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MICROSCOPIC IDENTIFICATION OF *PUCCINIA STRIIFORMIS* RR-01 ISOLATED FROM AN INFECTED WHEAT VARIETY

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ABSTRACT

Wheat (*Triticum aestivum*) is an important food crop grown all over the world, its production is decreasing day after day due to fungal attacks. Present experimental investigation was designed with an aim to synthesize organic fungicide to control these pathogens in future. Therefore, focus of present manuscript was to isolate the fungal strains of *Puccinia striiformis tritici* causes rust disease of wheat. Morocco as a check was grown in field conditions favorable for fungal growth on 24 November (2020). Experimental plants were inoculated with fungal strains *P. striiformis* RR01 at stages 55 to 65 according to Zadok scale. Fungal strains (RR-01) were isolated from the experimental crop after the appearance of symptoms of leaf rust disease and were processed further for identification. Identification was done microscopically confirming that the isolated strains belong to *Puccinia*. Further molecular analysis of RR-01 also confirmed that isolates have close resemblance with the *Puccinia striiformis.* Identified sequences of the strains were submitted to NCBI which were assigned the accession number for more confirmation dendrogram were also prepared. After complete identification, infected dried leaves were kept in envelopes as rust is biotrophic fungi, while fungal spores were also stored as glycerol stocks.

Keywords: *Puccinia striiformis, Pst,* wheat, RR-01

INTRODUCTION

Plants are the most important source of nutrients while wheat is among most important food crops which, covers almost 93% of man's food globally. Wheat, maize and rice are the major food crops. Wheat (*Triticum aestivum*) is cultivated in almost 28 countries, due to which it is known as the largest cultivated crop around the world (Afzal *et al*., 2008). For sustainable food production, it is crucial to maintain a link between the quality and production of food, as about 80% of the world's population depends upon it (Ahmed *et al.,* 2012). As one of the most important food source, the farmers and experts cultivate wheat equally. With the increase of human population growth, the demand for this crop is extensively increasing daily (Shewry and Hey, 2015). Current production of this crop is facing decline in the yield due to which we are only left with the option of import which could have serious impact on economy of a country. Around 50 different biotic and abiotic stresses are the limiting factors towards production of *T*. *aestivum* (Anonymous, 2000). Fungal pathogens are the most important biotic factors which are becoming the cause of loss in yield. Wheat rusts, smuts, blights and leaf spots are the most common pathogens (Iftikhar *et al.,* 2010). Out of other all fungal pathogens rust diseases can cause serious yield losses that ravages this crop since ancient times. There are three most common types of wheat rusts namely stripe or yellow rust (*Puccinia striiformis tritici*), leaf or brown rust (*Puccinia triticina tritici*) and stem

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black rust (*Puccinia graminis tritici*). All of these three can cause heavy losses in case of epidemic outbreak but stem and stripe rusts are among most harmful, causing losses ranging from 60% to 100% (Aboukhaddour *et al*., 2020).

It is reported that 100% of yield losses are attributed to stripe rust which is spread due to attack of *P. tritici* (*Pst*) specially in case of vulnerable wheat varieties when provided with suitable conditions (Chen *et al*., 2014). Around 88% of the wheat growing area is subjected to this threatening disease which is reported in more than 60 different countries of the world (Schwessinger, 2017). Attack of stripe rust on wheat crop spread rapidly as this is foliar disease its uredinia spores are carried by wind from one region to the other (Zeybek and Yigit 2004) such as from America to Australia (Wellings, 2007; Wellings *et al*., 2009). The differences in the yield losses and the severity of disease are due to the environmental features and the plant growth stage. This disease is causing yield losses globally. Many developed countries are also under attack of this devastating disease such as United States of America, Australia, India, Pakistan, Ethiopia, Kenya and the UK (Chen, 2020).

It had been noted that these pathogens start sporulation within 12 to 14 days of the inoculation in case of favourable conditions such as moisture, temperature and host resistance (Chen *et al*., 2014). After that stripe rsut starts showing its symptoms which woud be clearly observed as orange, narrow stripes which appear usually between veination of leaf. It starts producing telia as host approaches senescence (Chen *et al*., 2014). It can infect both the awns and glumes in severe cases but in case of resistant wheat variety *Pst* appears as a minute small flecks with chlorosis, necrosis with the negligble spore count or some times there is no visual symptoms. At seedling stage *Pst* covers all leaf area showing visible spots (Chen *et al*., 2014). Its alternate host is *Berberis* spp. yellow to orange flask-shaped pycnia appear on leaves which are turned in to oblong-shaped pycniospores afterwards. The objective of current study was to isolate, identify and preserve Puccinia striiformis (*Pst*) RR-01 from an infected wheat variety namely Morocco.

MATERIALS AND METHODS

Study design: Present experimental study was conducted at PCSIR, Lahore, Pakistan. The land was prepared according to the requirements set by national standards for wheat cultivation (Figure 01). Wheat (Morrocco) was cultivated during growing season 2020, provided all the required optimum conditions for wheat crop (Figure 01).

A

B

Figure 1. A presents land preparation before cultivation of Morrocco while (B) presents the crop before inoculation with pathogenic fungi.

Wheat cultivar: For the isolation of strains of wheat rust, fungus susceptible wheat variety (Morocco) was grown as this wheat variety is susceptible to this fungal pathogen (Joshi *et al*., 2017).

Purification and preservation of Teliospores: Affected leaves of wheat crop showing clear symptoms of rust fungus were collected from the experimental plot. These samples were then left in their envelopes overnight to reduce the moisture. These samples were further kept in desiccators having calcium chloride at 10-12 ℃ in refrigerator till further usage (Stubbs, 1988). Collected samples were rinsed in 2% solution of Calcium Chloride and distilled sterile water thereafter. Sterile samples were further transferred to sterile plates having moist filter papers under aseptic conditions. These dishes were kept at 6-10 ℃ for about 24-48h to stimulate the germination of uredospore. Stripes were picked individually to the first leaf of 7-10 days seedlings of susceptible Morocco (ARM) through aseptic swabs, which were sown in 10cm diameter pot. To avail the complete growth of fungus these pots were incubated at 10℃ in darkness and 100% relative humidity for 24 h. These pots were kept under recommended conditions till 20-25 days. Collection and purification of rust strains was carried out by following the methods described by Stubbs 1988 with slight modifications (Elsawy *et al*., 2015). Isolates samples (RR-01) were again collected from affected wheat crop after the appearance of identical symptoms of stripe rust, which was preserved again for further use.

Morphological and anatomical identification of teliospores: Stripe rust damages wheat leaves, sheaths, awns and glumes, so in order to go for complete identification first morphologcal identification was done. Chlorotic flecks on primary host leaves were noted which appeared 6–8 days after inoculation (Chen *et al*., 2014). Length and diameter of these flecks were measured. These uredospores were visually analyzed and were confirmed on the basis of color and size. Photographs of leaves of wheat crop infected with stripe rust were taken using digiporo-Labomed (PX 5) stereomicroscope. Furthermore mounts of the infected parts of crop were seen through Labomed CSM2 stereomicroscope, after making free-hand sections. Then compound microscope (MX4300H, Meiji Techo Co., Ltd., Japan) was used to visualize spores of each stage.

Molecular identification: Collected uredospore's were processed further for molecular identification at Pablo Alvarado Garcia Spain and couple ITS-specific primers (ITS4 reverse and ITS1 forward) (Table 01) were used for the amplification of ITS. Sequence similarity analysis was carried out through NCBI with already identified strain of rust species. The submitted sequences were assigned voucher No. SUB11905421. Moreover, dendrogram was prepared through phylogenetic analysis for further confirmation using Mega cluster (Al-Yassiry and Al-Alwani, 2022).

Table 1.Primers for molecular identification

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

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

Preservation of fungal strains: After complete identification, infected dried leaves were kept in envelopes as rust is biotrophic fungi, while fungal spores were also stored as glycerol stocks.

RESULTS

Symptoms: Symptoms of stripe rust appeared after 6 - 8 days of inoculation with sample (RR-01). It was observed that the uredospores which were yellow to orange in color. Both types of rust (Stripe and Leaf) could be differentiated on the basis of colour and shape of pustules. Clear yellow-orange pustules (smaller in size and circular in shape on upper surfaces of leaves) indicated the presence of *P. striformis* (Figure 1).

Microscopic species identification: The rust species identified was *Puccinia striiformis*. The uredinial stage of infection was observed, while spermagonia and aecial stages were not recorded. Uredinia were epiphyllous, dark orange brown or rusty colored, looking like granules around 1 to 3 mm in diameter. Urediniospores were subglobose or globose to slightly fusiform, yellowish-orange and thin walled (Figure 1).

Molecular identification: Molecular study of the experimental Sample (RR-01) indicated clealy that these strains belong to *Puccinia striiformis*. Acession number OP164710 was assigned to submitted voucher number SUB11905421. Dendrogram was prepared through Mega cluster using available data of NCBI for further confirmation (Figure 2).

Figure 1. A presenting Wheat crop infected with *P. striformis* (RR-01) B presenting the severity of leaf rust.

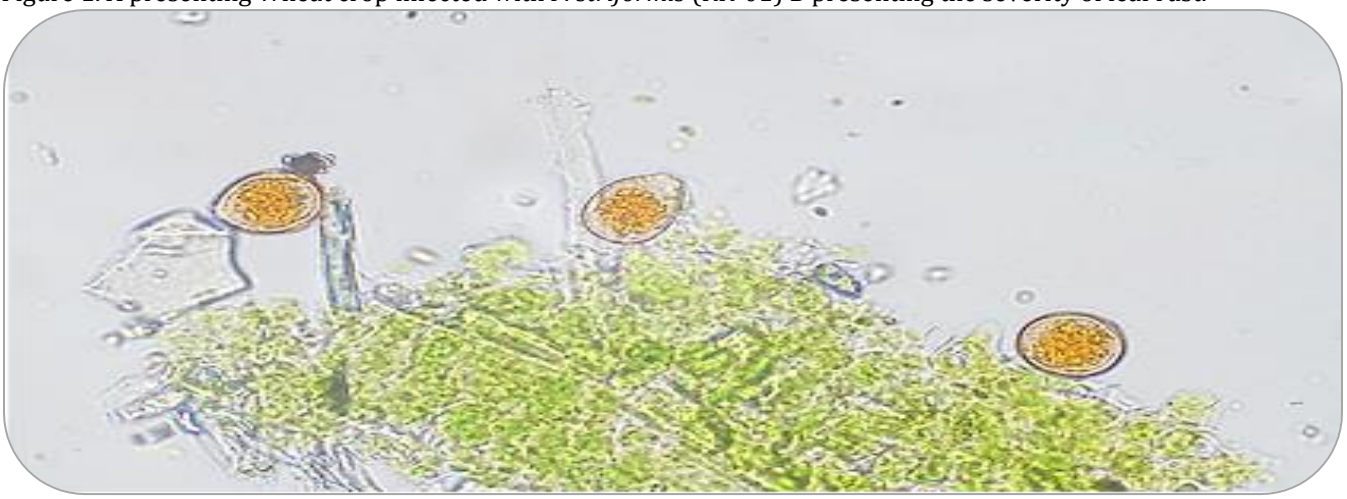

Figure 2. Illustration of microscopic image of Sample (RR-01)

DISCUSSION

Wheat is among the most important crops of the world. Stripe rust is the common disease of this crop which is caused by the pathogenic biotrophic fungi. The disease-causing-pathogens are distributed all over the world, and spread from one country to another (Ali *et al*., 2014; 2017 Poudyal *et al*., 2020). The present study was designed to isolate the pathogenic fungal strains to preserve for future research. Moreover the purpose of present study was to optimize the parameter for the isolation, identification and preservation of *pst* and to set a standarized protocol for the anti-fungal studies against these strain in future. Due to the biotrophic nature of such fungal pathogens, these are difficult to grow on artificial media therefore were stored on the infected plant parts.

Pathogenic *pst* were isolated from Morocco which were grown as a check, this is susceptible variety of wheat against stripe rust. It had been reported by Kiani *et al*., 2021 that Morocco is vulnarable to stripe rust. Presence of wheat rust is pre-confirmmed on the

appearance urediniospores in yellow to orange color which were clearlly seen on narrow stripes of leaves. Similar findings were also reported by Shabana *et al*. (2017). Isolated strains of fungus (RR-01) were further identified by microscopic examinition which suggested that these spores belong to the member of fungal genus *Puccinia.* The diameter of collected uredinia was around 1 to 3 mm.

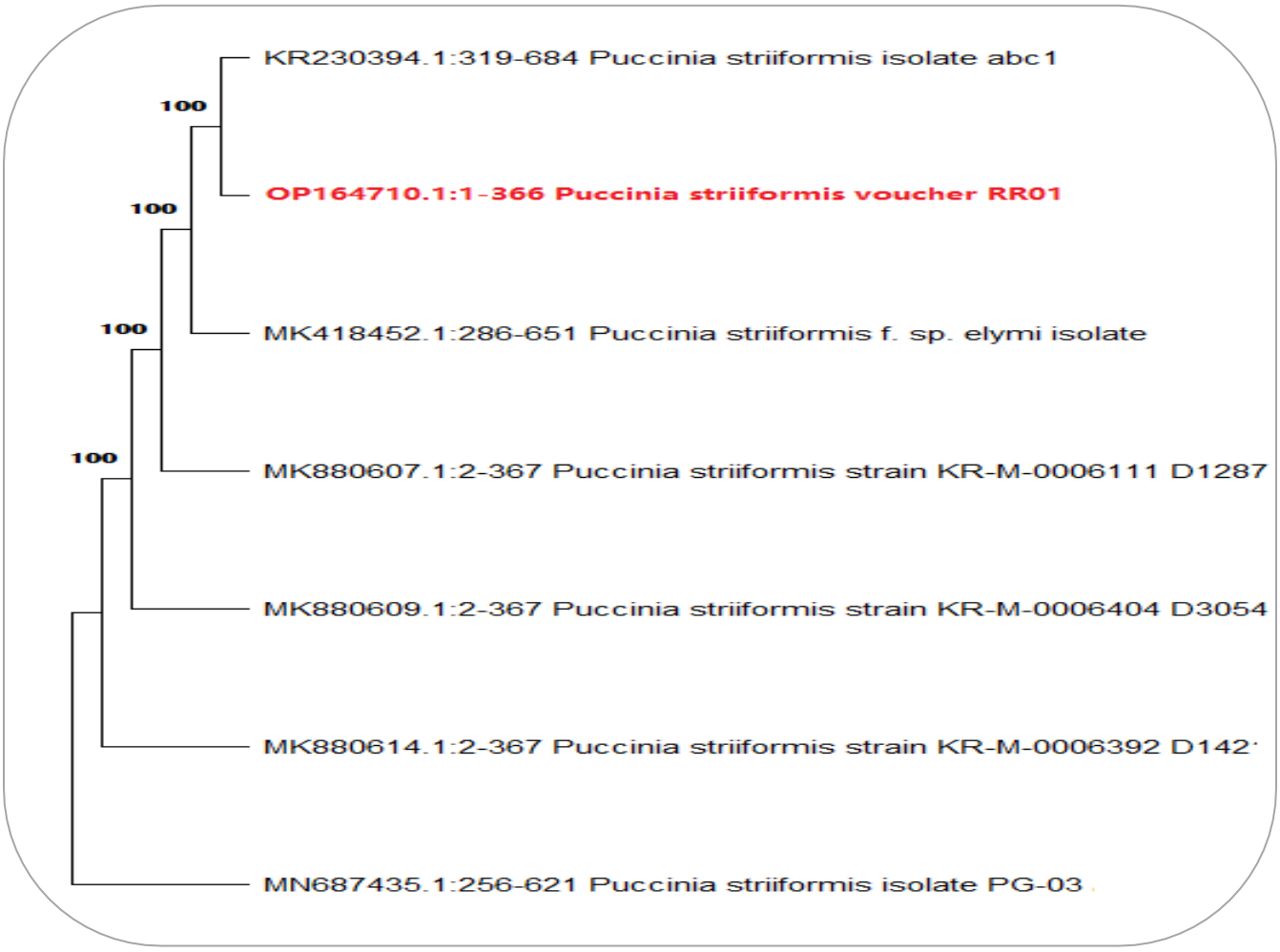

Figure 3. Illustration of phylogenetic analysis of *P. striformis* with accession number of experimental Sample (RR-01) highlighted, (OP164710_RR01), this dendogram was prepared by Mega Cluster.

Stripe rust of wheat is devastating disease of wheat which is distributed all around the world (Wellings, 2011). This disease spreads more under favorable environmental conditions. It has been reported by many experts that environmental factors are key components for the spread and development of stripe rust infection (Orabey *et al*., 2019). This disease is more dangerous in case of susceptible wheat variety if grown in field conditions (Chen, 2020). Rate of disease severity depends upon time and duration of disease attack (Chen, 2017). These urediniospores were seen flurishing in moist environment, which is also reported by another study (Chen *et al*., 2014).

Phlogenetic tree was constructed after obtaining voicher number from NCBI and dendogram was also prepared to find out the similarity of experimental species of rust with already identified species of wheat stripe rust. Kinship analysis of the fungal stain was carried out for the confirmation of experimental species (RR-01). Similar findings are also reported by experts. They also used neighbor-joining phylogenetic tree which is based on ITS sequence of nucleotides that are supposed to show phylogenetic relations among pathotypes of *P. striiformis tritici*, *P. graminis tritici*, and *P. triticina* (Aggarwal *et al*., 2018).

CONCLUSION

It had been concluded from the present experimental analysis that Morroco is the most susceptible wheat

variety and had a great potential to get infected by stripe rust. Crop showed 100% symptoms after artificial inoculation with pathogenic strains *P. striformis* (RR-01). Both Morphological and molecular identification of isolated strains made it clear that these strains have close relationship with *Puccinia*. Identified sequences were submitted to NCBI which showed close similarity with the *Puccinia striiformis* and obtained accession number was assigned OP164710_RR01.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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