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Investigation on the biotrophic interaction of *Ustilago esculenta* on *Zizania latifolia* found in the Indo-Burma biodiversity hotspot

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ABSTRACT

Ustilago esculenta is a uniquely flavored biotrophic smut fungus that forms a smut gall on the top internodal region of *Zizania latifolia*, a perennial wild rice found in the Indo-Burma biodiversity hotspot. The smut gall is an edible vegetable locally called "kambong" in Manipur, India. The life cycle of the fungus was studied *in vitro* and its biotrophism was observed during different stages of the plant growth starting from the bud stage to decaying stage using light, fluorescent and electron microscopy. The size of the smut gall and the number of internodes below the apical smut gall varied significantly ($P < 0.05$). Examination of various parts of infected plants using culture methods, microscopy and polymerase chain reaction revealed that *Ustilago esculenta* colonized *Zizania latifolia* in a non-systemic manner. Spores and fragmented hyphae of *U. esculenta* were present in the rhizome of infected plant throughout the year, but shoot interiors were without any fungal structures from April until September. The smut region of infected plants in early September to December were heavily sporulated with fragmented hyphae, while the nodal regions of infected plants had no spores and fragmented hyphae. Hyphae and spores were also absent in the internodes and membranes aboveground up to smut region of infected plants but were present in the old rhizomes.

1. Introduction

Zizania latifolia is a perennial wild rice whose distribution is now restricted to few habitats of South East Asia [23]. In India, this plant is prevalent in swampy habitats and wetlands of Imphal valley in Manipur state where it is called "kambong" or "ishing kambong" [1]. It is a tall, erect herb with a height of up to 2 m and possesses well-developed stolon and fibrous roots and in the past used to occupy large stretches in the wetlands of Imphal valley. Although not valued as a grain crop, its apical internode is a delicacy food item. The apical internode is swollen due to the occurrence of hyphae and teliospores of fungus *U. esculenta* [3,24]. The swollen part, also called smut gall due to the smut fungus in sporulated state, is eaten as a delicacy by native people and sold in local markets of Manipur, India. In few other South East Asian countries such as China, Japan, and Taiwan the smut gall of *Z. latifolia* has been used traditionally as an aquatic vegetable and is commonly called "jiaobai" in China or "gausun" in Taiwan [5,6,21], and "makomotake" in Japan [14]. In China and Japan, it is cultivated as a commercial food item due to its unique flavor and delicacy [8]. Interestingly, the smut gall consumed in China as a vegetable contains the fungus in the mycelial state and the dark sori of *U. esculenta* seen in a small number of "jiaobai" are discarded [11]. Contrary to China, the gall is always seen in sporulated state in India, and consumed after harvesting from the wild. Unfortunately, *Z. latifolia* bearing the smut gall that once used to be splendidly growing, predominantly at the fringes of Loktak Lake, is now on the verge of extinction. One of the main reasons driving extinction of *Z. latifolia* is attributed to the conversion of its natural habitats for

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aquaculture, paddy cultivation and also due to the lack of conservational efforts.

Although, some information on morphological, ultrastructural, biochemical and molecular aspects of *Z. latifolia* and *U. esculenta* are available from research carried out on this plant-fungus system prevalent in habitats of other Asian countries, there is very little information on "kambong" of Manipur. Even it has not been confirmed whether the interacting members found in Imphal valley are similar to *Z. latifolia* and *U. esculenta* reported from elsewhere. Furthermore, it is also not well understood as to how the fungus survives and moves through the plant during different stages of their growth and causes swelling only in its apical internode. It is also interesting to note that infection by *U. esculenta* interferes with inflorescence and seed production of the plant [3,24], but there is no available information on the molecular mechanisms involved in this process.

Previously it was reported that gall formation in *Z. latifolia* may be the result of variations in phytohormone content and resistance responses of the plant to the fungus during different developmental phases of the interaction [5,6]. In nature, *U. esculenta* is a member of smut fungi which are known as phytopathogens, and causes smut disease in a variety of plants. In diseased plants, it was observed that *U. esculenta* does not infect unswollen apex of the culm, roots and leaves [3,24]. The mode by which the teliospore originally invades the host is unknown and further, it is difficult to observe a complete life cycle of the fungus *in vitro* [3].

This study aims to generate comprehensive data on the morphological, ultrastructural and molecular basis of the interaction between *Z. latifolia* and *U. esculenta* present in the swamps and wetland areas. The specific objectives of this research were to (1) determine the morphological diversity of the plant and the swollen culm tip due to fungal infection and also identity of the fungus and plant, (2) decipher accurate stages in the life cycle of the fungus under *in vitro* conditions, and (3) describe the mode of survival, infection, and spread of the fungus in the plant by using advanced microscopic techniques.

2. Materials and methods

2.1. Survey and collection

The smut gall-bearing *Z. latifolia* plants were collected from the wetlands of Imphal valley in Manipur state during September through December 2013 and 2014. Plant materials with spindly galls were collected from 24 different sites distributed across four districts of Manipur adjoining Loktak Lake. Five plants from 1 m^2 area of natural habitat with intact smut gall were collected from each site. The total number of internodes between the base of the apical smut and the root were counted, and length of the first two internodes below the apical smut was measured. The smut galls were removed from the plant and they were weighed. The longitudinal and horizontal cross sections of the smut gall were examined under a stereo zoom microscope for the sporulation pattern, spore adherence and the cavities formed due to sporulation. Intact smut galls were stored in −80 °C until further use.

2.2. Total nucleic acid extraction and sequence analysis

Total nucleic acid (TNA) was extracted from the young leaf tissues of *Z. latifolia* and teliospores of *U. esculenta* using the CTAB extraction method [7]. The TNA was then 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 were monitored by BioSpec 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 [20]. The ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primer set (forward: 5'-tccgtaggtgaacctgcgg-3'; and reverse: 5'-tcctccgcttattgatatgc-3′) as earlier described [15].

Using 9 ng of TNA extracted from different portion of the plant such as stem (first and second above ground internodes, internode below smut and the smut region), young buds and roots, PCR was performed 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 deposited to GenBank and assigned to molecular species at 99–100% similarity.

2.3. Stages in the life cycle of the U. esculenta

Spores of *U. esculenta* collected from the infected *Z. latifolia* plants were used for the *in vitro* studies. These spores were grown in potato dextrose broth (PDB) in triplicates at three different temperatures of 20 °C, 25 °C and 30 °C in the stationary incubator and also in orbital shaker incubator at 25 rpm. The status of the spore germinations in broth were monitored at a time interval of 2 h. Post-germination changes were recorded at time intervals of 6 h for next 14 d. To study whether the fungal spores can infect the plant parts under *in vitro* condition, small pieces of surface sterilized plant parts from leaf, internode and rhizome were inoculated with the fungal teliospores in PDB and incubated in triplicates at 20 °C under shaking at 25 rpm and also in stationary conditions. They were then observed at 12 h interval for 14 d for any possible colonization.

2.4. Microbial culture analysis to confirm the movement of U. esculenta in infected plants

From a naturally infected plant, 2–3 mm diameter sections were made from leaves (the first to third leaf), stems (the first and second aboveground internodes, internode below smut and the smut region), young buds, rhizome and roots. 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 at the concentration 25 mg/L. The samples were incubated at 20 °C in the dark for 10 d 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 *U. esculenta* grew from the sections onto PDA.

2.5. Microscopy

2.5.1. Light and fluorescent microscopy

The movement of *Ustilago esculenta* to different parts of the plant at different stages of their growth was studied by phase contrast and fluorescent microscopes. Tissue samples from 100 plants, infected by mycelia of *U. esculenta* along with teliospore sori were sectioned with a Leica CM 3050S cryostat microtome to a thickness of (10–200 μm). Thin sections showing the mycelia and interaction zone with the plant, in smut region were stained with Calcofluor white stain (CWS) and some sections containing the structure of sori were mounted in lactophenol with aniline blue on a slide, covered with a cover glass, and examined. All the observations were carried out by fluorescent Olympus BX61 microscope fitted with fluorescent filters, coupled with image pro plus 5.1 as