Histopathological analysis of incompatible and compatible interaction of *Puccinia striiformis* f.sp. *tritici* on wheat at early infection stage

Deepika Kulshreshtha*, Rashmi Aggarwal† and Narendra Kumar‡

Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India.

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**ABSTRACT**

Stripe rust of wheat (*Puccinia striiformis* f.sp. *tritici*; Pst) incurs substantial yield losses in wheat worldwide. Two Indian Pst pathotypes 38S102 (I) and 46S119 (*Yr*9 virulence) showed incompatible and compatible interaction respectively in variety PBW 343. The histopathological study using confocal microscopy revealed that during incompatible interaction comparatively longer abortive germ tubes are produced with a rapid response at early stages of infection but with no fungal colonization. Whereas, in compatible interaction, germ tubes were shorter with infection peg formed above the stomata subsequently leading to colonization. The detailed microscopic observations showed that necrotic regions were more prominent in incompatible interaction than compatible interaction. This information would aid in understanding the growth and virulence mechanism of stripe rust pathogen in wheat.

**Key words:** Confocal microscopy, Compatible interaction, Incompatible interaction, *Puccinia striiformis*, Stripe rust.

**INTRODUCTION**

*Puccinia striiformis* f. sp. *tritici* (Pst) is one of the major biotic constraints of wheat causing stripe rust or yellow rust that affects wheat (*Triticum aestivum*) production by causing several epidemics in many parts of world (Chen, 2005). Globally, 10-70% yield losses have been reported due to yellow rust and in some cases nearly 100% yield loss occurs, if the infection continues from a very early stage to the later stage of the plants (Chen, 2005). Stripe rust appeared in severe form in plain areas in J & K, foot hills of Punjab and Himachal Pradesh, parts of Haryana and tarai regions of Uttarakhand during 2010–11 (Sharma and Saharan, 2011). Several wheat varieties have also been checked for the yielding ability and disease parameter (Singh and Tewari, 2006; Umeozor and Pessu, 2006). The use of chemicals is prefered approach (Line, 2002; Chen, 2007) for disease management in USA but chemicals are costly and hazardous to environment (Rosewarne et al. 2013). Above all, the major constraint of this approach is chemical resistance of the pathogens. The most promising approach is introgression of resistance genes in high yielding varieties of wheat to develop resistant cultivars (Roelfs et al. 1992). However, in recent years the emergence of new resistance breaking Pst pathotypes has hampered these approaches. For example, large regional epidemics and crop losses have been reported because of Pst pathotypes with virulence for *Yr*2, *Yr*9 and *Yr*27 during 1970s, 1990s and in 2010 respectively (Wellings, 2011). Thus, the combined knowledge of plant and pathogen genotype and traditional Pst virulence profiling will accelerate the identification of virulence-causing mutations (Hubbard et al. 2015) and aid in identifying novel strategies to manage this disease. This will better explain how new Pst races emerge and encourage the development of improved epidemiological models and durable resistance strategies.

A wheat variety that has been resistant in the past may not remain resistant to new races of rusts during the course of pathotype evolution (Brian, 2006). With stripe rust-resistance gene *Yr*27, PBW 343 (*Yr*9, *Yr*27) had withstood the breakdown of *Yr*9 resistance (McDonald et al. 2004; McIntosh et al. 2003). The spread and increasing adaptation of *Yr*27 virulence to local conditions became evident across the Himalayan foothills in 2007 (Prashar et al. 2007). Therefore, when it was deployed in 1995, the variety PBW 343 was shown to confer resistance to a virulent pathotype 46S119 (virulence on *Yr*9) of yellow rust, which came into India *via* Iran, Afghanistan and Pakistan during the early nineties (Nayar, 1996) but when temperature was low during infection and post infection, the resistance of PBW 343 succumbed to 46S119 making it virulent to this genotype at seedling stage due to the breakdown of *Yr*9 gene. Wheat rust surveys in different wheat growing zones of India during 2011-15 led to identification of pathotype 46S119 in 65.16% samples (Gangwar et al. 2016). Occurrence of stripe rust in high severity in India during last two decades was due to 46S119 and 78S84 that overcame widely used *Yr*9 resistance in wheat (Prashar et al. 2007). While pathotype 38S102 is

*Corresponding author’s e-mail: deepikakul12@gmail.com

†Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India.

‡Amity Institute of Biotechnology, Amity University Haryana, Manesar, Gurgaon-122 413, Haryana, India.
avirulent on Yr9 and it is restricted to the Southern Hill Zone (Nilgiri and Pulney Hills) of India.

Applying propagules to leaf tissue and observing germings and plant response at infection site is a common practice to study the interaction of plants and pathogenic fungi. Commonly large differences in response are observed among infection sites in such histological studies. Differences observed in spore germination and germ tube penetration of *Puccinia striiformis tritici* in susceptible and resistant wheat varieties suggested inhibiting spore germination and retarding germ tube penetration in resistant variety (Stubbs and Plotnikova, 1972). Further studies on Pst development in resistant and susceptible hosts included confocal microscopy to give more detailed insights (Moldenhauer, 2006). These studies referred to how different genotypes of the wheat affect growth of Pst pathotype but no study has been performed to explain the differences in germination and development behaviour of virulent and avirulent Pst pathotypes on the same wheat genotype. Pathotype 46S119 is one of the recently evolved pathotypes and has shown virulence to Yr9 whereas, pathotype 38S102 was avirulent to Yr9 (Prashar *et al.* 2007); therefore, the objective of this study was to analyse the growth responses of these Pst pathotypes having different nature of pathogenicity towards wheat genotype using confocal microscopy.

**MATERIALS AND METHODS**

**Wheat cultivars and fungi:** The wheat cultivar taken for the study was PBW 343 as it contains Yr9 gene for which Pst pathotype 46S119 is virulent and 38S102 is avirulent. *P. striiformis* f. sp. *tritici* pathotype 46S119 prevalent during 2011-15 and pathotype 38S102, restricted to the Southern Hill Zone were taken to understand colonizing behavior. These pathotypes were collected from IIWBR, Flowerdale, Shimla in February 2017 and earlier studies have shown their virulence/avirulence behavior (Nagarajan *et al.* 1983; Bhardwaj *et al.* 2012).

**Fungal inoculation and disease development:** A total of 8-10 seeds per pot of wheat variety were sown (in year 2017) in a total of 20 pots with 4" diameter and filled with sterilized soil. Seedlings were raised in pots placed in trays and covered with spore proof chambers at 15±2°C. A total of 6-7 (10-11 days old) seedlings per pot were selected for inoculation when the primary leaf was completely open. Inoculation was carried out in 10 pots for each pathotype under temperature controlled glasshouse at 10-14°C in National Phytotron Facility, IARI, New Delhi, India. Two suspensions were prepared by suspending the urediniospore from each pathotype (2 mg/ml; 1×10^4 to 1×10^6 spores/ml) in sterile distilled water with Tween-20 (0.2%, v/v) and shaken vigorously for uniform mixing for inoculation. Prior to surface inoculation, the seedlings of PBW 343 were sprayed with fine mist of distilled water using hand sprayer followed by smooth rubbing of leaves by fingers to disturb the wax layer and to open the stomata of leaves and then the urediniospore-water-tween20 suspension was sprayed over 10 pots for each pathotype. After inoculation, pots were then transferred to a moisture chamber at 15±2°C. Between 0-96 hours post-inoculation (hpi) (viz., 8, 16, 20, 24, 36, 48, 72, 96 hpi and 14 dpi), 5-7 leaves (one from each pot) were harvested at each time point and stored at -20°C for further analyses. A total of 5-7 leaves (one from each pot) from symptomatic stage (14dpi) were also harvested for microscopic observations. Five leaves from each pathotype inoculation were taken for germ tube length measurement at 20 hpi.

**Sampling and staining:** Inoculated leaves were cut into small bits and fixed in ethanol: chloroform (3: 1, v/v) containing 0-15% (w/v) trichloroacetic acid for ~18 h (Moldenhauer, 2006). The leaf samples were kept in 0.5M sodium hydroxide at 90°C for 30 min, subsequently rinsed with sterilized water and dipped in 0.1M Tris–HCl buffer (pH 5.8) for 30 min. The leaves were stained in 0-1% (w/v) Uvitex 2B (Polysciences Inc., Warrington, PA) followed by washing with sterilized water (four times; 10 min. each) and finally in 25% (v/v) glycerol (Niks, 1990).

**Microscopy and imaging:** Specimens were prepared for mounting by placing them over a slide with a drop of water and observed immediately. Mounted segments were analysed for the structural morphology with a confocal laser scanning microscope Leica TCS SP5 (Leica Microsystems) equipped with epifluorescence optics using 10x (air) and 40x (air) magnification. The leaves were excited with UV-laser beams at 351 and 364 nm and scanned with filter settings at 400–500 nm for Uvitex stained fungal infection structures and autofluorescing necrotic tissue. A total of 5 leaves per pathotype were analysed for germ tube length measurement. Statistical significance of germ tube length was determined using Student’s t-test at 95% confidence interval in R. Differences were regarded as significant if p < 0.05.

**RESULTS AND DISCUSSION**

Wheat genotype PBW343 expressed resistance (incompatible interaction) against stripe rust pathotype 38S102 causing no symptoms (Fig 1a), whereas pathotype 46S119 showed symptoms (Fig 1b). Wheat genotype PBW343 expressed resistance (incompatible interaction) against stripe rust pathotype 38S102 causing no symptoms (Fig 1a), whereas pathotype 46S119 showed symptoms (Fig 1b).
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Fig 2: Differences of wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) pathotypes 46S119 (virulent; a) and 38S102 (avirulent; b) and germ tube development in wheat genotype PBW343 at 20 hpi.

46S119 resulted in susceptibility (compatible interaction, Fig 1b) under controlled conditions. Symptoms of stripe rust disease on genotype PBW 343 inoculated with virulent pathotype 46S119 observed at 14 dpi consisted of linearly grown small, yellow orange uredinia parallel to the leaf veins.

Confocal microscopy and Uvitex-2B staining showed the differences in colonization and infection by two pathotypes (38S102 and 46S119) of *P. striiformis* f. sp. *tritici* on genotype PBW 343. The fungal infection structures were visualized together with structures of plant leaf tissue. The early stage of the interaction of Pst with host leaf tissue is represented by urediniospore germination. The germ tube started to appear 16 hpi in leaves inoculated with both the pathotypes (38S102 and 46S119). The germ tubes of both the pathotypes of Pst showed penetration through stomatal pore. However, longer germ tubes were observed in incompatible interaction as compared with the compatible reaction at 20 hpi (Fig 2 a, b). In pathotype 38S102, these longer germ tubes (54.6 μm) resulted in abortive germination (at 20 hpi) followed by hypersensitive response and no disease establishment (Table 1). During incompatible interaction longer germ tubes were observed showing less penetration sites. During compatible interactions the germ tube length was observed to be 35.86μm, which was significantly (p<0.05) shorter than the germ tube length in incompatible interactions. Earlier, Niks (1990) also observed that formation of longer germ tube on resistance genotypes was associated with more energy consumption leaving less energy for disease establishment during infection process of *P. hordei* in barley. After urediospores germination, the germ tubes enter into the leaf tissue through stomata and Zhang et al. (2008) demonstrated that leaves of susceptible wheat cultivar Mingxian 169 infected with Yilipu can also form appressoria during incompatible interaction.

During compatible interaction i.e., 46S119 inoculated on PBW 343, substomatal vesicle (ssv) and formation of infection hyphae (ih) were observed (Fig 3a, b, c). Fig 3a shows the penetration of germ tube through stomata in host infected with pathotype 46S119 at 24 hpi. We observed that germ tubes penetrated the stomata at 20 hpi to further form a substomatal vesicle at 24 hpi in pathotype 38S102 inoculated leaves while in pathotype 46S119 inoculated leaves, the response for penetration and substomatal vesicle formations was observed later at 24 hpi and 36 hpi, respectively. The substomatal vesicle usually formed 2-3 (upto 4) infection hyphae at 36 and 48 hpi in 38S102 and 46S119, respectively and hyphae later developed branches to form secondary infection hyphae in compatible interaction at 72 hpi (Fig 3b). Infection hyphae separated the haustorial mother cells by a septum in compatible interaction at 96 hpi (Fig 3c). In contrast, aborted sporelings were observed in incompatible interaction at 48 hpi (Fig 3d). The haustoria were regular with a spherical body and a slender neck separated from haustorial mother cell by a
Table 1: Difference in Germ tubes length at 20 hpi under incompatible and compatible interactions.

| Germ tubes length (in μm) | PBW343 inoculated with pathotype 38S102 (Incompatible interaction) | PBW343 inoculated with pathotype 46S119 (Compatible interaction) |
|---------------------------|------------------------------------------------|---------------------------------------------------------------|
|                           | P<0.05, significant difference between mean germ tube length in incompatible interaction (38S102) and compatible interactions (46S119). |
| Standard deviation        | 54.6*                           | 35.86*                          |
| Student's t-test           | 1.875                           | 2.206                           |
| p-value                   | 6.53e-07                        |                                 |

*Mean of five replications.

Septum at 96hpi (data not shown). Thus the fungal infection structures appeared more quickly in incompatible interaction than compatible interaction similar to earlier reports by Moldenhauer et al. (2006). In incompatible interaction, some necrotic regions were completely surrounded by callose and the host organelles around the infection structures were disintegrated into vesicles (Fig 3e). The compatible interaction is enabled with a complex mechanism in which the virulent Pst pathotype 46S119 was able to prevent hypersensitive response (HR) in PBW 343 during infection process. While, during an incompatible interaction, hypersensitive response was observed wherein the infected cell or its neighbouring cells showed disintegration to restrict further growth of the avirulent pathogen. This hypersensitive response resulted in formation of necrotic spots as reported earlier by Kang et al. (2003). The detailed microscopic observations showed that necrotic regions were more prominent in incompatible interaction than in compatible interaction at 72 hpi (Fig 3 f, g). The release of toxic metabolites from necrotic regions might hinder the hyphal branching and haustorial growth and further result in the long unbranched hyphae (Zeilinger et al. 2016). The elongated hyphae in compatible interaction (Fig 3f) were previously called ‘runners’ having long branches that grow over the leaf surface and thought to have role in rapid sporulation (Bozkurt et al. 2010). Microscopic observation of symptomatic stage of compatible interaction showed sporulated colonies of pathotype 46S119 at 14 dpi (Fig 3h).

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During infection process on a host, pathogen germ tubes have to overcome sequential barriers which may inhibit the infection process which are established from the host R gene and pathogen Avr gene interaction. These hurdles may be after stomatal penetration, after substomatal vesicle formation or after haustorium formation. Differences in fungal infection structures of pathotype 38S102 and 46S119 on wheat cultivar PBW343 were compared, leading to incompatible and compatible reaction respectively. Pathotype 38S102 is avirulent on Yr9, therefore showed incompatible interaction (HR) on PBW 343 having Yr9 gene, while 46S119 is virulent on Yr9 leading to compatible reaction on PBW 343 which was structurally correlated through confocal microscopy.

Conflict of interest
The authors declare no existence of any conflict of interest.

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