

Confined Enzymatic Activity of *S. scitamineum* and *U. esculenta* at the Smut Gall during Infection

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Abstract

We aimed to evaluate the enzymatic abilities of *U. esculenta* and *S. scitamineum* to degrade cellulose, pectin and lignin and explain the possible confinement of the smut - fungi in the smut-gall. *S. scitamineum* and *U. esculenta* were isolated from the smut gall of *S. spontaneum* and *Z. latifolia* plants, respectively. The teliospores were inoculated into a culture flask containing various concentrations (3%-10%) of pure cellulose powder to check the degradation ability of the fungi. The fungi were sub-cultured onto modified Czapek Dox Agar (CDA) supplemented with 0.3% carboxy methyl cellulose (CMC) followed by Gram's Iodine test to determine the relative cellulolytic activity index (RCAI). Extracellular proteins were harvested and profiled by SDS-PAGE and subsequently zymography was carried out with regular 0.1% substrate infiltration to determine cellulolytic, pectinolytic and laccase activities. The fungi were capable of producing cellulase and pectinase but lacked the laccase enzyme required to degrade the lignin, perhaps as an essential evolutionary requirement for the completion of their life cycle in the host. Importantly, the lack of laccase activity by *U. esculenta* and *S. scitamineum* could partially explain why the fungal colonization remains localized in the topmost part of *S. spontaneum* and *Z. latifolia*.

Keywords: Smut; Enzyme; SDS-PAGE; Zymogram; PCW

Introduction

Cellulose, hemicellulose, pectin, and lignin are the major components of plant cell wall [1]. The organization and interactions of cell wall components are still not known with certainty and there is still considerable debate about how cell wall organization is modified to allow cells to expand and grow particularly in the presence of biotrophic fungal endophytes. Cellulase, pectinase and ligninase are produced extracellularly by most microbes and play a significant role in degrading various polysaccharides to produce glucose for their growth and survival [2,3].

It is a well-known fact that the living organisms try to adapt themselves with the environment by adopting different strategies to the prevailing conditions. Based on field observations and data generated in the Indo-Burma biodiversity hotspot on *S. scitamineum* and *U. esculenta* [4], it is thought that smut fungi often undergo unique adaptation in their biota, where they evolved to become associated with various plants of the family Poaceae.

Zizania latifolia is a wild rice which does not give out rice grains due to the presence of a fungal endophyte *U. esculenta* [4-6] restricted to few selected habitats of South East Asia [7]. Strangely, occurrence of *Z. latifolia* in India is found to confine only in the swampy habitats and wetlands of Imphal valley, Manipur [8]. Similarly, *S. scitamineum* is another smut fungus that harbors inside *Saccharum spontaneum* and is also found in the Indo-Burma biodiversity hotspot of India [8]. The prevalence of *S. scitamineum* in the field results in the swelling of the

upper parts of *S. spontaneum* in the form of smut. The infection of both *U. esculenta* and *S. scitamineum* interferes with inflorescence and seed production by their respective host [4,5].

The fungi *S. scitamineum* and *U. esculenta* often form localized infection in *S. spontaneum* and *Z. latifolia* during biotrophic interactions that develops into smut-gall. Advanced microscopic studies revealed that these fungi do not degrade the sclerenchymatous cells found in the vascular bundles. It is not clear whether these fungi lacks cellulolytic, pectinolytic and laccase activities to invade their host systemically and thus confine only to the smut-gall portion of the infected plant. In this present study, we determined the cellulolytic/pectinolytic/laccase activities of *S. scitamineum* and *U. esculenta* and correlate the differences in their enzymatic potential with evolutionary divergence.

Materials and Methods

Microorganism

The fungi *S. scitamineum* (Genbank accession gi|744827319|gb|KP068082.1) and *U. esculenta* (Genbank accession gi|643431282|gb|KJ627848.1) were isolated from the smut gall of *S. spontaneum* and *Z. latifolia*, respectively and were used in this study.

Fluorescence microscopy

The distribution and the area colonized by fungus inside the plant after the formation of smut gall were studied by fluorescence microscopy. Tissue sections of thickness 10-250 µm from smut-gall

were generated by using Leica CM-3050S cryostat microtome. The sections containing *U. esculenta* and the interaction zones were stained with 100% v/v calcofluor-white stain (CWS; Sigma[®], Saint Louis, USA) that binds to the cellulose materials and was performed according to the manufacturer's instructions. All the observations were carried out by fluorescent Olympus BX61 microscope fitted with fluorescent filters, coupled with image pro plus 5.1.

Screening for cellulase activity

The fungus was grown on modified Czapek Dox Agar (CDA) medium consisting of the following (*gL-l*): NaNO₃, 2.0; K₂HPO₄, 1.0; NaCl, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O 0.001; supplemented with 0.3% carboxy methyl cellulose (CMC). After 2 days of incubation at 20°C and 30°C, the culture plates were flooded with Gram's Iodine for 2-3 min. Positive activity was determined based on the relative cellulolytic activity index (RCAI).

Cellulose degradation

Teliospores of *S. scitamineum* and *U. esculenta* were inoculated into a culture flask containing various concentrations (3%-10%) of the pure cellulose powder along with control flask with no inoculation. Fluorescent microscopic images of the Cellulose powder (Sigma[®], Saint Louis, USA) with different inoculated strains were stained with CWS after 21 days of stationary incubation at 30°C.

Protein quantification

The protein content was estimated using the dye-binding method [9]. 15 µl protein sample was mixed with 500 µl of Bradford reagent (five diluted) (Bio-rad[®] Laboratories, Hercules, CA, USA) and allowed for 15 min. The absorbance was taken spectrophotometrically at 595 nm. Bovine serum albumin (BSA) dissolved in ReadyPrep rehydration buffer was used for establishing the standard curve.

Profiling extracellular proteins

Extracellular proteins from culture supernatant precipitated using (NH₄)₂SO₄ was used to study the protein profile. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 5/12% and 5/14% gels.

To determine the protein bands with cellulolytic, pectinolytic and laccase activity, regular SDS-PAGE was performed with 0.1% substrate infiltration but avoiding boiling and β-mercaptoethanol to the sample buffer. After the run, gels were incubated in 1% Triton-X-100 for 30 min to remove SDS (repeated twice). The gel was then washed extensively in distilled water to remove the excess Triton-X-100 and then again incubated for 1 h at 50°C in 50 mM sodium citrate buffer at the pH optimum of each enzyme [10].

Phylogenetic analysis

Phylogenetic analysis was performed based on rDNA sequences for *U. esculenta* and *S. scitamineum*. Sequence alignment was performed using Muscle program. Best substitution model parameters were determined based on Akaike information criterion, corrected (AICc) and Bayesian information criterion (BIC). The evolutionary history was inferred using the maximum likelihood (ML) method and rooted as previously described [11] and all the analysis were performed in MEGA 6 software [12]. The strength of the internal branches of the ML tree was statistically tested by performing 1000 bootstrap replications.

Results and Discussion

This study shows the ability of two smut fungi to produce cellulase, pectinase and the evidence explaining the need of various enzyme secretions in varying amounts depending upon different circumstances a fungus encounters during its growth *in vivo* and *in vitro*. It was noted that *S. scitamineum* (herein referred as gi|744827319) and *U. esculenta* (herein referred as gi|643431282) isolated from the smut gall of *S. spontaneum* and *Z. latifolia*, respectively (Figures 1 and 2), produced white mats on PDA plates after 14 days of growth (Figure 3).

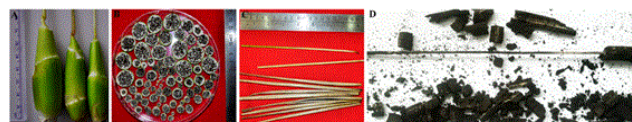


Figure 1: Morphological features of the smut galls on *Zizania latifolia* and *Saccharum spontaneum*. A-B Morphology of the *Z. latifolia* smut gall after *U. esculenta* infection and its horizontal cross sections. C-D Morphology of the *S. spontaneum* smut gall after *S. scitamineum* infection and the longitudinal inner view of smut, Bar=0.5 cm.

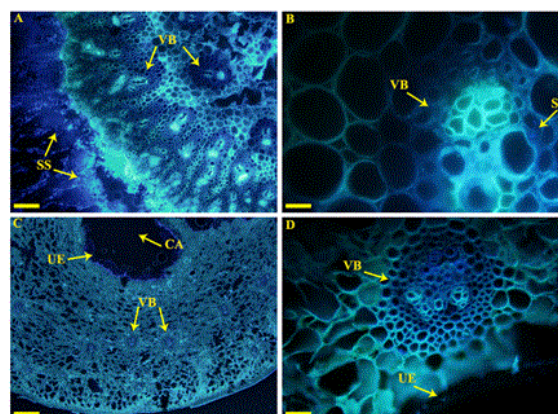


Figure 2: Fluorescent microscopic images of the cross sections, showing the vascular bundle of the infected *S. spontaneum* stalk with *S. scitamineum* and that of *Z. latifolia* with *U. esculenta* stained with CWS. A-B, horizontal cross sections showing the intact vascular bundle (VB) and the fungal material of *S. scitamineum* (SS) mainly teliospores and sporidia and hyphae on the peripheral epidermal and inner cortical cells (CC), scale bar A=200 µm and B=50 µm. C-D, Horizontal cross sections showing the intact vascular bundle (VB), and the fungal material of *U. esculenta* (UE) mainly teliospores, with the formation of cavity (CA) scale bar A=500 µm and B=25 µm.

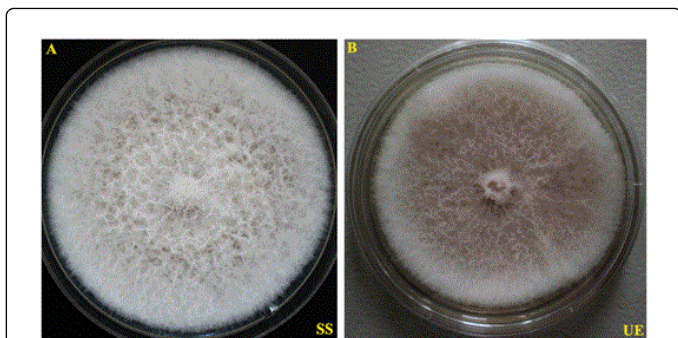


Figure 3: Colony feature of *Sporisorium scitamineum* (SS) and *Ustilago esculenta* (UE) fungal isolate from the smut gall of *S. spontaneum* and *Zizania latifolia* respectively grown in PDA plate for 14 days containing chloramphenicol at the concentration of 25 mg L⁻¹.

These cultures were subsequently subcultured and used for studying their cellulolytic, pectinolytic and ligninase activity. Fluorescent microscopic images of the cellulose powder (Sigma, Saint Louis, USA) were stained with CWS after 14 days of inoculation with different fungi. It was found that the smut fungi converted cellulose powder into small subunits compared to uninoculated control (Supplementary Figure S1). The *S. scitamineum* was able to carry out more breakdown of the cellulose compared to *U. esculenta* from the microscopic images obtained (Supplementary Figure S1). In this study, we found that the cellulose breakdown capabilities of the two smut fungi and zymogram assays further confirmed that *S. scitamineum* had better abilities compared to *U. esculenta* as evident from the relative clearance zone formed (Supplementary Figure S2). The extracellular protein profiles of *S. scitamineum* and *U. esculenta* obtained on 12% gel showed distinct bands of cellulase and pectinase. It was found that *S. scitamineum* and *U. esculenta* differentially secreted extracellular proteins (Supplementary Figures S3-S6 and Figure 4).

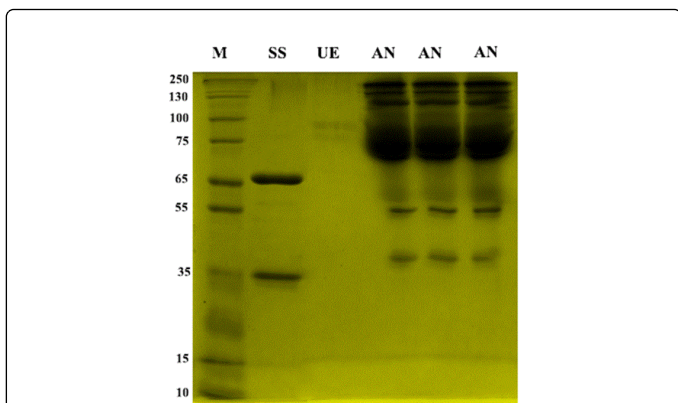


Figure 4: SDS-PAGE Gel of 12% showing the extracellular protein profile of the fungus *Sporisorium scitamineum* (SS) and *Ustilago esculenta* (UE) along with the positive control *Aspergillus niger* (AN). Note; M is the molecular weight marker in kDa.

The smut fungi extracellular proteins did not show any activity on the zymogram when lignin was used as a supplement in the gel.

On the basis of the divergent results observed on the enzymatic activity, we generated the phylogenetic history for the two species (Figure 5). The best substitution method was K2+I [13] based on BIC=2560.54 and AICc=2178.73. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 76.6839% sites). The transversion/transition rates were [C↔G]=0.06, [A↔G]=0.14, [T→G]=0.06, [T→C]=0.14, [C→A]=0.06 and the estimated transition/transversion bias was 1.27. Within the dataset, 325 patterns were found. Out of a total of 1450 sites, 1242 sites were without polymorphism (85.66%). The tree was generated at a highest log likelihood of -1032.53 (Figure 5). Two distinct clades (I and II) were formed and the overall distance (d) among taxa was 0.057. The studied species were distant from each other, reaffirming the disparity in their enzymatic profile although both form smut-gall in the same ecosystem. Thus, indicating that, they must have evolved differently yet conserve the traits to cause localized infection in their host plants.

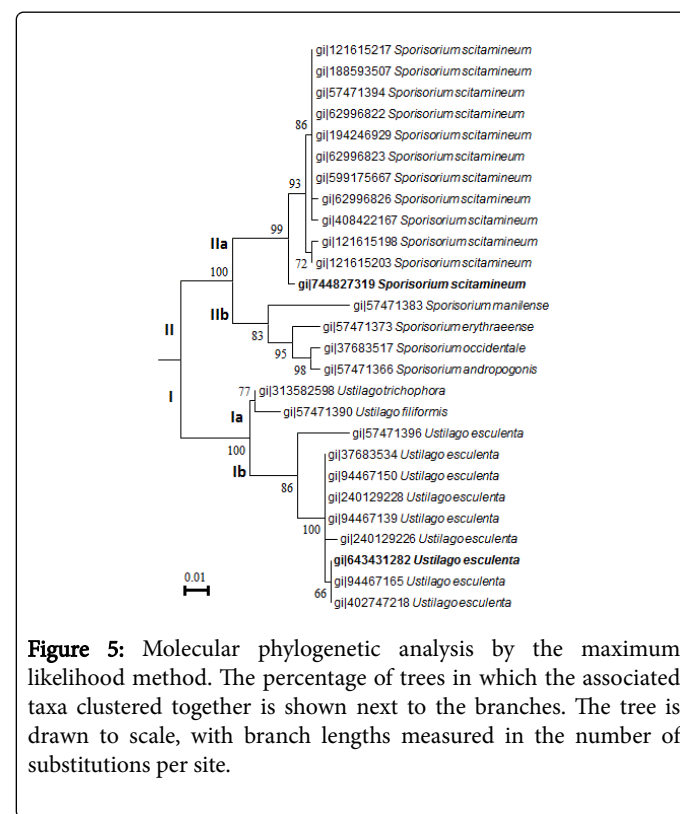


Figure 5: Molecular phylogenetic analysis by the maximum likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Previous study showed that fungi flagellum was lost early during evolution; thus, the majority of fungi species lacks flagellum [14]. By default, absence of flagellum could have prompted some fungi to depend on their enzymatic potential to explore their immediate niche or associate with non-selective hosts to derive their food, protection and other conditions to complete their life cycle. This might have encouraged certain fungi to modulate its enzyme secretions to find a strategic host without harming it drastically. A fungus generally secretes less amount of enzyme while establishing a biotrophic relationship with the plant as found in *U. maydis* - *Z. mays* interaction [15].

Because of the absence of fungal material inside the vascular bundles of the smut galls and the presence of hyphae in the intercellular spaces of nearby ground tissue in *Z. latifolia* and *S. spontaneum* [8], two hypotheses could be proposed. Firstly, the

observation could be due to the fact that smut fungi are naturally incapable of producing the laccase enzyme required to degrade the sclerenchymatous cells of the vascular bundle containing significant amount of lignin. Secondly, the plant could mount counter defence mechanism aimed at suppressing the fungal activity. We observed that when the fungi are taken out of the host and deprived of their natural source of food, triggers in them the trait to express various enzymes. Hence, the smut fungi when grown on media devoid of any plant material showed more cellulase and pectinase enzymes which we believe were suppressed previously by the plant's defense system and/or due to easy availability of nutrition.

After 2 days of incubation at 20°C and 30°C, the culture plates of *U. esculenta* and *S. scitamineum* flooded with Gram's Iodine for 2-3 min showed positive cellulolytic activity in the form of clear zones. The cellulolytic and pectinolytic activity of *S. scitamineum* and *U. esculenta* were further confirmed with the bands on SDS-PAGE along with a positive control of extracellular protein of *Aspergillus niger* and further by a zymogram assay. This study reveals that smut fungi generally does not produce cellulase and pectinase and other PCW (Plant Cell Wall) degrading enzymes when inside or on the plant at significant levels so as to evade plants counter defense system.

Our findings confirm that smut fungi are naturally incapable of degrading the lignin material, while it has the ability to degrade the cellulose and pectin material of the plant and complies with microscopic and zymographic results (Supplementary Figures S5 and S6). The fungi *U. esculenta* and *S. scitamineum* failed to produce any clear band in the zymogram when lignin was supplemented to the gel and validated the inference derived from the *in vivo* microscopic observations. It was observed that the lipid content was higher in *U. esculenta* than the *S. scitamineum*. This could be one of the reasons which complemented the energy requirements of *U. esculenta* whereas *S. scitamineum* had to depend more on its enzymatic activity for the energy (Supplementary Figure S7).

The cellulose and hemicellulose components of the plant cell walls are intimately associated with the lignin moiety in most cases and consequently may present a barrier to the hydrolytic enzymes catalyzing the degradation of the polysaccharide. Although the cellulolytic and pectinolytic capacity of smut fungi is clearly evident from this study the fungi aversion for lignified substrate unquestionably revealed their apparent inability to synthesize any of the recognized lignin-transforming enzymes in the plant as well as in the media.

Lignin has always been associated with reduced digestibility of the overall plant biomass, which helps defend against pathogens and pests [16]. Since smut fungi appear to lack a lignin lytic or lignin transforming system, we suggest they could have been evolved for the proper incubation time required for the fungus on the plant to complete their life cycle without killing the plants prematurely prior to inflorescence. Thereby accumulating sufficient nutrition for the next generation and ensuring progeny existence and spread of smut fungi.

Phenotypically, the studied fungi form smut-gall but genetically placed in different clades and sub clades (Figure 5). Although they have evolved differently based on rDNA analysis, they have conserved similar enzymatic potentials to cause localized infection that form smut-gall. Interestingly, neither *S. scitamineum* nor *U. esculenta* clustered with other taxa in subclade IIa and IIb, respectively, suggesting that the smut fungi have evolved differently. It is now accepted that fungi evolution has been successful since they diverged

from other life around 1,500 million years ago [17], with the glomaleans branching from the "higher fungi" at ~570 million years ago [18], providing clues that the process is dynamic. It could be interesting to mine the signalling molecules exchanged between the fungi and the host in order to affirm whether this rDNA divergence is maintained at the chemical level [19].

Conclusion

This study reveals the cellulase and pectinase activity of the smut fungi *U. esculenta* and *S. scitamineum* and stressed on the lack of ligninase activity and the possible evolutionary background to this phenomenon. It was found that the two smut fungi produce more cellulase at 30°C than at 20°C. The various smut fungi found on various hosts can be assumed to be a potential candidates for the cellulase and pectinase enzymes. It is proposed that, confine development of smut-gall at the topmost part of *S. spontaneum* and *Z. latifolia* could be due to lack of ligninase activities by *S. scitamineum* and *U. esculenta* preventing systemic colonization of their host.

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Conflict of Interest

The authors declare no conflict of interest.

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