Micron 81 (2016) 8–15

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Biotrophic interaction of *Sporisorium scitamineum* on a new host—*Saccharum spontaneum*

Robinson C. Jose[a,b,c,*], Bengyella Louis[a,d], Sailendra Goyari[a], Sayanika D. Waikhom[a,d], Pratap J. Handique[b], Narayan C. Talukdar[a,b,c,*]

[a] *Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal 795001, Manipur, India*
[b] *Department of Biotechnology, Guwahati University, Guwahati 781014, Assam, India*
[c] *Division of Life sciences, Institute of Advanced Study in Science and Technology, Guwahati 781035, Assam, India*
[d] *The University of Health and Allied Sciences, School of Basic and Biomedical Science, Department of Biomedical Sciences, PMB 31Ho Volta Region, Ghana*

ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 5 November 2015
Accepted 10 November 2015
Available online 17 November 2015

Keywords:
Biotrophism
Smut-gall
Teliospores
Electron microscopy
rDNA
Phylogeny

ABSTRACT

Sporisorium scitamineum is a biotrophic smut fungus harbored inside the smut gall on the top internodal region of *Saccharum spontaneum*, a wild relative of sugarcane (*Saccharum officinarum*). The interactions of spined conidia of *S. scitamineum* with *S. spontaneum* were examined during the different stages of plant growth starting from the bud stage to the decaying stage. The spores in the soil from the polyetic inocula grew into confined epidermal cells of the buds and finally sporulated in the topmost internodal region. Hyphae invasion of the plant tissues were restricted to the point of infection. Culms of infected plants in late October sporulated, notably; hyphal sporulation produced shorter hyphal stolons. Remarkably, the nodal regions of infected plants had no spores and fragmented hyphae. On the basis of microscopic analyses, hyphae and spores were absent in all internodes above the ground till the topmost smut gall region. This result indicated that, *S. scitamineum* undergoes tissue-confined invasion of *S. spontaneum*. By associating culture medium method with polymerase chain reaction (PCR) on plant portions void of smut gall, *S. scitamineum* was not detected, indicating that colonization was not systemic. It was observed that the biotrophic interaction resulted in structural reorganization in the restricted region of infection forming erect cylindrical structure, in which the fungus was sandwiched between the central stalk and sheath, and possibly played a key role in preventing inflorescence. Comparatively, a significant difference in the rate of teliospores germination between reference *Ustilago esculenta* (26.6%, $P<0.05$) and *S. scitamineum* (62.9%, $P<0.05$) at 20° C was observed. This study also provides insights on the effect of different temperature regimes on the germination of *S. scitamineum* teliospores *in vitro*.

1. Introduction

The first report of smut incidence caused by *Sporisorium scitamineum* on sugarcane (*Saccharum officinarum*), was observed in Natal, South Africa in 1877 (Luthra et al., 1940). Infected stems in sugarcane often develop a large whip-shaped apical section replacing the inflorescence. The most recognizable diagnostic feature of smut infected sugarcane is the emergence of a "smut-whip" (Comstock, 2000). A smut-whip is a curved, pencil-thick growth, gray-to-black in color that emerges from the top of the infected sugarcane plant. The smut-whip arises from the terminal bud or lateral shoots on infected stalks. They can vary in length from a few inches to several feet long and are mainly composed of host and fungal tissues (Rott and Comstock, 2015). Sugarcane smut produces microscopic spores that are adapted to aerial dispersal by wind current (Ferreira and Comstock, 1989), allowing standing sugarcane plants to become infected in the buds (Comstock, 2015).

Recently, it was found that *S. scitamineum* is also a biotrophic fungus of *S. spontaneum* and ecologically endemic to the Indo-Burma biodiversity hotspot of North Eastern regions of India. Under field conditions, the interaction of *S. scitamineum–Saccharum spontaneum* results in swelling of the topmost internodal region of *S. spontaneum* to form smut gall, which are locally called "Singmut–kambong", and commercialized as edible vegetables. Comparatively, smut formed from *S. scitamineum–S. officinarum* interaction results in thinner unhealthy stems associated with high yield loses (Piepenbring et al., 2002). Nonetheless, it is still unclear

* Corresponding authors at: Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal 795001, Manipur, India.
E-mail addresses: robinsoncjose@yahoo.co.in (R.C. Jose), nctalukdar@yahoo.com (N.C. Talukdar).

http://dx.doi.org/10.1016/j.micron.2015.11.002
0968-4328/

how *S. scitamineum* colonizes a new host such as *S. spontaneum.* The aim of this study was to provide new understanding on the colonization mechanism of *S. scitamineum* on a new host—*S. spontaneum.*

2. Material and methods

2.1. Survey and Collection

The smut gall bearing *S. spontaneum* plants were collected from the wetlands of Imphal valley adjoining the Loktak Lake in Manipur State, India during January, 2013 through December, 2014. Twenty plants with infected slender galls and 20 non-infected plants were randomly collected from 5 different sites. Experimental plants domesticated in water tank of the Institute of Bioresources and Sustainable Development (IBSD) were also collected. The smut galls were removed from the infected plants and the length and weight were recorded. The smut gall with the teliospores was stored in −80 °C for further studies.

2.2. Total nucleic acid extraction and sequence analysis

Total nucleic acid (TNA) was extracted from plant sample and pure isolate of *S. scitamineum* using the CTAB extraction method (Doyle and Doyle, 1987). The TNA was treated with 1 μL of RNAse A (10 mg/mL) and 1 μL proteinase K (1 mg/mL) at room temperature for 4 h. The quantity and quality of DNA was checked on nanodrop spectrophotometer (Thermo Scientific®, USA) and on 0.8% w/v agarose gel electrophoresis, respectively. The trnL-F intergenic spacer of chloroplast genome was amplified using the primer set (forward: 5′-ggttcaagtccctctatccc-3′; reverse: 5′-atttgaactggtgacacgag-3′) as earlier described (Taberlet et al., 2007). The ITS1-5.8S-ITS2 region of the rDNA was amplified using the primer set (forward: 5′-tccgtaggtgaacctgcgg-3′; reverse: 5′-tcctccgcttattgatatgc-3′) with PCR conditions as earlier described (Korabecna et al., 2003).

We used PCR method with the TNA from portions of the stem (first and second aboveground internodes, internode below smut and the smut region), young buds and roots to check for fungal presence so as to determine whether colonization was systemic or not. The PCR products were purified and sequenced in ABI370X1 Cycler Sequencer (ABI, USA) using the same set of primers. Sequences were automatically trimmed and assembled in DNAbaser 3.5.3 software. Following BLAST homology search in DNA public repository and annotation, the sequences were submitted in NCBI nucleotide database and assigned to molecular species at 99–100% similarity. The phylogenetic placement for *S. scitamineum* type isolate causing smut in *S. spontaneum* was determined by the maximum likelihood (ML). The best substitution model was determined based on Akaike information criterion, corrected (AICc) and Bayesian information criterion (BIC). The analysis and tree editing was performed in MEGA6v.1 (Tamura et al., 2013).

2.3. Germination rate and pattern of teliospores in vitro

To determine whether the germination rate and pattern of teliospores from *S. scitamineum* had any similarity with teliospores of *Ustilago esculenta* which cause smut gall disease in *Zizania latifolia*, we performed *in vitro* temperature-dependent germination experiments. The teliospores were grown in 50 mL potato dextrose broth (PDB) in Erlenmeyer flask at 20 °C, 25 °C and 30 °C at 100 rpm and stationary conditions with three replicates for each condition. Temperatures >30 °C and <20 °C was avoided due to insignificant growth. The teliospores were observed at time intervals of 2 h till the emergence of hyphae. Post germination changes and germination percentage of teliospores were recorded.

2.4. Culture approach to confirm the distribution of S. scitamineum in infected plants

From a naturally infected plant, 2–3 mm diameter sections were made from leaves (first to third leaf), stems (first and second aboveground internodes, internode below smut and the smut region), young buds and roots. The sectioned tissues were used throughout the experiment otherwise mentioned. All sectioning was performed with a Leica CM-3050S cryostat microtome. The dissected tissues were surface sterilized for 45 s in 10% sodium hypochlorite, rinsed in sterile water with three changes and plated on PDA containing chloramphenicol 25 mg/L. The samples were incubated at 20 °C in the dark for 10 days and examined daily by bright field microscopy. This experiment was replicated three times, with each replicate encompassing section of 5 different plant portions. The different plant portions were considered infected when *S. scitamineum* grew from the sections on PDA.

2.5. Microscopy

1.5.1. Light and fluorescent microscopy

An attempt was made to study the movement of *S. scitamineum* in the plant at different stages of growth by phase contrast and fluorescent microscope. Tissue sections of thickness 10–200 μm from leaf, stem and root of plants showing smut-gall and no smut gall were generated. The sections showing characteristics *S. scitamineum* and the interaction zone were stained with 100% v/v calcofluor-white stain (CWS; Sigma®, Saint Louis, USA) following the manufacturer instructions. Wheat germ agglutinin-Alexa fluor-488 (WGA, Life Technologies®, USA) and Propidium iodide (PI, Life Technologies®, USA) staining was performed according the manufacturer protocol with slight changes. Tissues were stained with 1 mg/mL WGA at 37 °C for 10 min, followed by washing with sterile water with three changes. The stained WGA-tissues were further stained with 100% v/v PI for 10 min followed by washing with sterile water with three changes. All samples were examined using a fluorescent microscope (Olympus BX61, Japan) coupled with image lab 5.1 software.

1.5.2. Scanning electron microscope

Samples for scanning electron microscopy (SEM) were prepared as previously described (Louis et al., 2015; Waikhom et al., 2013). Teliospores were placed on double-sided adhesive tape, mounted on a specimen stub, sputter-coated with gold–palladium, *ca* 20 nm and examined in a SEM (JSM-6360, JEOL) at 20 kv.

1.5.3. Transmission electron microscopy

Samples for transmission electron microscopy were prepared as previously described (Williams and Carter, 1996). Infection sites at precise stages of development were identified by light microscopy and ultrathin sections (approximately 50 nm in thickness) were generated with an ultra-microtome (MT-X; RMC Inc., Tucson, USA). The ultrathin sections were mounted on copper grids, and stained with 2% uranyl acetate and Reynolds lead citrate for 7 min (Reynolds, 1963). Stained samples were examined with a transmission electron microscope (JEM-2100, USA) operated with a Gatan 832CCD camera.