedusa sauvagii* skin) and cecropin A.[20] However, in contrast to the lack of activity against the tested *Aspergillus* species by the wheat histones, these peptides significantly reduced the germinating conidial viability of *A. flavus*, *A. fumigatus*, and *A. niger*.

The wheat histones had their greatest activity against *F. graminearum*, the causative agent of FHB in wheat and barley. The concentration needed to achieve 100% viability loss for this species is, approximately, only 25% of that needed to achieve the same degree of viability loss with the other tested *Fusarium* species. These results suggest that wheat histones are designed to prevent the growth of the *Fusarium* spp., especially *F. graminearum*, which are wheat phytopathogens.[27]

It was not surprising that the wheat histones were ineffective against the tested *Aspergillus* species and *G. uvicola*, which are not pathogens of wheat.[27] However, the significant lethality of these histones for only the germinating but not the non-germinated conidia of *P. digitatum* and *P. italicum* was not expected. This suggests that the germinating conidia of the tested *Penicillium* species share binding sites with *Fusarium* species or that the histones are capable of attaching to several types of binding sites.

The time-course study showed that, *in vitro*, the wheat histones act very rapidly and significantly reduce *F. graminearum* non-germinated and germinating conidial viability. Significant viability loss for both non-germinated and germinating conidia was achieved immediately with a concentration of 2.5 μM.

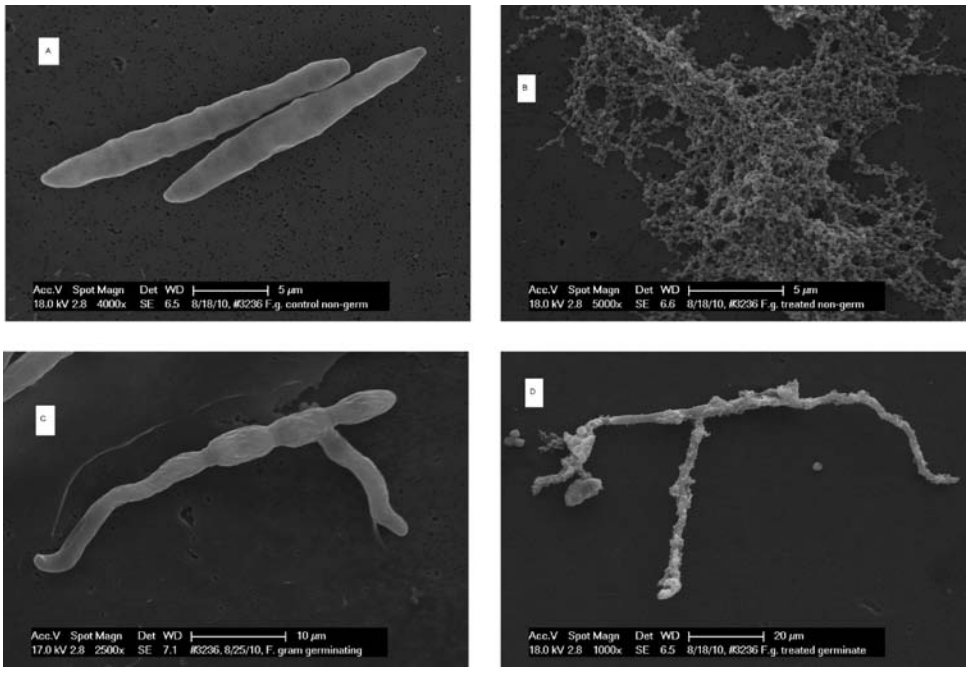

Figure 8. (A) SEM image (4000×) of the non-germinated conidial control of *F. graminearum*. (B) SEM image (5000×) of non-germinated conidia of *F. graminearum* after incubation with 2.5 μM wheat histones (H1−H4) for 30 min. (C) SEM image (2500×) of a germinating conidial control of *F. graminearum*. (D) SEM image (5000×) of a germinating conidium of *F. graminearum* after incubation for 30 min with 2.5 μM wheat histones (H1−H4).

Nearly 100% loss of viability was observed for both conidial types at 2.5 μM after 20 min of exposure to the histones. The rapid histone action suggests pore formation in the conidial or hyphal outer wall.

Although plant histones are highly conserved, the literature shows that there are sufficient structural differences among them to suggest that antimicrobial activity is not uniform between plants.[11] The antimicrobial properties of the respective plant histones may depend upon recognition of the pathogen(s), against which it must defend itself. For example, *Fusarium* species are serious pathogens of wheat but not cotton. In contrast, *A. flavus* is a problem in stressed or damaged cottonseed, whereas *Fusarium* is not known to infect the seed.[28] This may be the reason that our studies with wheat histones showed activity against *Fusarium* spp. but not *Aspergillus* spp. However, few studies have shown that histones are involved directly in plant defense.[29] It is possible that histone H2B may be involved in the regulation of the defense responses against *Verticillium dahliae* toxins,[29] while in *Arabidopsis*, histone acetylation and deacetylation may play a key role in the regulation of plant responses, such as the production of jasmonic acid and ethylene signaling to pathogens.[30]

Nevertheless, *in vitro*, wheat histones H1−H4 show significant viability reduction for both the non-germinated and germinating conidia of several *Fusarium* species, especially *F. graminearum*, a wheat pathogen. The production of these wheat proteins, which have no role in wheat-related allergies, outside of the cell may enhance host plant resistance to this fungus. It also may be possible to employ these histones as antifungal agents under certain conditions.

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