Journal of Phytopathology and Pest Management 5(2): 108-128, 2018
pISSN:2356-8577 eISSN: 2356-6507
Journal homepage: http://ppmj.net/

Biology of rice kernel smut disease causal organism *Tilletia barclayana* and its molecular identification

Rabie A.S. Elshafey*

Rice Research and Training Center, Rice Research Department, Field Crops Research Institute, Agricultural Research Center, 33717 Sakha, Kafr El-Sheikh, Egypt

Abstract

In Egypt, Rice kernel smut disease is a new production biotic constrains, the kernel smut of rice is caused by *Tilletia barclayana*, which started in appearance in different rice growing governorates, especially in Belkas, Dakahlia governorate whereas the cultivation of cultivar Giza 178 was concentrated in this area. The study aimed to investigate the life cycle of the causal organism of rice kernel smut disease "*Tilletia barclayana*" and determine all growth stages of this fungus and its relationship with the host " *Oryzae sativa*, L", in addition, molecular identification of this fungus. The life cycle of this disease was investigated; teliospore was germinated as emerging germ-tube and bear a promycelium. This promycelium bear from 20-100 filliform basidiospores at the tip of the promycelium. The non-conjugated basidiospores were germinated and produced mycelium. Both filliform and allantoid sporidia (Crescent-shaped conidia) generally develop from that mycelium on short sterigmata. The allantoid sporidia discharged from strigmata, then was germinated and produced binucleate mycelium which able to infect the different floral tissues, ovary and produced smut balls of teliospores. Concerning the proper time for artificial inoculation, Inoculation at flowering stage induced the highest infection with all cultivars. Inoculation at milking stage recorded the second rank of infection with the cultivar Giza 171. The lowest infection was recorded from inoculation at seedling and maximum tillering. In addition, both the highest infection at flowering stage proofed that the infection was local not systemic. For the proper spore type to artificial inoculation, Allantoid spores induced the highest infection % because it germinate and directly gave the binucleate mycelium which able to invasive the ovary and all tissues of opening spikelets, followed by the filliform sporidia which recorded the second rank of infection %, while teliospores induced the lowest infection %. For the survival of teliospores, the teliospores can survive for more than 2 years and provided the initial inoculum source from season to season. For host resistance, there are a significant source of resistance in the breeding program such as Sakha 101, Giza 179, Sakha 105, Sakha 106, some GZ lines; GZ 10101, GZ10144, GZ 10154 and GZ10305. So, detection of new resistance resources allow to further progress in establishing a successful breeding program of kernel smut For molecular identification based on ITS region, the Egyptian isolates reflected high level of identity with kernel smut from USA, China and India ranged from 80-100%.

Keywords: rice, kernel smut disease, *Tilletia barclayana*, teliospore, resistance, varieties.

* **Corresponding author:** Rabie A.S. Elshafey,
E-mail: relshafey13@yahoo.com

Introduction

Rice kernel smut disease is fungal disease caused by *Neovossia horrida* (Takah.) Padwick & A. Khan (syn. *Tilletia barclayana* (Bref.) Sacc. & P. Syd.) Tilletiales (Vánky, 2001; Castlebury et al., 2005), which, like *T. indica*, causes a partial bunt that affects both yield and quality. Rice kernel smut disease is a new production constrains specially in some locations of Egypt such as Belkas, Dakahlia governorate that had a wide cultivated areas of Giza 178 rice cultivar (Elshafey, 2013). Kernel smut that causes a partial or complete replacement of rice grains contents of panicle with teliospores masses as a direct effect on grain yield and quality (Webster & Gunnell, 1992). Rice kernel smut, recognized as caryopsis smut, black smut (Biswas, 2003), was widespread and presents almost in all rice-grown ecosystems worldwide. It has found in upland or irrigated rice growing countries (Elshafey, 2013; Farr et al., 2005; Biswas, 2003; Chahal, 2001; Webster & Gunnell, 1992; CMI. 1991; Ou, 1985). It was considered as an endemic disease, extended to epidemic level, chronic problem, and persistent in southern USA rice production areas especially with parboiled rice. In addition, It consider as minor disease but can be changed to major in some rice production areas such as India and southern USA and recorded remarkable yield losses (Brooks et al., 2009; Carris et al., 2006; Chahal, 2001). Kernel smut considered as minor with sporadic nature but became important economical rice fungal diseases with severe quality and yield losses in Texas (Uppala et al., 2017). The direct grain yield losses can be reached to 15% in addition to a significant loss of rice grain quality (Gravois & Bernhardt, 2000; Sharma et al., 1999; Webster & Gunnell, 1992; Whitney 1992). Kernel smut was an air-borne whereas secondary sporidia, forcibly discharged into the air and infect floral tissues through open florets as a local infection. While, a soil-borne teliospores play an important role in infection process as primary inoculum (Whitney, 1992). *Tilletia barclayana* can be able to infect many grasses in addition to rice host, highly specific fungus on their hosts and organs and localized in its infection on florets and ovary (Singh & Pavgi, 1972; Whitney, 1992). Survival of pathogen play a crucial role in epidemiology and disease development, whereas, teliospores as a primary source of infection with thick-wall it provided the ability to survive at least wintering from season to another. Therefore, starting rice cultivation with healthy seeds is main point in management (Chahal, 2001). For resistance of kernel smut, many cultivars are resistant under natural field infection but few germplasm resistant using boot inoculation technique (Cartwright et al., 1996; Lee et al., 1991). Therefore, screening under natural infection with providing a source of inoculum could improve discovery of field resistant germplasm. Under the threat of climate change could be kernel smut shifted from minor to major disease and causes severe problems. Therefore, understanding biology of pathogen was the critical point in host-pathogen interaction and integration disease management. The main objectives of current study were: isolation, clarify the growth nature of this fungus, life cycle and host pathogen interaction relationship throughout varietal resistance. Molecular identification with ITS region and compare sequence analysis of some Egyptian isolates with worldwide through NCBI website.

Materials and methods

Isolation of the causal pathogen: Full matured rice grains that showed characteristic kernel smut symptoms were collected. The mass of mature teliospores of *T. barclayana* were surface-sterilized 0.5% NaClO (5 % vol/vol commercial bleach) for 2-3 minutes and rinsed with sterilized water several times. Then, teliospores were streaked on 2% water agar media to germinate. Individual teliospores was picked and transferred into an antibiotic-containing potato dextrose agar media and incubated in an incubator at 29° C. Two weeks later, teliospores were examined for full germination. Each of germinated single colony was transferred to Potato Sucrose Agar (PSA) medium again to maintain pure isolates of causal organism and cultured in incubator. For long preservation, 1 ml of allantoid sporidia suspension on potato sucrose broth of 7-days old culture covered with 1 ml sterilized glycerol 35% in cryptovail at -80˚c.

***Tilletia barclayana* life cycle and long-term storage:** The entire germination process of teliospores was illustrated in details. Teliospores were examined microscopically at ×40 magnification to determine time of germination. Teliospores were considered germinated once emerging of germ-tube and basidiospores had formed on promycelium. Each type of spores and all life cycle was distinguished and each stage illustrated. All special microscopic features and measurements were determined based on fifty randomly samples of fully matured spores. For impact of long-term storage, one hundred mature teliospores were examined in interval 6-month for each of three replicates throughout two years and half under room temperature during 2015-2017 seasons. In addition, based on samples of 100 teliospores, germination % was calculated during different storage periods to determine teliospores survival and overwinter.

Detection of resistance resources: During 2015 and 2016 seasons, field experiments were carried out in Rice Research and Training Center (RRTC) experimental farm, Sakha, Kafr EL-Sheikh to evaluate the varietal resistance of kernel smut disease. Twenty rice Egyptian varieties, with a wide variation in their duration period and types, were artificially inoculated under open field conditions and allocated in a randomized complete block design with four replicates. Twenty-five days old seedlings of each cultivars was transplanted in plots measured 2×2 m^2 at 20 × 20 cm. Urea (46 % N) was added in accordance to the recommendation package of each cultivar. All cultural practices were applied as recommended. During flowering stage with opening florets, all cultivars were inoculated at (10-1 pm) with allantoid sporidia suspension of 4×10^5 sporidia/ ml of the most aggressive isolate Eg 01. Gelatin 2.5 g/L was used to induce smut infection through increase adhesion of spores on florets (Elshafey et al., 2015). Samples were collected at the end of season and infection scored based on infected panicles and grains /m^2.

Effect of inoculation date and type of spores on disease infection: For preparation of spore suspension, five

colonies of 15-days growing culture of *Tilletia barclayana* fungus were inoculated into 150 mL potato sucrose broth medium in 250 ml Erlenmeyer flasks, The inoculated flasks were incubated at 28 c for 7 days in an orbital shaker (120 rpm) under continuous fluorescent light. Each type of spores was separated based on the age of culture and their pigment. The highly susceptible cultivar Giza 171 was transplanted in plots measured 2×2 m^2 at 20 × 20 cm in a randomized complete block design with four replicates. Rice plants were inoculated at different growth stages; seedling stage, maximum tillering, late booting, flowering and complete heading with allantoid spores at 4×10^5 sporidia/ ml from 14-days old culture of isolate Eg 01 to investigate the impact of inoculation date on severity of kernel smut. In additional trail, Japonica rice cultivar Giza 171 was inoculated with different types of spores; teliospores, filliform and allantoid sporidia after complete flowering at 12 pm. Plants were inoculated by spraying a suspension of secondary sporidia (10^5-10^6 / ml) of the smut fungus onto the opening florets during flowering stage. All infected panicles/m^2 were counted.

Disease parameters: The response of different rice varieties to kernel smut disease was evaluated according to disease parameters as follow with Slaton et al. (2004) and Elshafey (2013): Infection percentage as number of infected panicles /m^2. Infection severity as number of infected spikelets /m^2. Differentiation among various isolates of *Tilletia barclayana*: All isolates were grown on potato sucrose media (200g potato, 40g sucrose, 20g agar/ 1 distilled water) incubated in incubator at 29° C for two weeks. Then, fully growth cultures were categorized in different categories based on compare morphological traits specially secretion of specific pigment and nature of growth on the media.

Extraction of fungal DNA: Two isolates of Kernel smut fungus (Eg 01, Eg 02,) were selected based on morphological variation such as red and violet pigments and virulence. Mycelium of each isolate was scraping from the surface of a 10-day old culture. The mycelia (approx. 100 mg) were ground in liquid nitrogen. DNA was extracted from the powdered tissue using i-genomic plant DNA extraction Mini Kit (iNtRON Biotechnology, Inc, Cat. No.17371) according to manufacturer's instructions. The eluted DNA was stored at -20 ºC until use.

Amplification of ITS region: Amplification of internal transcribed spacer (ITS) region was conducted in an automated thermal cycler (C1000 TM Thermal Cycler, Bio-RAD) using the primer pair: ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) and ITS5 (5´ GGAAGTAAAAGTCGTAACAAGG 3´) primers (White et al., 1990). The following parameters were applied: 35 cycles of 94ºC for 30 s, 51ºC for 1 min, 72ºC for 1.5 min, and a final extension at 72ºC for 3 min. Each PCR mixture (25 µl) as follow, (1 µl) of 25 ng nucleic acid, 1 µl of each primer (10 pmol), (12.5 µl) of GoTag®Colorless Master Mix (Promega Corporation, USA) and 9.5 µl of Nuclease free water (Promega). Negative control contained

the same PCR reagents, but no DNA. 15 µl of all PCR products were analyzed by electrophoresis through a 1% agarose gel (Sigma), stained with ethidium bromide, and DNA bands were visualized using a UV trans-illuminator.

DNA sequencing and data analysis: The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, California) and both strands were sequenced with an ABI377 automated DNA sequencer (Applied Biosystems Inc., Foster City, California) in both directions with the same primer sets (ITS4- ITS5). Raw sequence chromatograms were assembled and edited using GAP4 (Bonfield et al., 1995) to correct ambiguous bases or remove low quality stretches from the termini of the sequences. Homologies to known sequences were detected using the BLASTN algorithms (Altschul et al., 1997) against the non-redundant GenBank database at http://www.ncbi.nlm.nih.gov/blast. Multiple alignments were performed using Cluster (Thompson et al., 1994) and the phylogenetic analyses were conducted with MEGA7 using the maximum parsimony method (Tamura et al., 2007). The ITS4/ITS5 sequences of different *Tilletia barclayana* species used for comparisons and were submitted to GenBank (www.ncbi.nlm.nih.gov). Sequence analysis was carried out in LGC group, Germany.

Statistical analysis: All data were subjected to analysis of variance according to Gomez and Gomez (1984). Treatments mean were compared by LSD 5%. All statistical analysis was performed using analysis of variance technique by means of Genstat 5 computer software package.

Results and Discussion

Characteristic symptoms of kernel smut under artificial inoculation: During full maturity, kernel smut characteristic symptoms appeared and become evident. Some individual panicle grains were partially smutted or completely transformed to sori of black powdery teliospores mass (Figure 1). Black kernel smut sours of the fungus could replace partial or entire grain starch content. Teliospores could be remain covered with grain glumes or discharged and scattered from rupturing sori and surface contaminated of grains and leaves. Therefore, it is easy to detect kernel smut disease visually in field. Severity and infection development depend on level of cultivars resistance. Kernel smut disease has no direct effect on vegetative growth of infected plants. All diagnostic symptoms are in agreement with Ou (1985), Whitney (1992) and Biswas (2003).

Figure 1: Kernel smut symptoms, left was severe smutted grains and right healthy ones of Giza 171 cultivar under artificial inoculation.

Morphological characteristics of *Tilletia barclayana*: Mature Teliospores are light to dark brown, globose, 18-32.9µm, whereas, sterile cells hyaline in color 18-31µm. Teliospores comprises three layers; the perisporium, episporium and endosporium. Teliospores have outer thick wall to maintain spore dormancy state. Promycelium is frequently non-septate, 34-523×6-7.3 µm with long branched ones. The primary sporidia or basidiospore are aseptate, filliform to needle in shape, 52-75 x 1.1-2 µm. A terminal whorl of primary sporidia bear 20 to 100 basidiospores (Table 1 and Figures 2-5). The number of basidiospores varied from isolate to another in accordance with Castalbury et al. (2005) and Carris, et al. (2006). *T. barclayana* recorded a significant variation in different isolates teliospores size and this trait could be used to identify specific specie, in agreement with Matsumoto et al. (1985). Filliform secondary sporidia are septate**,** 37.5-62.5 × 2.4-2.6 µm, while, Allantoid secondary sporidia are hyaline, septate and have crescent shape, 10-21.2 x 2.9-3.8 µm in diameter (Table 1 and Figures 2-5) (Carris et al., 2006).

Table 1: Morphological characteristics of *Tilletia barclayana*.

Type of spores	Measurements of diameter (µm)	Development period	Appearance
Tiliospore	18-32.9 µm	20-30 days from infection of open florets	Black
Sterile cell	18-31µm	20-30 days from infection of open florets	hyaline
Promycelium	34-523× 6-7.3 µm	Germ-tube of germinated teliospores	hyaline
Basidiospore	45-70 × 1.1-2 µm	1-2 days after of tiliospore germination	hyaline
Filliform sporidia	37.5-62.5 × 2.4-2.6 µm	3-4 days after of teliospores germination	Colony was cottony and white powdery
Allantoid sporidia	10-21.2 x 2.9-3.8 µm.	7 days after teliospores germination	Production of dark purple pigment

Figure 2: A, colony morphology of Eg 01 isolate with reddish pigment of tilletia. B, Eg 02 with violent pigment. C, no pigment. D, growth nature of kernel smut fungus colony, left creamy embedded in the media, right, white cottony and convex raised colony.

Based on morphological traits, growth nature of some isolates have convex raised cottony colony from media surface (Figure 2D right), while second type was leathery embedded colony in media (Figure 2D left). For pigment production, there are three categories of pigments, the first type of isolates produced reddish color (Figure 2A), and the second have violet (Figure 2B), and the last isolates have white colony and creamy color without any pigments (Figure 2C). Therefore, *T. barclayana* fungus reflected a wide variation in morphological traits and these results in agreement of Levy et al. (2001) and Elshafey (2013).

Life cycle: On PDA media, the teliospores were germinated, emerging short germ-tube and formed a non-septate promycelium (Figure 3A-B). On the tip of promycelium, almost 20-100 primary sporidia were developed in whorls. In the beginning, it folded and assembled together to form candle-flame shape (fig 3 E). Then with the progress of time, the attatched sporidia matured and become unfolded to form a brush shape such as an opening flower (Figure 3D-E). Also, primary sporidia could be formed on the tip of very elongated and branched promycelium (Figure 3F). The primary sporidia developed and differentiated to basidiospores with length 85-127 µm that discharged or still attached to promycelium (Figure 3H). the non-conjugated basidiospores that attached to promycelium germinated and produced mycelium which carried two types of secondary sporidia; filliform and allantoid sporidia with Crescent-shaped (Figure 4I-K). Both filliform and allantoid sporidia are arise from lateral short sterigmata called sporogenous cells on this dikaryon mycelium (Figure 4L-P) (Carris et al., 2006; Ingold, 1996). The filliform and allantoid sporidia was curved usually have 7-9 septa, while allantoid sporidia divided by 12-19 septa (Figure 4Q1). Each cell of filliform and allantoid sporidia could be germinated. These sporidia were discharged and germinated in turn formed binucleate mycelium (Figure 4Q). Binucleate mycelium that responsible to infection of florets tissues, ovary and produced smut sori of teliospores inside rice grains. Finally, teliospores can be produced on Potato Sucrose Agar (PSA) media after formation of filliform and allantoid sporidia to complete life cycle (Figure 4R, S). In Tilletia indica, sexual compatible pairs of basidiospores were coupled and conjugated through short conjugation-tube. The conjugated pairs of basidiospores were formed H-shape and still attached to the promycelium. After plasmogamy and karyogamy, basidiospores produced binucleate mycelium that carried both filliform and allantoid sporidia on short strigmata. Therefore, the strains of this fungus are heterothallic (Castlebury & Carris, 1999). In Tilletia barclayana, germination of teliospores lead to production of large numbers of non-conjugating primary basidiospores. Basidiospores without conjugation developed mycelium that bears both of filliform and allantoid sporidia. So, the strains of this fungus were homothallic. The obtained results in accordance with those of Goates (1988), Durán (1987), Vánky and Bauer (1992), Vánky and Bauer (1995), Castlebury and Carris (1999). *T. horrida* is homothallic based

on the presence of two nuclei in detached basidiospores and the lack of conjugation between basidiospores (Ou 1985; Singh & Pavgi, 1973). Moreover, inoculation with single and paired sporidia of *T. horrida* demonstrated the heterothallic nature of this pathogen (Whitney & Frederiksen, 1975). Allantoid sporidia could germinated and produce germ tubes directly (Figure 4Q1, Q2) (Ingold, 1997; Ingold, 1996). In culture, Allantoid and filiform sporidia passively dispersed, are formed from short, lateral sporogenous cells on the hyphae (Ingold, 1996). In conclusion Tilletia barclayana fungus produced 4 types of spores, Tilliospores, basidiospores, Filliform and allantoid sporidia, (Figure 5).

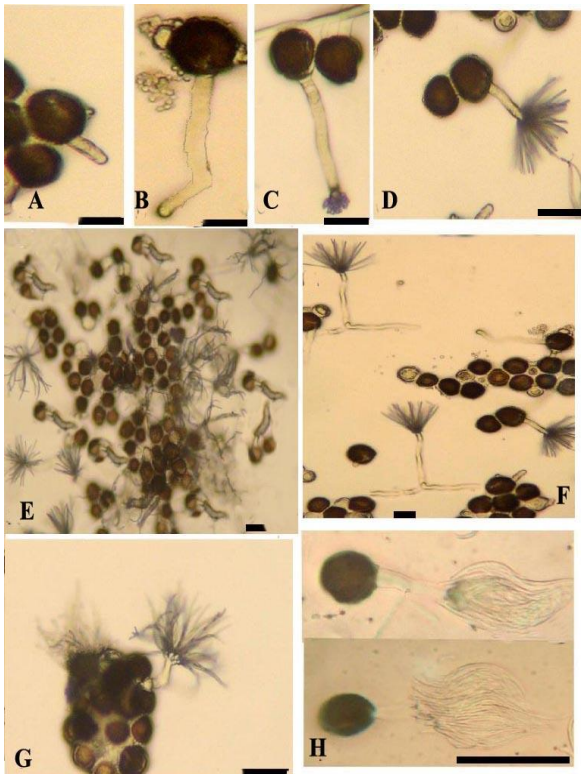

Figure 3: A, germinated tiliospore with short germ tube. B, promycelium of tiliospore. C, start of basidiospores production on the top of promycelium. D, brush shape of promycelium, E-G, different shapes of basidiospore production. H, non-conjugating basidiospores. Scale bars: a-d, G 20 µm, e, f 10 µm, H 50 µm.

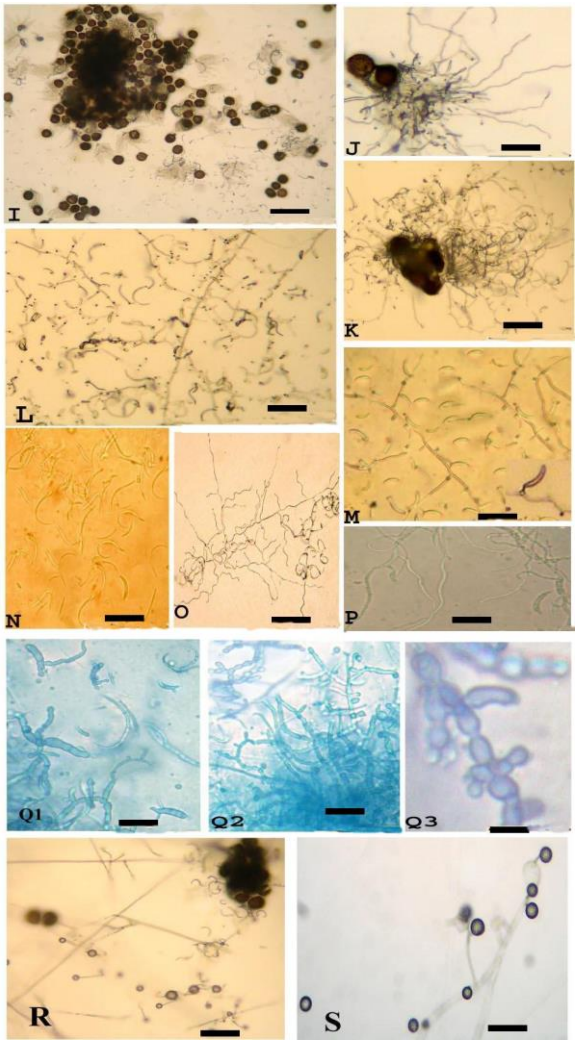

Figure 4: I-K, germination of basidiospores and production of filliform and allantoid spores. L, filliform spores on mycelium, M, allantoid spores on mycelium, N, mass of filliform spores, O, germination of individual filliform spore. P, germination of individual allantoid spore. Q1-Q3 different shapes of germinating filliform and allantoid spores. R, teliospores combined with allantoid spore. S, terminal teliospores produced on sporogenous mycelia on culture. Scale bars: a-s 20 µm.

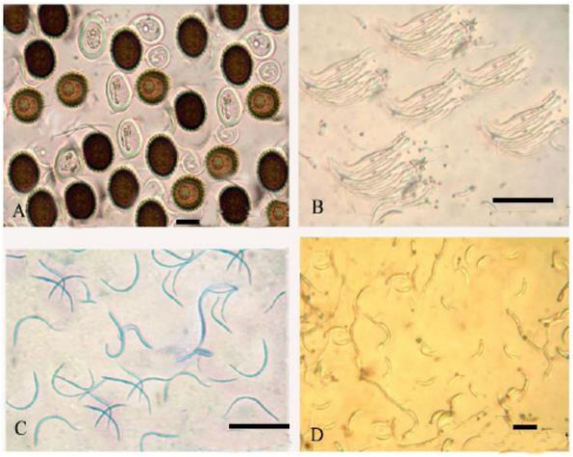

Figure 5: A, tiliospore of kernel smut at various stages of maturity; sterile body, immature and mature teliospores. B, basidiospore. C, Filliform sporidia. D, allantoid sporidia. Scale bars: a 20 µm, b, c 50 µm, d 20 µm.

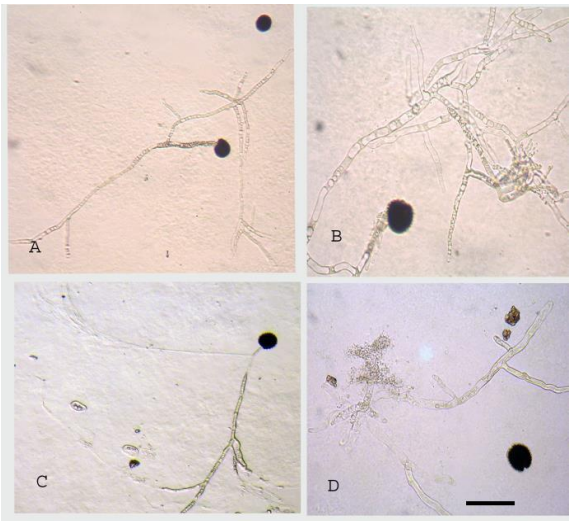

Figure 6: A, germination and promycelium formation. B, start of autolysis of mycelium. C, autolysis of some mycelium branches. D, full autolysis of promycelium of kernel smut fungus. Scale bars: a-d 20 µm.

Under specific conditions, some germinated spores of kernel smut produced promycelium that start to autolysis with time, until full lysis. On PDA media, the teliospores were germinated, emerging short germ-tube and formed promycelium (Figure 6A). Some fungal mycelium could be autolysis and enhanced by different factors and conditions and some electrolytes could be liberated on media. Therefore, the kernel smut mycelium could subject to autolysis due to lytic enzymes from any bacteria and other fungi or natural autolysis. The mycelium of kernel smut was severely affected during autolytic phase and totally lose their constitution (Figure 6D). The major cell wall constituents of mycelium could be hydrolyzed by degradation enzymes such as chitinase and glucanases which produced from lytic microorganisms or present naturally in mycelium itself (Lloyd & lockwood, 1966). Therefore, soil lytic microorganism could be shared in reduction of infection and inoculum capacity from soil for this fungus by induced autolysis of promycelium (Ko & lockwood, 1970).

Varietal resistance of kernel smut disease: Some local rice cultivars in addition to Egyptian hybrid rice (H1), beside GZ line were evaluated against kernel smut resistance. For kernel smut resistance, highly significant differences were found among rice varieties. Old japonica short grains varieties namely; Giza 171 was the most susceptible, Whereas, it recorded the highest infection percentage almost 18 and 20 as infected panicles/ m^2 followed by Giza 178 which exhibited (12 and 14 infected panicles/ m^2) with highly significant differences. Late exertion of some panicles after complete heading in Giza 178 and H1 from unproductive tillers, induced the severity of smut infection, it extended the period of opening florets to receiving smut spores compared with

some japonica tolerant varieties that their panicles have high uniformity level in exertion and maturation. Moreover, Giza 177 as an early maturing cultivar recorded high infection level (6.7 and 8.6 infected panicles/ m^2). Concerning the widely grown varieties, Sakha 101 appears to be more tolerant than others and exhibits some field resistance to kernel smut, which recorded 1.37 and 1.77 infected panicles/ m^2 compared with highly susceptible Giza 171. In addition, Sakha 104 as second extensively grown varieties exhibited low level of kernel smut infection, 2.33 and 2.57 infected panicles/ m^2. Indica rice varieties that distributed in constricted area such as, Giza 182 recorded lowest infection 2 and 3.7 infected panicles/ m^2. The new release rice cultivars Sakha 105, Sakha 106 and Sakha 107 were exhibited low level of infection 2.1 to 2.43. Concerning hybrid rice varieties, H1 exhibited, a moderate infection 4.1 compared with the highly susceptible cultivar Giza 171. severity of infection reflected the same levels of infection with different varieties, 2015 season (Table 1). The same trend of results was recorded in 2016 season. Giza 171 as a japonica short grain the oldest rice cultivar which is earlier distributed on large scale all over rice governorates so the pathogen produced different compatible races. As a result of long period of interaction with the host it became highly susceptible. In addition, Giza 171 was very late maturing cultivar, consequently the high primary inoculum intensity was provided from early maturing cultivars. Giza 178 was highly susceptible specially in belkas location whereas it was grown on large areas for many years and the fungus produce highly host-specific races. The obtained results are in agreement with those results of Templeton (1961**)** who mentioned that some cultivars are susceptible, while zenith, Tauching netve 1 and Vista are resistant. Also, Cartwright et al. (1997) identified some susceptible to highly susceptible rice cultivars Cypress, LaGrue, M204, and Newbonnet as a temperate japonica. For long grains such as Giza 182 that restricted in small scattered areas so these environments not provided an opportunity for the pathogen to high perform interaction with the host, as a result of this weak interaction this cultivar exhibited a lowest infection level. Although, Sakha 101 and Sakha 104 as a japonica short grain were covered now almost 50 % of total cultivated areas, they recorded the lowest level of infection. The obtained results are in contrary with Tempelton (1967), Biswas (2003). They found that the long-grain cultivars are most susceptible as predominant in USA than short and medium grains that recorded low and intermediate reaction respectively. In addition, heading late cultivars exhibited severe infection than early maturing ones. In addition, kernel smut caused severe smut for more 1000 acre in Texas that cultivated with Presidio, Cheniere and XL 753 as long grain cultivars (Uppala et al., 2017).

Table 2: Evaluation of rice cultivars resistance to kernel smut disease, 2015 and 2016 season.

Cultivar	Rice type	Duration days	2015		2016	
			No. of infected		No. of infected	
			panicles/m^2	grains/m^2	panicles/m^2	grains/m^2
Giza 171	J	160	17.67	21.23	19.70	22.67
Giza 177	J	125	6.77	8.33	8.67	9.23
Giza 178	IJ	135	12.33	14.10	14.23	15.67
Giza 179	IJ	125	1.37	1.33	1.23	2.43
Giza 182	I	126	3.57	3.33	2.33	2.90
H1	IJ	135	4.10	4.67	3.90	5.90
Sakha101	J	145	1.37	1.47	1.77	1.57
Sakha104	J	135	2.33	1.566	2.57	2.77
SAKHA105	J	125	2.43	2.90	1.57	2.33
SAKHA106	J	126	2.10	2.57	1.57	2.67
SAKHA107	J	125	2.43	3.21	1.23	2.00
GZ-7112-6-20	J	125	2.33	2.67	2.23	3.67
GZ 9807-6-3-2-1	J	120	1.33	1.33	1.90	1.00
GZ9399-4-1-1-2-1-2	IJ	120	3.90	4.23	2.23	3.43
GZ9461-4-2-1-2	IJ	125	2.33	3.77	2.90	2.43
GZ10101-5-1-1-1	J	125	1.37	2.00	2.23	1.67
GZ10144-14-4-4-1	J	125	1.37	1.33	1.57	1.43
GZ10154-3-1-1-1	J	125	1.03	1.33	1.57	1.77
GZ10305-14-1-1-2	J	125	1.37	1.00	1.23	1.43
GZ10365-2-4-1-2	J	125	1.70	1.00	1.23	1.43
L.S.D. 5%			0.790	1.248	1.258	0.896

Giza 177 was cultivated on large area and exhibited a highly susceptible reaction although it was the earliest maturing cultivar. The obtained results are in harmony with Chouhan and Verma (1964) who noted that early maturing cultivars were more susceptible than later maturing ones in India. Shorter anthesis period contributes in the direction of resistance (Anita, 2000). There are wide variations in resistance levels among rice varieties (Biswas, 2003; Tempelton, 1971; Singh & Pavgi 1970). From previous results it concluded that, the successful crosses for management of rice kernel smut must depend on all tolerant cultivars; Sakha 101, Sakha 104, Sakha 105, Giza 182 and GZ10101-5-1-1-1, GZ10144-14-4-4-1, GZ10154-3-1-1-1,and GZ10305-14-1-1-2 promising lines. Depending on high resistant varieties in past years could explain the high progress achieved to manage kernel smut with currently promising lines. Therefore, the detection of new resistance resources allow to further progress in establishing a successful breeding program of kernel smut. Rice differ in their susceptibility to *T. barclayana* but in general, short-grain cultivars are more resistant than medium and long grain (Carris et al., 2006).

Cluster analysis of disease parameters: From cluster analysis of disease parameters (Figure 7), the cluster divided to two main clusters. The first main cluster compromised three cultivars as a highly susceptible group, Giza 171, Giza 177 japonica type and Giza 178 indica japonica. The second main cluster included two sub-main clusters. The first sub-main group have H1 Egyptian hybrid 1 as moderately

susceptible, Sakha 105, Sakha 106, Giza 182, Gz7112, GZ9461, and GZ9399. The second sub-main cluster involved highly resistant varieties to kernel smut diseases. Giza 179, Sakha 101, Sakha 107, Sakha 104 in addition to some GZ lines, GZ10144. GZ10154, GZ10305, GZ10365, GZ9807 were located in highly resistance group. These results in agreement with Gravois and Bernhardt (2000), reported that Katy, Drew and Kaybonnet, were identified as stable, low susceptible cultivars to rice kernel smut. There are wide variations among the response of different Egyptian varieties to kernel smut inoculation. The availability of highly resistant resources of germplasm and efficient screening program has a net return in disease reduction and breeding progress.

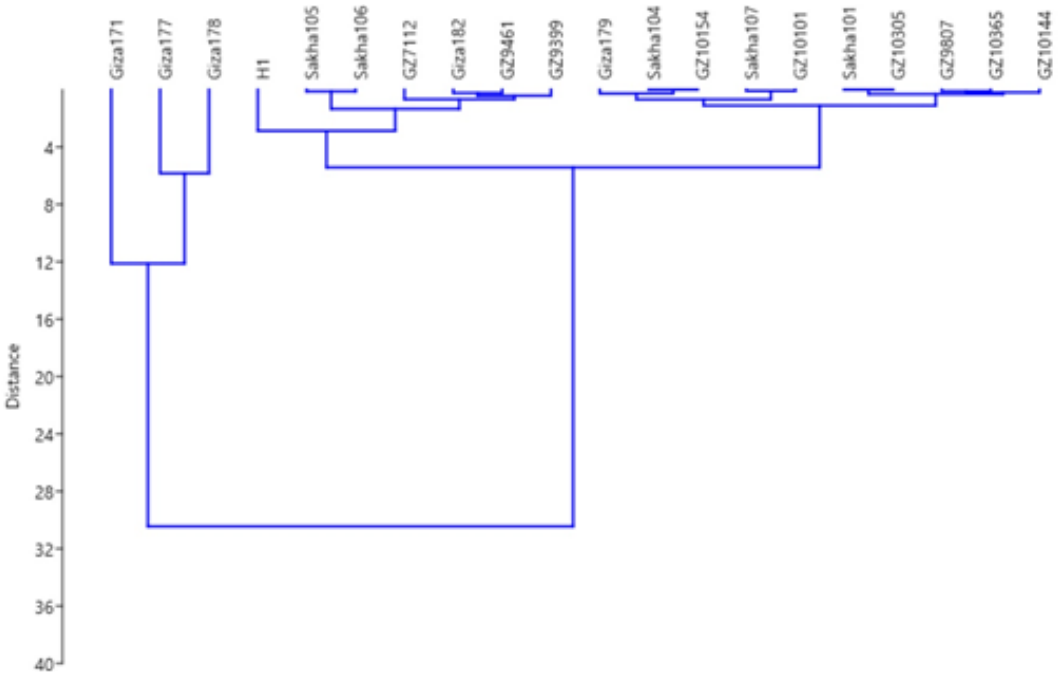

Figure 7: cluster analysis dendrogram of different Egyptian varieties based on their response to infection of *Tilletia barclayana* kernel smut fungus.

Inoculation date: Giza 171 was artificially inoculated at various growth stages, there are highly significant differences among inoculation dates. Low infection was recorded from inoculation at seedling stage, whereas, the highest infection induced at flowering stage, subsequently booting stage recorded the second rank of infection. Therefore, these results in agreement with Uppala et al. (2017), they inoculated field plots with sporidia of smut during booting stage to ensure infection and evaluate fungicides. The lowest infection was recorded from both inoculation at seedling and maximum tillering. Infection depends on the viability of sporidia during the season, so inoculation with allantoid sporidia at seedling stage induced low infection due

to lose some of their viability during this period. However, a recent study with *T. indica*, *T. horrida*, *T. walkeri*, and *T. caries* demonstrated that sporidia are remarkably durable. Sporidia were viable for 31-49 days under (10–20% RH at 20–22˚C) and 56-88 days (40–50 % RH, 18ºC) (Goates, 2010; Goates, 2005). Therefore, sporidia can survive within reproductive stage of numerous rice varieties. Also, both the highest infection at flowering stage and the lowest through inoculation at seedling proofed local infection not systemic. The pathogen can infects developing florets of rice plant, growing within embryo "milk" until the kernel enters soft dough stage. These results in agreement with behavior of *Tilletia barclayana* that infects the open rice flowers only at anthesis, grows within developing rice kernel as mycelium and eventually consumes endosperm content, converting it into black teliospores that survive on seed and residue in soil (Cartwright et al., 1994; Whitney & Frederiksen, 1975). Other research has determined that the fungus was capable of infecting florets before anthesis and confirmed that the infection process was enhanced by high moisture during the heading phase and disease more prevalent during rainy years (Uppala et al., 2017; Cartwright et al., 1995). During harvest, Teliospores of *T. barclayana* are released from smutted grains and survive in contaminated soil. After rice cultivation, teliospores were germinated and produce primary and secondary sporidia, which air forcibly discharged onto ovaries through open florets and repeat disease cycle (Whitney, 1992). Since teliospores serve as soil-borne primary source of inoculum for the initiation of this disease. Singh and Pavgi (1973) reported that local infection of florets start with grown of sporidia on stigma, and penetrate through style to chalazal end of ovary, the hyphae persist between aleuron layer and seed coat, consume endosperm and developing sorus. The embryo is not invaded.

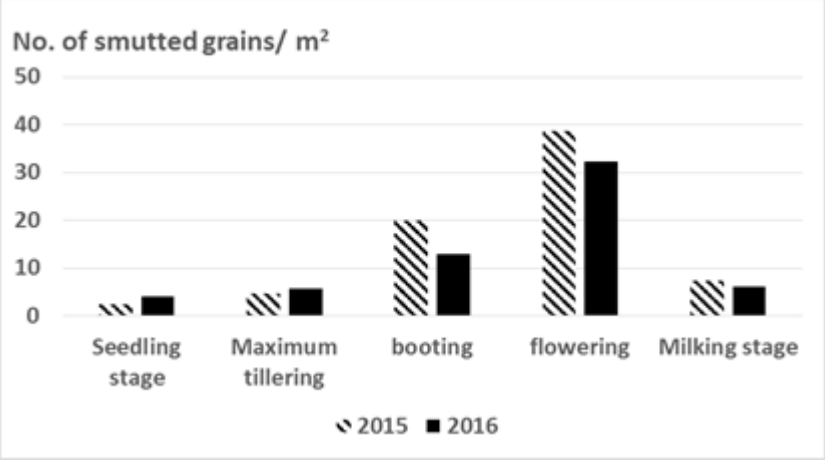

Figure 8: Effect of inoculation date on severity of kernel smut infection (no. of smutted grains/m^2) on rice cultivar Giza 171, 2015 and 2016 seasons.

kernel smut fungus not transmitted by systemic infection and not infects seedlings, but primary infection develop from a local inoculation at anthesis by airborne secondary sporidia (Whitney & Frederiksen, 1975; Tullis & Johonson 1952). From the current results, infection of smut reached the peak during complete heading, but before anthesis onset and decreased gradually with inoculation after anthesis, in agreement with Goates and Jackson (2006). In addition, flowering stage was the most susceptible stage for smut infection. This supported by some others.

Effect of spore types on kernel smut infection: Japonica rice cultivar Giza 171 was inoculated with different types of smut spores during flowering stage. Data in Figure (9) illustrated that there are a wide variation in infection among types of spores. Artificial inoculation with allantoid spores induced the highest infection percentage since it can germinate and directly gave binucleate mycelium that able to invasive ovary and all tissues of opening spikletes. Filliform sporidia recorded second rank of infection percentage whereas, teliospores prompted the lowest infection percentage. Allantoid secondary sporidia are simply airborne that deposited on the open florets and possibly are responsible for most infections. The production of infected grains was much higher with allantoid sporidia than filliform sporidia inoculation (Chahal., 2001). Results from several studies indicate susceptibility only during specific periods within boot swelling to anthesis stage (Rush et al., 2005; Bonde et al. 2004; Kumar & Nagarajan, 1998; Bains, 1994). However, a recent study with *T. indica*, *T. horrida*, *T. walkeri*, and *T. caries* demonstrated that sporidia are remarkably durable. Sporidia were viable for 30-88 days (Gates, 2010).

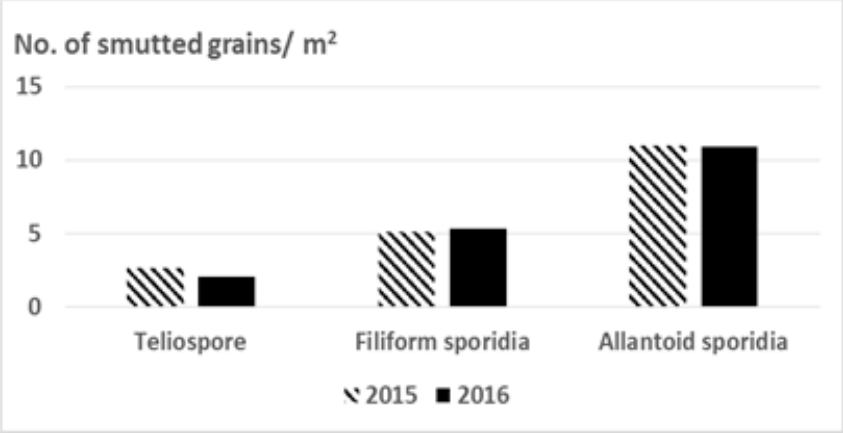

Figure 9: Effect of artificial inoculation with different type of kernel smut spores on infection (no. of smutted grains/ m^2) of rice cultivar Giza 171, 2015 and 2016 seasons.

Allantoid sporidia are considered as primary and infective agent for both *T. indica* and *T. horrida*. Natural infection by *T. indica* occurs via airborne

inoculum during heading. In addition, *T. horrida* and *T. indica* have same local infection, morphological traits and distantly related (Carris et al., 2006; Ingold, 1996). Also, Singh and Pavgi (1973) speculated that sporidia lodge on the feathery stigma and penetrate through the style to the chalazal end of the ovary. Therefore, for successful varietal resistance evaluation, reproducible and efficient inoculation technique, allantoid sporidia must be used in artificial inoculated cultivars.

Survival of teliospores during two years: Teliospores that collected directly from fresh harvested grain commonly germinate poorly compared with germination after stored for several months to a year or longer. Therefore, the germination percentage reached 1-4 % after grain harvest of various cultivars. The germination percentage increased to be 15 % after store for 6 months. Extended the store period to 1 year induced the germination to 32%. One year and half later, the germination percentage can reach a peak to more than 95%. The teliospores can survive for more than two years, thus teliospores of *T. barclayana* successfully overwintered to permanent fields within infected rice grains. More than 90% of total teliospores annually transferred in dormancy state as a source of inoculum. So, it is recommended to eradicate kernel smut must use healthy seeds free from this fungus. The teliospores can survive for more than 2 years as a seed-borne fungus**.** Spores of the fungus persistent viable for a year or two in soil and several years in infected grain. Once the rice field is flooded, these spores produce a series of secondary sporidia, which are forcibly discharged and infect the developing grains. whereas under laboratory and field conditions, *T. horrida* can survive form 2 to 3 years (Ou, 1985; Singh 1975). Teliospores of *T.indica* and *T. horrida* have three type of dormancy (Chahal et al., 1993); first type is postharvest dormancy whereas fresh harvested teliospores are poorly germinated than stored for months or years. For one-year storage, germination not reached more than 50% of teliospores under optimum stored conditions.

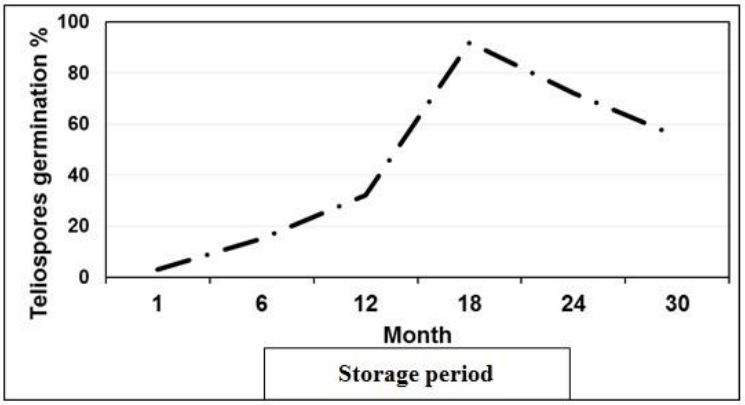

Figure 10: Survival of kernel smut on rice grains of different rice cultivars under lab conditions. MAH month after harvest.

Second type, is long-term dormancy and survival of teliospores in open field contributes to this type. The third type due to cold conditions (Thinggaard & Leth, 2003; Rattan & Aujla 1990; Smilanick et al., 1985; Bansal et al., 1983; Mitra, 1935). Finally, germination rate and ability exhibited a remarkable increase with increasing of storage period. Ustilospores have been found viable following 3 years in stored grains. They also survive passage through the digestive tracts of domestic animals (Ou, 1985).

Molecular identification of kernel smut fungus: Two isolates; Eg 01, Eg 02, were utilized for molecular identification and morphological features that involved pigment color and colony growth nature. The ITS region of rDNA was amplified with primers ITS4/ITS5 and sequenced (Arruda et al., 2005). The ITS4/ITS5 sequences of the tested isolates were submitted and get accession numbers in NCBI Genebank as follows; MH042043 for isolate Eg 01, whereas MH042044 of isolate Eg 02. The product size was within the range 574-699 bp of both isolates. BLAST sequence analysis of the consensus sequence for Egyptian isolates was compared with worldwide ones (GenBank Accession No.: AY837516.1, AY837518.1, AY837519.1 and HQ317521.1 Tilletia barclayana from USA rice paddy fields matched with identity of almost 74-81%. These four USA isolates were more closely related to MH042043.1 Eg 01. While, DQ827708.1 from Asian isolate of Pakistan was close to isolate Eg 01 with name *Tilletia horrida*. For isolate Eg 02 Accession No. MH042044.1, was more associated and close to accession AF39894, AF399892, from china with identity 76-80%, in addition, isolates from India have the same trend of identity AY425727, AY4560053 as 80%. Also, isolates of Mexico highly matched with Egyptian isolates by 100%, accessions no. AY818970, AY818971, AY8189774, (Figure 11 & 12A, B).

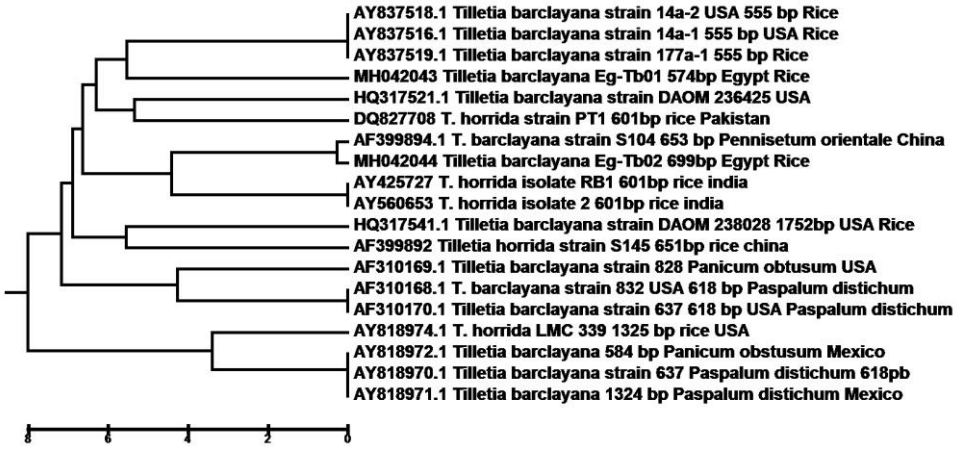

Figure 11: Phylogenetic tree constructed based on ITS region using ITS4/ITS5 primers of *Tilletia barclayana* Egyptian isolates and other worldwide isolates.

A.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tilletia barclayana strain S104 18S ribos	443	464	92%	2e-127	76%	AF399894.1
Tilletia barclayana strain DAOM 238028	205	323	45%	1e-55	80%	HQ317541.1
Tilletia barclayana strain DAOM 236425	199	309	47%	5e-54	80%	HQ317521.1
Tilletia barclayana strain 637 18S ribosc	197	316	46%	2e-53	79%	AF310170.1
Tilletia barclayana strain 832 18S ribosc	197	294	44%	2e-53	79%	AF310168.1
Tilletia barclayana strain 828 18S ribosc	192	310	46%	7e-52	78%	AF310169.1
Tilletia barclayana voucher WSP 68466	21.1	42.2	3%	2.6	100%	AY818971.1
Tilletia barclayana voucher WSP 68658	21.1	42.2	3%	2.6	100%	AY818970.1
Tilletia barclayana strain 14a-1 60 kDa	331	331	98%	1e-93	74%	AY837516.1
Tilletia barclayana strain 177a-1 60 kDa	309	309	95%	4e-87	74%	AY837519.1

B.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tilletia horrida isolate 2 internal transcribed spac	214	290	42%	2e-58	81%	AY560653.2
Tilletia horrida strain PT1 18S ribosomal RNA ge	214	290	42%	2e-58	81%	DQ827708.1
Tilletia horrida strain S150 18S ribosomal RNA g	214	323	47%	2e-58	81%	AF399893.1
Tilletia horrida internal transcribed spacer 1, part	208	284	42%	1e-56	80%	AY425727.2
Tilletia horrida strain S145 18S ribosomal RNA g	208	318	47%	1e-56	80%	AF399892.1
Tilletia horrida strain 358 18S ribosomal RNA ge	208	284	42%	1e-56	80%	AF310173.1
Tilletia horrida strain K01 18S ribosomal RNA ge	205	281	42%	1e-55	80%	DQ827714.1
Tilletia horrida strain YN1 18S ribosomal RNA ge	205	281	42%	1e-55	80%	DQ827713.1
Tilletia horrida strain D97 18S ribosomal RNA ge	205	281	42%	1e-55	80%	DQ827705.1
Tilletia horrida strain S080 18S ribosomal RNA g	205	314	47%	1e-55	80%	AF398435.1
Tilletia horrida strain 338 18S ribosomal RNA ge	205	281	42%	1e-55	80%	AF310172.1
Tilletia horrida strain WSP69539 18S ribosomal I	205	281	42%	1e-55	80%	AF310171.1
Tilletia horrida strain US1 18S ribosomal RNA ge	201	277	42%	2e-54	80%	DQ827709.1
Tilletia horrida strain D95 internal transcribed spa	179	307	48%	5e-48	77%	DQ827704.1
Tilletia horrida strain CN1 internal transcribed sp	179	308	48%	5e-48	77%	DQ827699.1
Tilletia horrida voucher LMC 339 28S ribosomal	21.1	42.2	3%	2.9	100%	AY818974.1

Figure 12: Matched accessions of Egyptian isolates through NCBI website for *Tilletia barclayana* A, and *Tilletia horrida* B, and their identity values.

The DNA sequence analysis demonstrated that sequence of Egyptian isolates was matched with isolates from different ecological and hot spots areas worldwide. The ITS region is most useful for molecular systematics at the species level, and even within species in fungi (Meenupriya & Thangaraj, 2011). It combines the highest discrimination ability to closely related species with a high PCR and sequencing success rate across abroad range of fungi (Schoch et al., 2012). Therefore, the Egyptian isolates R01, R18 and R21 were clustered together in the same clade and closely related to some isolates of China, USA, Japan and Vietnam. Whereas, isolate R02 and R05 more divergent and

separated in different clades and closely related to isolates of Iran, India, USA and Australia. The internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. Egyptian isolates of *Tilletia barclayana* were highly matched and have high identity score with USA, India, and China pathogen, these results in harmony with Chahal (2001), Carris et al. (2006) and Brooks et al. (2009), reported that this fungus considered as minor disease but can be changed to major in some rice production areas such as; India and southern USA and recorded remarkable yield losses, In addition, Kernel smut considered as minor with sporadic nature but became important economical rice fungal diseases with severe quality and yield losses in Texas (Uppala et al., 2017). Therefore, In Egypt must deal with this fungus in a proper IPM approaches under climate change conditions.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST (Basic Local Alignment Search Tool) and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research **25**: 3389–3402.

Anita, 2000. Studies on factors affecting development of kernel smut of rice. M.Sc. Thesis, Punjab Agriculture University, Ludhiana, India.

Arruda MA, Bueno CRNC, Zamprogno KC, Lavorenti NA, Urashima AS, 2005. Reacao do trigo ` a Magnaporthe grisea nos diferentes estadios de desenvolvimento. Fitopatologia Brasileira **30**:121–126

Bains SS, 1994. Influence of wheat spikes maturity on susceptibility to infection and growth of sporidia of *Neovossia indica*. Indian Journal of Mycology and Plant Pathology **24**: 111–15.

Biswas A, 2003. Kernel smut disease of rice: current status and future challenges. Environment and Ecology **21**: 336–351.

Bonde MR, Berner DK, Nester SE, Peterson GL, Olsen MW, Cunfer BM, Sim T, 2004. Survival of Tilletia indica teliospores in different soils. Plant Disease **88**(4): 316–324.

Bonfield JK, Smith KF, Staden R, 1995. A new DNA sequence assembly program. Nucleic Acids Research **24**: 4992–4999.

Brooks SA, Anders MM, Yeater KM, 2009. Effect of cultural management practices on the severity of false smut and kernel smut of rice. Plant Disease **93**:1202–1208.

Carris LM, Castlebury LA, Goates BJ, 2006**.** Nonsystemic bunt fungi *Tilletia indica* and *T. horrida*: a review of history, systematics and biology. Annual Reviews Phytopathology **44**: 113–33.

Cartwright RD, Lee FN, Crippen DL, Eason R, Templeton GE, 1996. Artificial inoculation of kernel smut of rice in Arkansas. p. 107–110. In R.J. Norman and B.R. Wells (ed.). Research Series, Arkansas Agricultural Experiment

Station, Hamblen Ave, Fayetteville, AR 72704, USA.

Cartwright RD, Lee FN, Crippen DL, Templeton GE, 1994. Kernel smut research in Arkansas. In*:* B.R. Wells (ed.). Research Series, University of Arkansas Agricultural Experiment Station Research Series **439**: 71–77.

Cartwright RD, Lee FN, Hunter RL, Crippen DL, Tempelton GE, 1995. Research in Arkansas on kernel smut of rice. In: B.R. Wells (ed). Research Series, Arkansas Agricultural Experiment Station, University of Arkansas, **446**: 115-124.

Cartwright RD, Parsons CE, Ross WJ, Lee FN, Templeton GE, 1997. Rice disease monitoring in Arkansas. In*:* R.J. Norman and T.H. Johnston (eds.). Arkansas Rice Research Studies 1996. University of Arkansas Agricultural Experiment Station Research Series **456**: 81–89.

Castlebury LA, Carris LM, 1999. *Tilletia walkeri*, a new species on *Lolium multiflorum* and L. perenne. Mycologia **91**: 121–31

Castlebury LA, Carris LM, Vinky K, 2005. Phylogenetic analysis of Tilletia and allied genera in order Tilletiales (Ustilaginomycetes: Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. Mycologia **97**(4): 888–900.

Chahal SS, 2001. Epidemiology and management of two cereal bunts. Indian Phytopathology **54**: 145–57

Chahal SS, Aulakh KS, Mathur SB, 1993. Germination of teliospores of *Tilletia barclayana*, the causal agent of kernel smut of rice, lation to some physical factors. Journal of Phytopathology **137**: 301–308

CMI. 1991. *Tilletia barclayana* (Bref.) Sacc. & Sydow. CMI Distribution Maps of Plant Diseases 75 pp.

Durán R, 1987. Ustilaginales of Mexico. Taxonomy, Symptomology, Spore Germination, and Basidial Cytology. Pullman, Whitman, Washington State University, USA, 331 pp.

Elshafey RAS, 2013. Evaluation of varietal resistance and physiological characters of false and kernel smut diseases of rice in Egypt. Mansoura Journal of Plant Protection and Pathology **4**(1): 125–142.

Farr DF, Rossman AY, Palm ME, McCray EB, 2005. Fungal databases, Syst. Bot. Mycol. Lab., ARS, USDA. http://nt.ars-grin.gov/fungaldatabases.

Goates BJ, 1988. Histology of infection of wheat by Tilletia indica, the Karnal bunt pathogen. Phytopathology **78**:1434–1441.

Goates BJ, 2005. Durability of secondary sporidia of floret infecting Tilletia species: implications for epidemiology. Phytopathology **95**: 961.

Goates BJ, 2010. Survival of Secondary Sporidia of Floret-Infecting *Tilletia* Species: Implications for Epidemiology. Phytopathology **100**(7): 655–662.

Goates BJ, Jackson EW, 2006. Susceptibility of Wheat to *Tilletia indica* during stages of spike development. Phytopathology **96**(9): 962–966.

Gomez KA, Gomez AA, 1984. Statistical procedures for agricultural research. 2nd ed., John Wiley and Sons, USA.

Gravois KA, Bernhardt JL, 2000. Heritability and genotype x environment interactions for discolored rice kernels. Crop Science **40**: 314–318.

Ko WH, Lockwood JL, 1970. Mechanism of lysis of fungal mycelia in soil. Phytopatholoy **60**: 148–154.

Kumar J, Nagarajan S, 1998. Role of flag leaf and spike emergence stage on the incidence of Karnal bunt in wheat. Plant Disease **82**:1368–1370.

Levy LL, Castlebury A, Carris LM, Meyer RJ, Pimentel G, 2001. Internal transcribed spacer sequence-based phylogeny and polymerase chain reaction-restriction fragment length polymorphism differentiation of *Tilletia walkeri* and *T. indica*. Phytopathology **91**: 935–940.

Lloyd AB, Lockwood JL, 1966. Lysis of fungal hyphae in soil and its possible relation to autolysis. Phytopatholoy **56**: 595–602.

Matsumoto TT, Whitney NG, Bonde MR, Royer MH, 1985. Morphological variation of teliospores of *N. horrida*. Phytopathology **75**: 1365.

Meenupriya J, Thangaraj M, 2011. Analytical characterization and structure elucidation of metabolites fromAspergillusochraceus MP2 fungi. Asian Pacific Journal of Tropical Biomedicine **1**: 376–380.

Mitra M, 1935. Stinking smut (bunt) of wheat with special reference to *Tilletia indica* Mitra. Indian Journal of Agricultural Science **1**: 51–74.

Ou SH, 1985. Rice Diseases. CMI, Kew, UK, 380 pp.

Rattan GS, Aujla SS. 1990. Survival of Karnal bunt (*Neovossia indica*) teliospores in different types of soil at different depths. Indian Journal of Agricultural Science **60**: 616–18

Rush CM, Stein JM, Bowden RL, Riemenschneider R, Boratynski T, Royer MH, 2005. Status of Karnal bunt of wheat in the United States 1996 to 2004. Plant Disease **89**: 212–223

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA bar- code marker for fungi. Proceedings of the National Academy of Sciences USA **109**: 6241–6246.

Sharma RC, Gill SS, Joshi DP, Rang A, Bassi G, Bharaj TS, 1999. Kernel smut—a major constraint in hybrid seed production of rice and its remedial measures. Seed Research 27: **82**–90.

Singh R, Pavgi M, 1972. Cytology of teliospore germination and development of *Neovossia horrida*. Riso **21**: 259–268.

Singh RA, 1975. Source of inoculum and the epidemiology of bunt of rice. Riso **25**: 77–80.

Singh RA, Pavgi MS, 1970. Varietal Reaction and resistance to kernel bunt of rice. Indian Phytopathology **33**: 51–53.

Singh RA, Pavgi MS, 1973. Development of sorus in kernel bunt of rice. Riso **22**: 243–50.

Slaton NA, Gbur EEJ, Cartwright RD, DeLong RE, Norman RJ, Brye KR, 2004. Grain yield and kernel smut of rice as affected by preflood and midseason nitrogen fertilization in

Arkansas. Agronomy Journal **96**(1): 91–99.

Smilanick JL, Hoffmann JA, Royer MH, 1985. Effect of temperature, pH, light, and desiccation on teliospore germination of *Tilletia indica*. Phytopathology **75**: 1428–31.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24**: 1596–1599.

Templeton GE, 1961. Local infection of rice florets by the rice kernel smut organism, *Tilletia horrida*. Phytopathology **51**(2): 130–131.

Thinggaard K, Leth V, 2003. Use of the fluorochrome vital dye acridine orange to determine viability and germination of *Tilletia indica* teliospores in soil. Seed Science and Technology **31**: 329–40.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research **22**: 4673–4680.

Tullis EC, Johanson AG, 1952. Synonymy of *Tilletia horrida* and *Neovossia barclayana*. Mycologia **44**(6): 773–788.

Uppala S, Liu G, Liu B, Wu M, Zhou XG, 2017. Texas Rice Kernel Smut Research. 10.13140/RG.2.2.10801.28006.

Vánky K, 2001. Taxonomical studies on Ustilaginales. XXI. Mycotaxon **78**: 265–326.

Vánky K, Bauer R, 1992. Conidiosporomyces a new genus of Ustilaginales. Mycotaxon **43**: 427–436.

Vánky K, Bauer R, 1995. Oberwinkleria, a new genus of Ustilaginales. Mycotaxon **53**: 361–368.

Webster RK, Gunnell PS, 1992. Compendium of rice diseases. American Phytopathological Society, Minneapolis, MN

White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Eds.) PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, California, USA, 315–322 pp.

Whitney NG, 1992. Kernel smut. In: Compendium of Rice Diseases. R.K. Webster and P.S. Gunnel (eds.). American Phytopathological Society, St. Paul, MN, USA, 28–29 pp.

Whitney NG, Frederiksen RA, 1975. Kernel smut of rice. Publication 1231, College Station, Texas Agricultural Experiment Station, Texas, USA.