Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

IDENTIFICATION OF ISOLATED STRAINS OF *USTILAGO TRITICI* RS-01 FROM AN INFECTED WHEAT CROP

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A B S T R A C T

Loose smut has a wide distribution and can occur anywhere wheat is cultivated and produced. It is caused by the fungus *Ustilago tritici*. Mild symptoms may be present prior to heading, including yellowish leaf streaks and stiff, dark green leaves. Spores are not enclosed by the seed coat, so are quickly dispersed by rain or wind after emergence. These spores infect other wheat plants at flowering, causing seed infection. Wheat (*Triticum aestivum* L.) is an important crop and staple food of human population throughout the World. It is widely cultivated in Pakistan and in most of the other agricultural countries like China, India and Bangladesh. Therefore, an experimental study was designed to cultivate Galaxy-2013 for the isolation of pathogenic strains of *tritici* having potential to cause loose smut of wheat, after natural attack during cropping year 2019-2020. Main purpose of study was to isolate samples of *Ustilago*, which would be used for the testing of anti-fungal products for future studies during cropping years (2020-2022) after complete identification, isolated strains (RS01) were identified as *Ustilago tritici* and an accession number (OP164708_RS01) was obtained after blast the obtained sequences at NCBI, which were further confirmed by phylogenetic analysis. Therefore, it was expected that some wheat varieties those are vulnerable to the attack of *Ustilago* required careful cultivation by adapting suitable strategies to protect crops especially wheat from certain disease to enhance food requirement in the country.

Keywords: Loose smut, *Ustilago tritici*, wheat and agriculture.

INTRODUCTION

Wheat is among the most important cereal crops cultivated globally for achieving global food security. It is commonly cultivated worldwide and is second highest in production, but its production is affected by *T. aestivum*is among essential cereal crops which are cultivated largely around the globe after rice (27%) and maize (25%), which accounts 30% of financial records worldwide. This crop has attained the great importance and attention of farmers as well as researchers being a vital source of energy and protein for human diet. This crop is basic source of diet for humans especially for the citizens of a country like Pakistan, India, and Bangladesh (Arshad and Shafqat 2012). No doubt wheat crops are cultivated on large scale in most countries it had been estimated that 600 million tons (MT) of wheat is grown around the world covering an area of almost 2-5 million hectares.

The contribution of underdeveloped countries is 55% to the total production of wheat worldwide. It had been reported that the cultivation of this food crop in Pakistan is 25.09 million tons (MT) which covers the area of 8.8 million hectares annually. Value and the importance of this crop could not be denied and ignored since this crop is contributing about 20% world's food calories. Wheat crops are susceptible to numerous devastating diseases such as rust and smut which causes enormous damage and yield losses. The loose smut of wheat is an infectious wheat disease which is caused by the species of *U. tritici*, a

Submitted: June 28, 2023
Revised: July 17, 2023
Accepted for Publication: December 05, 2023
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biotrophic fungi. This disease is common among cereal crops and flourishes during historic times, which were recognized by Roman people who designated it *Ustilago* a word from Latin meaning burns (Wilcoxson and Saari, 1996; Gad *et al*., 2019); (Ahmed *et al*., 2022).

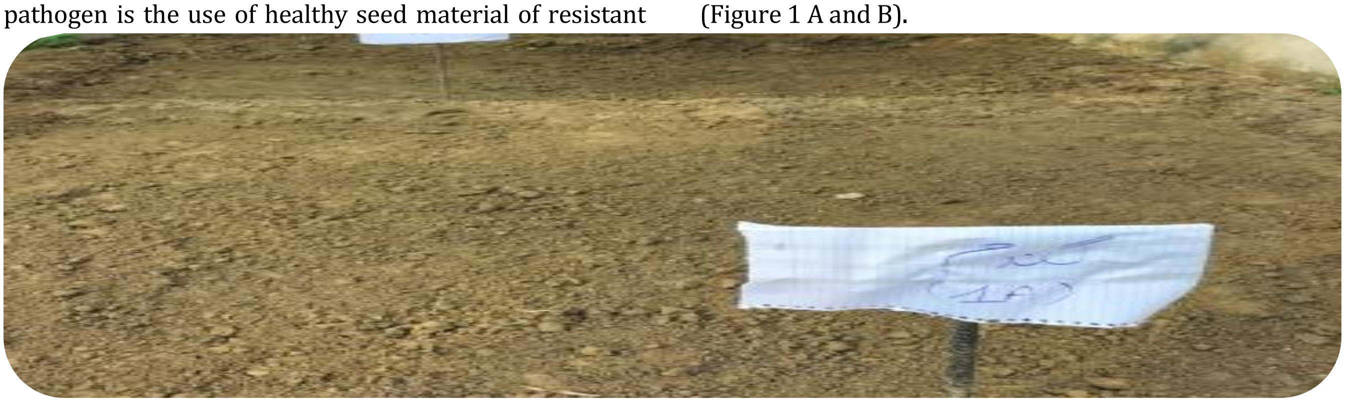
U. tritici is a fungal pathogen which can cause loose smut of wheat which is commonly considered as a threatening plant disease which causes 40% of yield losses (Quijano *et al*., 2016; Esse et al. 2020). It had been a great threat to produce seeds especially for developing countries which is a major concern for the landowners of small lands cultivating wheat to meet their food requirements and uses seeds which were stored last growing season. Loose smut of wheat flourish more if found favorable environmental conditions such as cool and humid climate at anthesis stage of crop therefore, the chances for the losses could be significant in the regions having favorable environmental conditions for fungal spread. But losses due to this disease are also reported in some other regions of dry and warm climate as well (Roland *et al*., 2016; Zandalinas et al.,2022). The chances for the attack of this disease are between early and mid-anthesis stages of crop but successful infection can occur even after anthesis. Attack of this disease is easily recognized physically at the emergence of ear as each grain becomes completely or partially replaced by a mass of blackish fungal spores. These fungal spores are released after the emergence of the ear which leaves bare remains of the ear rachis behind, which is the clear indication of disease severity. Spores of these pathogens spread passively at the growing points of crop and develop into a smutted ear (Malik and Batts, 1960); Mohdly *et al*., 2023. The problem with the infected seed is that it never showed any kind of sign or mark which is obvious or unambiguous until the appearance of infected ears on crop (Eibel *et al*., 2005; Abrahim, 2019).

The only recommended solution to overcome this fungal pathogen is the use of healthy seed material of resistant varieties. Cultivation of resistant varieties are the most effective measures in controlling the disease, which is preventive measure, while chemical treatment is the protection method. There are numerous other chemical-based fungicides which are commonly available in the market to treat this disease. The use of chemical based fungicidal material to control these pathogens is a common practice which had great adverse results on the environment so for that's why an alternative ways and tools are needed to overcome the use of chemical-based material to improve cropping system (Badar *et al*., 2020; Badar *et al*., 2022).

Both protective and preventive management strategies for loose smut of wheat are not effective for farmers cultivating wheat at all low scale for their own. Cultivation of resistant varieties for local farmers growing wheat for meeting their food requirements is not in practice as they are using seeds stored from their previous year crops. Moreover, they are not familiar with the advanced and resistant varieties. The use of chemical-based fungicide is not affordable for small scale producers and has numerous toxic effects on the environment. Therefore, the present experimental investigation was designed for the evaluation of economic and environmentally friendly plant based fungicidal material.

MATERIAL AND METHODS

Experimental layout: Loose smut is a common disease of wheat, having a greater potential of reducing yield, which is caused by member of *tritici*. Therefore, seeds of Galaxy-2013 obtained from Punjab Seeds Cooperation Sahiwal, Pakistan were grown in three different agricultural field of Kotmoman, District Sargodha, Punjab, Pakistan. Pathogenic strains of *tritici* causing loose smut disease of wheat were isolated from an infected wheat spike. A small piece of land was prepared for the cultivation of wheat, provided all the essential conditions for wheat growth (Figure 1 A and B).



A

B

Figure 1. A and B Land preparation for the cultivation of Galaxy-2013

Basic preparation for wheat cultivation: The present study was conducted using clay loamy soil, to provide crop with all the required constituents soil analysis (Table 1) was performed before the application of any fertilizer. After those fertilizers were applied in the form of urea (46%), DAP (18%) and NPK in the ratio of 100:75:50 (N: P: K) kg/ha[1]. The source of fertilizers was urea and source of Zinc was $ZnSO_4.7H_2O$. NPK fertilizer was applied as a basal dose, crop was irrigated at tillering, booting and milking stage.

Symptoms of disease: Symptoms of this disease were not recorded before heading; therefore, detection of disease was not possible until crop reached at heading. But the most interesting fact is that smutted heads of infected crops usually emerge earlier than those of healthy ones. After of appearance of symptoms, it is easy to recognize the emergence of disease, as kernels becomes blackish, and heads contain olive black masses of teliospores.

Collection and preservation: After the appearance of disease symptoms ten samples of teliospores (Sample RS-01, RS-02, RS-03, RS-04, RS-05, RS-06, RS-07, RS-08, RS-09 and RS-10) from freshly smutted spikes, were collected from an experimental crop. Collected samples were shade dried, gently scraped thoroughly by 53 µm mesh sieve, sealed in pre-labelled cellophane plastic bags and samples were stored at 10 °C. Potential of these strains were tested using water agar and those showed viability >70% were saved for further uses (Nasr, 1977; Rajput *et al*., 2019).

Identification of teliospores: Plant infected spikes were photographed using digiporo-Labomed (PX 5) which were further illustrated through the photographs taken from microscope attached camera in the field. While free-hand sections were made, and mount of infected parts were observed under a Labomed CSM2 stereomicroscope. At least 30-35 spores of each spore stage were examined under a compound microscope (MX4300H, Meiji Techo Co., Ltd., Japan) for spore measurement. Each spore stage was photographed (Gupta *et al*., 2009).

Molecular identification: Molecular identification of the collected teliospores were carried by Pablo Alvarado Garcia Spain, while the amplification of Internal Transcribed Spacer Region (ITS) has been done via couple ITS-specific primers (ITS1 forward and ITS4 reverse) (Table 2). The obtained sequences were BLAST on NCBI for sequence similarity with already identified strain of *U. tritici*. Genetic association and phylogenetic analysis have been done by neighbor-joining of multiple isolates of local *U. tritici*, which has been compared with multiple sequences of nitrogenous bases by depending on ITS interface region. Dendrogram was constructed for more clarity and understanding of the isolated strains (Al-Yassiry and Al-Alwani 2022).

Table 1. Physio-chemical analysis of experimental soil

Characteristics	Value	Unit
Soil texture	Clay loam	---
pH	7.70	---
EC	0.03	dS m-1
Organic matter	2.75	%
Sand	37.5	%
Silt	35	%
Clay	27.5	%
Carbonate	Absent	----
Nitrogen (N)	0.014	mg/kg
Potassium (K)	4.1	mg/kg
Phosphrous (P)	110	mg/kg
Zn (Zn)	18	mg/kg

Note: Soil analysis of experimental sample suggested that soil is fit for cultivation of Wheat crop.

Table 2. Primers for molecular identification

Primer name	Primer sequence (5'-3')	rRNA operon binding site
ITS1F (F)	CTTGGTCATTTAGAGGAAGTAA	Small subunit
ITS4 (R)	TCCTCCGCTTATTGATATGC	Large subunit

ITS1F= forward primer, ITS4=reverse primer, F=forward, R= reverse and rRNA=Ribosomal Ribonucleic acid

Thousand Grain Weight (TGW): To assess the impact of loose smut disease on crop yield thousand grain weight (TGW) g of wheat grains were recorded, buy weighing the grains manually collected from infected plots.

RESULTS

Symptoms of loose smut were recorded at ear emergence as individual grains were completely replaced by a mass of blackish fungal spores (Figure 2; Figure 3 A and B). Ears of affected crop were also seen partly and fully affected. In the case of affected crops spores were seen released with emerges of disease symptoms which left the bare remains of the ear rachis. After the attack of disease only blackish mass were seen left which clearly indicated the severity of disease. While experimental teliospores were seen single celled, olivaceous brown to dark blackish brown, spherical to ovoid shaped, thick walled with slight reticulate ornamentation (Figure 4), which clearly indicates that these teliospores belongs to *tritici*. All the isolated strains were found similar after primary and secondary screening therefore one strain RS-01 was selected for further studies.

Molecular identification: Isolated samples were further supported by the molecular analysis which suggested that it had a clear resemblance with the *U. tritici*. However, the submitted sequences (Table 3) were assigned the accession number OP164708 by NCBI, and data collected after blast was used to construct a dendrogram for phylogenetic analysis (Figure 5). Presented phylogenetic tree were based on 739-bp ITS 18S ribosomal RNA analysis of the smut order Ustilaginales.

Figure 2. An infected Wheat crop with loose smut disease caused by *U. tritici* Mass of spores replacing spike

Figure 3. A and B Infected spikes of wheat (Galaxy-2013) due to the loose smut of wheat at maturity

A B

Figure 4. A and B Microscopic image of an isolated strain (RS-01) from an infected crop of *T. aestivum* (L.) showed that isolated spores of *U. tritici* were at telial stage

Table 3Sequences of 739-bp ITS analysis of the smut order Ustilaginales.

1 ttccgtaggg tgaaccctgc ggatgggatc atttcgatga aaaacctttt ttcagaggtg
61 tggctcgcac ctgtccaact aaacttgagc tacctttttt caacacggtt gcatcggtcg
121 gcctgtcaaa cagtgcgacg caaggagaaa atcctcgcgt ctgctgggcg acggacggac
181 aattttattt aacacttttg gatgatctag gatttgaagg agaaaaagtc atttttacga
241 atgaaatcgactggtaatgcggtcgtctaattttaaaaacaacttttggcaacggatctc
301 ttggttctcccatcgatgaagaacgcagcgaattgcgataagtaatgtgaattgcagaag
361 tgaatcatcg aatctttgaa cgcaccttgc gctcccggca gatctaatct ggggagcatg
421 cctgtttgag ggccgcgaat tgtttcgaac gacagctttt ttcttttgga aaaggttgac
481 ggatcggtattgagggtttttgccatttaccgtggctccctcgaaatagattagcgcatc
541 cattttataggcaagacggacgaaagctcgatttttgctctcttccctgccgggtttt
601 gatactatcaggacttcggagaggttgagatgggtaggagctggacgcaacggcttgctg
661 tttggagtgcttctgaaacccgcccatgccgagtttttaggaagctaggaagggattta
721 taataatcatcgcctcaga

Key: Base count 184 a 153 c 190 g 212 t

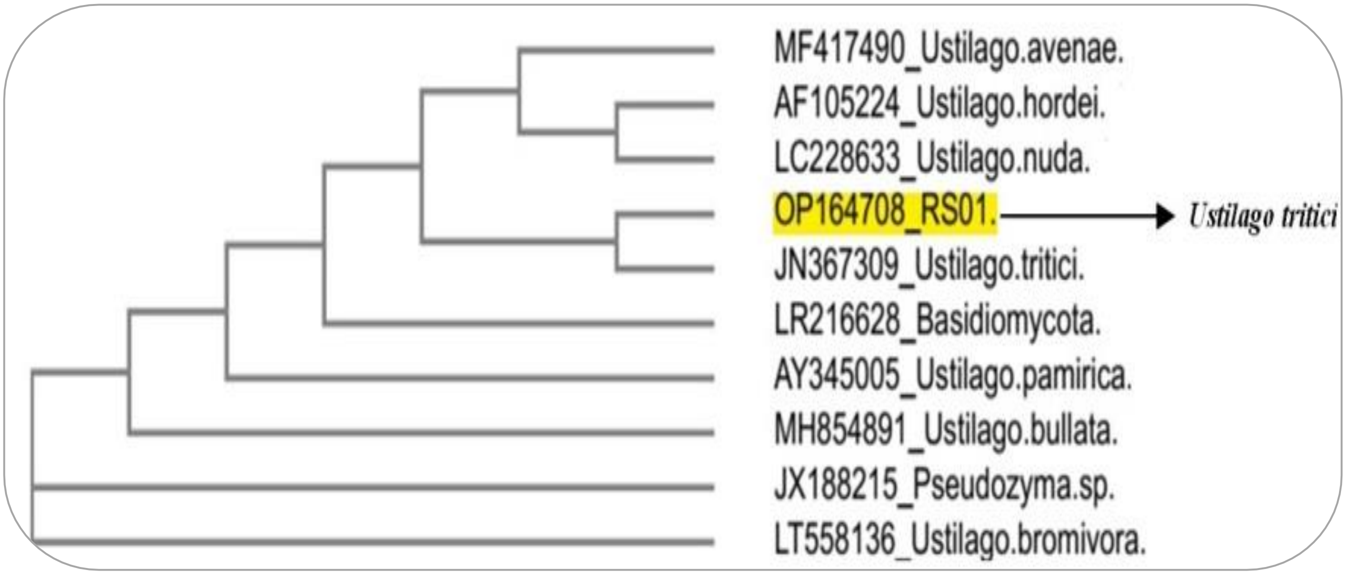


Figure 5. Phylogenetic tree of *U. tritici* on Minimum Evolution analysis, accession number of experimental Sample (RS-01) is highlighted, (OP164708_RS01). The obtained accession number for the isolated sample was used for the present course of study.

Thousand Grain Weight: Outcomes of study showed the lowest thousand grain weight 30g, which had greater impact on yield.

DISCUSSION

U. tritici belongs to the basidiomycetes, a well-known pest of wheat, usually appears on crops at flowering. This is also marked as air-born disease as the teliospores of the disease travel through air from one of the infected flowers and settled down at the shoulder of the ovary of the other. After the germination of spores dikaryotic infectious hyphae enter in the germinating seed. Therefore, fungal pathogens start growing through the seed coat into the scutellum and embryo at the base of the seed (Badr *et al*. 2009; Sammour *et al*. 2015). Once mycelia grow in the developing seed in start penetrating and reached till the top position in crop, initially these pathogens travel slowly and eventually develops into a smutted ear (Abrahim, 2019). It had been reported by experts that a single basidiospore is thin walled, uninucleate and oval to round, similar results were recorded by present study. It produces a fine tube before germination, which is usually called as infection tube or infection thread either on the soil or on the young host plant. This germ tube is usually haploid or monokaryotic, which is the indication of presence of fungal pathogens of *Ustilago*. It becomes dikaryotic after the fusion with the other partner, which develops into dikaryotic mycelium (Patil,2021).

The disease appears at heading when dark brown to black, "smutted," seed heads appear among newly emerged, healthy, green heads. The seeds in these diseased heads are converted into a mass of dry, dark brown to black spores (teliospores) (Gangwar *et al*. 2018). The disease is called loose smut because the spores produced on smutted heads are easily removed by wind and rain. Typically, after a short while, only the bare, sooty, flower stocks remain. In some cases, the seed head is only partially infected. Prior to heading, diseased plants may have dark green leaves with chlorotic streaks, but this symptom generally goes unnoticed. Seed with no visible symptoms can be infected with the fungus (Woldemichael, 2019).

The most important point about these pathogens is that these can be detected easily through microscopy, as the infected seed and developing plants did not showed any obvious or unambiguous macroscopic symptoms before it reached the attack. There are numerous other diseases that had been an important pathogen of crops such as rusts, smuts, mildews, and some others, which had deleterious impact on crops (Draz *et al*. 2015). Yield and crop losses due to loose smut of wheat in not new in the agricultural sector, it was first time recorded in 1888 due to the seed exchange between countries (Banziger *et al*. 2022). Disease spread with the passage of time and was recorded in 1949 again. With the passage of time due to the efforts of experts and technology disease records are seen diminished in some countries. But it was again appeared in the duration of

1984/1985, keeping in view the loses due to this disease an annual survey was performed 1985-1989, in about 16 Governorates, which reported that disease incidence was about 0.1% on the commercial varieties (Al-Tamimi *et al*. 2015). The loose smut of wheat is of great importance, due to having a great potential to cause heavy yield loss to various crops of economic importance.

Morphologically an infected seed of loose smut looks like healthy seeds and that also adds to non- adoption of seed treatment. Therefore, for better production and protection of wheat cultivation of resistant wheat varieties are the best solution, even to overcome the overuse of fungicides.

CONCLUSION

Study concluded that there are some susceptible wheat verities that can show the symptoms of loose smut of wheat at heading. The loose smut of wheat is caused by *Ustilago tritici* which are the pathogenic enemies of wheat. It had a great impact on yield as grains were replaced by blackish mass after the attack. Due to this disease wheat crop in Pakistan is under threat. Therefore, cultivation of disease-free seeds, along with effective treatments are required for better production. While cultivation of wheat, better production along with protection strategies should be focused.

ACKNOWLEDGEMENT

All the authors are highly acknowledged to the support of Prof. Dr. Muhammad Gulfraz for his continuous support.

REFERENCES

Ahmed, A., R. Badar, A. Arshad, M. Munazir, S. Younas and R. Mehmood. 2022. Microscopic identification of *Puccinia striiformis* RR-01 isolated from an infected wheat variety. Pakistan Journal of Phytopathology, 34(2):341-347.

Al-Tamimi, A. J. T., N. A. Al–Badeiry and S. Q. Khayoon. 2015. Genetic characterization of 22 of Tomato (Lycopersicon esculentum L.) genotypes using SDS-PAGE method. International Journal of Scientific and Engineering Research, 6(1):897-901.

Al-Yassiry, Z.A. and B. Al-Alwani. 2022. Estimation of Botanical Diversity by Molecular Marker Methods. Biomedicine and Chemical Sciences, 1: 132-137.

Arif, A. 2019. Loose Smut of Wheat (Ustilago tritici) and Its Managements: A Review Article. journal of Biology, Agriculture and Healthcare, 9(8): 25-33.

Arshad, S. and A. Shafqat. 2012. Food security indicators, distribution, and techniques for agriculture sustainability in Pakistan. International Journal of Applied Science and Technology, 2(5):137-147.

Badar, R., A. Ahmed, M. Munazir, N. Khalique, H. Waheed, S. Munawar and H. Basheer. 2022. Aphicidal effects of organic formulations against tomato aphid *Myzuspersicae* (sulzer) to overcome crop damage/diseases. Pakistan Journal of Phytopathology, 34(1): 161-171.

Badar, R., A. Ahmed, S. Firdous, M. Munazir and F. Gulzar. 2020. Characterization of biopesticides as potential antifungal and antiaphid agents. British Journal of Pharmacology, 177(11):2515-2515.

Badr, S., A. E. Z. Mustafa, W. Taher and R. H. Sammour. 2009. Genetic variability in Lathyrus spp. as revealed by karyotype analysis. Cytologia, 74(1):101-111.

Bänziger, I., A. Kägi, S. Vogelgsang, S. Klaus, T. Hebeisen, A. Büttner-Mainik and K. E. Sullam. 2022. Comparison of thermal seed treatments to control snow mold in wheat and loose smut of barley. Frontiers in Agronomy, 3:775243.

Draz, I. S., M. S. Abou-Elseoud, A. E. M. Kamara, O. A. E. Alaa-Eldein and A. F. El-Bebany. 2015. Screening of wheat genotypes for leaf rust resistance along with grain yield. Annals of Agricultural Sciences, 60(1): 29-39.

Eibel, P., G. A. Wolf and E. Koch. 2005. Development and evaluation of an enzyme-linked immunosorbent assay (ELISA) for the detection of loose smut of barley (*Ustilago nuda*). European Journal of Plant Pathology, 111:113-124.

Esse, H. P. V., T. L. Reuber and D. V. D. Does. 2020. Genetic modification to improve disease resistance in crops. New Phytologist, 225(1):70-86.

Gad, M. A., A. E. Ghanam and D. E. E. Hefny. 2019. Management of loose smut disease (*Ustilago tritici*) and determination of fungicides residues in wheat matrices using QuEChERS methodology. Menoufia Journal of Plant Protection, 4(3):107-118.

Gangwar, O. P., S. C. Bhardwaj, G. P. Singh, P. Prasad and S. Kumar. 2018. Barley disease and their management: An Indian perspective. Wheat and Barley Research, 10(3):138-150.

Gupta M. K., S. Narwal, V. Umapathy, R. Mall and A. Kumar. 2009. Characterization of potential antigen (s) of *Tilletia indica* teliospore walls to develop a specific immunoassay for Karnal bunt detection. Food and

Agricultural Immunology, 20(2):79-94.

Malik, M. M. S. and C. C. V. Batts. 1960. The infection of barley by loose smut (*Ustilago nuda* (Jens.) Rostr.). Transactions of the British Mycological Society, 43, 117-125.

Mohdly, B. R., A. E. B. TE, A. El-Ati and H. G. Gomaa. 2023. Identification and control of pathogenic races of *Ustilago nuda* loose smut causal pathogen of barley. Zagazig Journal of Agricultural Research, 50(1): 67-80.

Nasr, I. A. 1977. Standardization of inoculation techniques for sugarcane smut disease. Sugarcane Pathologist Newsletter.

Patil, Y. P. 2021. A textbook of botany: Diversity of Microbes. Chapter No. 1. Darshan Publishers, Tamil Nadu, India

Quijano, C. D., F. Wichmann, T. Schlaich, A. Fammartino, J. Huckauf, K. Schmidt and C. Sautter. 2016. KP4 to control Ustilago tritici in wheat: enhanced greenhouse resistance to lose smut and changes in transcript abundance of pathogen related genes in infected KP4 plants. Biotechnology Reports, 11:90-98.

Rajput, M.A., I. A. Khan, R. N. Syed and A. M. Lodhi. 2019. Optimization of inoculation technique of Sporisorium scitamineum for the induction of smut disease in sugarcane propagative material. Pakistan Journal of Agricultural Research, 32: 275-281.

Roland, L., M. Drillich, D. Klein-Jöbstl and M. Iwersen. 2016. Invited review: Influence of climatic conditions on the development, performance, and health of calves. Journal of dairy science, 99(4):2438-2452.

Sammour, R. H., M. M. El Shamy, A. E. Z. M. Mustafa and E. S. Yousef. 2015. Inheritance of resistance to loose smut *Ustilago tritici* of wheat. Research and Reviews in Biosciences, 10: 137-146.

Wilcoxson, R. D. and E. E. Saari. 1996. Bunt and Smut Diseases of Wheat: Concepts and Methods of Disease Management (eds). CIMMYT, Mexico, pp. 1-11.

Woldemichael, M. D. 2019. Importance, biology, epidemiology, and management of Loose Smut (Ustilago nuda) of barley (Hordeum vulgare): A Review. East African Journal of Sciences, 13(1): 89-108.

Zandalinas, S. I., D. Balfagón, A. Gómez-Cadenas and R. Mittler. 2022. Responses of plants to climate change: Metabolic changes during abiotic stress combination in plants. Journal of Experimental Botany, 73(11):3339-3354.

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Asma Ahmed	:	Supervised the whole experiment
Nazia Kanwal	:	English editing service and reviewed the final version
Arisha Akram	:	Technical assistance and helped in lab work
Dilawar Abbas	:	Helped in sample collection
Nagina Janat	:	Reviewed the whole manuscript
Mehmooda Munazir	:	Reviewed the article
Tuba Riaz	:	Helped for reference setting and formatting