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Mechanism of leaf rust resistance in wheat wild relatives, *Triticum monococcum* L. and *T. boeoticum* L.

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Abstract

Triticum monococcum L. and *T. boeoticum* L., diploid wild relatives of bread wheat (*T. aestivum* L.), possess resistance to leaf rust (also known as brown rust) caused by *Puccinia triticina* Eriks. Haustorium formation-based resistance mechanisms (i.e. pre-haustorial and post-haustorial resistance) to leaf rust have been studied and reported in various *T. monococcum* accessions. In the present study, the mechanism of leaf rust resistance in *T. monococcum* and *T. boeoticum* accessions was studied using confocal laser scanning microscopy. Components of resistance studied at a histological level against leaf rust pathotypes, a Mexican pathotype (TCB/TD) and a Swiss pathotype (97512-19), indicated different types of resistance mechanism operative in the two accessions. The resistance in *T. monococcum* ranged from pre-haustorial resistance against 97512-19 to post-haustorial resistance against TCB/TD. The response in *T. boeoticum* was post-haustorial with necrosis against the two pathotypes. Pre-haustorial resistance observed in *T. monococcum* could serve as a potential source of durable rust resistance in wheat breeding.

Keywords: leaf rust, post-haustorial resistance, pre-haustorial resistance, *T. aestivum Puccinia triticina*, *T. boeoticum*, *T. monococcum*, wheat wild relatives

Introduction

Leaf rust caused by *Puccinia triticina* Eriks., is less damaging compared to stripe rust (caused by *P. striiformis* West.) and stem rust (caused by *P. graminis f. sp. tritici*) but due to its frequent and widespread occurrence it causes greater annual losses in bread wheat (*T. aestivum* L. em. Thell). Development and deployment of host genetic resistance is the most effective and economical approach to combat this disease. Although more than 78 leaf rust resistance genes (*Lr1* to *Lr78*) identified from cultivated wheat and its progenitor and non-progenitor species have been catalogued (McIntosh *et al.*, 2017; Kolmer *et al.*, 2018), leaf rust losses are occurring continuously worldwide as most of these genes deployed by breeders condition a hypersensitive reaction and are amenable to frequent breakdowns due to emergence of new virulences.

Resistance to a particular disease is attributed by genetic control, race-specificity and durability. The durability of resistance is related to the activation of haustorium formation-based resistance mechanism(s) (Niks and Rubiales, 2002; Singh *et al.*, 2005; Madrid *et al.*, 2008). Based on histological observations two types of resistance i.e. pre-haustorial and post-haustorial have been described in diploid wheat (Niks and Dekens, 1991). Pre-haustorial resistance prevents the formation of haustoria by the fungus despite the normal haustorium mother cells (HMCs) formation and a papilla often induced at the site of attempted cell wall penetration (Heath, 1981; Jacobs, 1989b; Niks and Dekens,

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1991). Pre-haustorial resistance is very common in non-host interactions as it reacts non-specifically to the different pathotypes (Heath, 1977, 1985), thus is expected to be long lasting (Niks and Dekens, 1991). This type of resistance does not allow the formation of hypersensitivity reaction or sporulating pustules, thus, imposing less selection pressure on the pathogen. In general, race-specific hypersensitivity resistance is post-haustorial.

Wheat wild relative, *T. monococcum*, has high levels of resistance to the wheat leaf rust fungus, *P. triticina*. Pre-haustorial and post-haustorial resistance to leaf rust has been studied and reported in various *T. monococcum* accessions (Niks and Dekens, 1991; Jacobs *et al*., 1996; Anker and Niks, 2001; Lind, 2005). Serfling *et al*. (2016) reported pre-haustorial resistance in Einkorn (*T. monococcum* accessions) against *P. triticina*.

One of the leaf rust-resistant recombinant inbred lines, designated as RIL101, derived from a cross involving A-genome wild wheat germplasm accessions *T. boeoticum* acc. pau5088 and *T. monococcum* acc. pau14087 at Punjab Agricultural University, Ludhiana showed a high level of resistance to various diseases including leaf rust, and was used to transfer rust resistance to the hexaploid wheat background. RIL101 was crossed to leaf rust susceptible *T. aestivum* cv WL711 using *T. durum* cv N59 as a bridging parent (Singh *et al*., 2007). The inheritance of leaf rust resistance from wild relatives has been worked out in the backcross populations derived from the cross N59/RIL101//3*WL711 and leaf rust resistance was found to be effective throughout the plant life (All stage resistance: ASR) (Sandhu, 2013). The present investigation was carried out to determine the mechanism of resistance in the *T. monococcum* and *T. boeoticum* parental accessions used to transfer leaf rust resistance to bread wheat background.

Materials and methods

Plant material

The plant material used for studying the mechanism of resistance for leaf rust consisted of diploid wheat wild relatives i.e. *T. monococcum* acc. pau14087 (hereafter referred to as *T. monococcum*), *T. boeoticum* acc. pau5088 (hereafter referred to as *T. boeoticum*), *Tb*5088/*Tm*14087 derived RIL101 and leaf rust susceptible *T. aestivum* cv WL711. The detailed information on *T. monococcum* and *T. boeoticum* accessions used in these studies and crossing scheme to generate BC_1F_8 introgression lines has been presented in Singh *et al*. (2007) and Chhuneja *et al*. (2008). Seedling and adult plant stage screening of these introgression lines identified leaf rust resistance gene(s), which was effective at all growth stages.

Screening against leaf rust pathotypes

The plant material was screened against different *P. triticina* pathotypes at the adult plant stage at the Institute of Plant Biology, University of Zurich, Zurich. Three *P. triticina* pathotypes including two Swiss pathotypes (97512-11, 97512-19) and a Mexican pathotype (TCB/TD) showed a prominent reaction on these germplasm lines and were selected for further studies. The avirulence-virulence formula for TCB/TD is avirulent to *Lr 3ka, 9, 10, 11, 16, 17, 19, 21, 24, 25, 27 + 31, 29, 30, 32, 33* and virulent to *1, 2a, 2b, 2c, 3, 3bg, 13, 14a, 14b, 15, 18, 20, 23, 26, 28*. Swiss pathotype 97512-19 is avirulent to *Lr 10*. The germplasm lines *T. monococcum*, *T. boeoticum*, RIL101 and WL711 were inoculated with rust at Zadoks growth stages 37–39 when most of the flag leaves developed using a fine mist of *P. triticina* urediniospores suspended in Tween 20 (as a surfactant). Infected plants were covered with a polythene hood to maintain high humidity and transferred to a growth chamber with a temperature of 16 °C and 90% humidity for 24 h in the dark. The climate conditions of the chamber were then changed to 25 °C/20 °C for 16 h/8 h day/night and 70% humidity. Leaf samples inoculated with each pathotype were harvested on the third, fifth, seventh, tenth and twelfth-day post-inoculation, and fixed for further analysis. For each genotype, three inoculated leaves were sampled. Disease severity was recorded as the percentage of leaf area covered by rust, following a modified Cobb's scale (Peterson *et al*., 1948) and infection type viz. 0 = immune, TR = traces of resistance, MR = moderately resistant, MS = moderately susceptible and S = susceptible.

Sample preparation

The samples for fluorescent microscopic analysis were prepared according to Moldenhauer *et al*. (2006). The flag leaf sections (±3 cm long) were fixed and cleared in ethanol: trichloromethane (3:1) solution containing 0.15% trichloroacetic acid for 24 h. The samples retrieved from fixative were washed with 50% ethanol and incubated in 0.5 M sodium hydroxide at 90 °C in an incubator for 30 min. After washing twice with water, the leaf samples were treated with 0.1 m Tris HCl buffer (pH 5.8) for 30 min. For staining, the leaf samples were soaked in 0.1% Uvitex 2B (Ciba-Geigy) for 5 min, rinsed four times (10 min each) with water and finally with 25% Glycerol for 15 min. The material was then stored in 50% glycerol at 4 °C until further use.

Microscopic observation

The Uvitex stained leaf samples of *T. monococcum*, *T. boeoticum*, RIL101 and WL711 were analysed under a

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fluorescent microscope for studying the leaf rust infection against two pathotypes i.e. TCB/TD and 97512-19 that showed differential phenotypic expressions. About 2 cm of leaf segments within the 2–10 cm region from tip was excised and examined using a Nikon Labophot fluorescence microscope equipped with epifluorescence optics. A UV-1A filter (excitation filter 330–380 nm, barrier filter 420 nm) was used to visualize fungal structures by their light blue fluorescence. The endophytically growing fungal infection structures in the leaf were observed using a confocal laser scanning microscope (Leica Microsystems) when excited with UV-laser beams at 351 and 364 nm and scanned with filter settings at 400–500 nm. The necrotic mesophyll cells were visualized with laser excitation at 514 and 543 nm and scanned with filter settings at 560–680 nm. The infection units in each leaf segment were recorded for four parameters (pre-stomatal exclusion of fungus, aborted penetration of infection units, early abortion and fully established mycelia colonies) as studied by Jacobs *et al.* (1996). Each leaf segment was screened for all the cells having infection structures which varied from 10 to 50.

Results

Phenotypic analysis

The phenotypic evaluation of *T. monococcum* and *T. boeoticum* at the adult plant stage showed resistance against the *P. triticina* pathotypes tested in this study, although the level of resistance varied in the two wild wheat accessions. *T. monococcum* produced nearly immune and immune reaction against TCB-TD and 97512-19, respectively. *T. monococcum* showed flecking with 5 MR type of reaction against pathotype 97512-11 (Fig. 1, Table 1). In *T. boeoticum*, the terminal disease severity was 10–20 MR against TCB-TD and 97512-11; however, a susceptible reaction was observed against 97512-19. In response to 97512-19, *T. boeoticum* showed medium to large size pustules surrounded by large chlorotic areas with more than 40% of leaf coverage (Table 1). RIL101 also showed a disease score of 10–20 MR to pathotypes TCB-TD and 97512-11 whereas gave a susceptible reaction against 97512-19. WL711, which is susceptible to Indian *Pt* pathotypes, exhibited a moderate type of susceptible reaction with some chlorosis against all the three pathotypes (Fig. 1, Table 1).

Microscopic investigation of haustorium formation

The progress of the infection process for rust pathotypes TCB/TD and 97512-19 was observed in *T. monococcum*, *T. boeoticum*, RIL101 and WL711 (Figs. 2 and 3).

Mechanism of infection against P. triticina *pathotype TCB/TD:*

On infection with TCB/TD, there was no pre-stomatal exclusion observed in any of the lines tested at 3 dpi as all the germinated spores formed appressoria over the stomata (Fig. 2). A few cells in the parental accessions (*T. monococcum*, *T. boeoticum*) and RIL101 showed aborted penetration of the fungi. *T. monococcum* significantly differed from *T. boeoticum* for the presence of infection sites with early aborted structures (Fig. 2). *T. boeoticum* had an almost negligible early abortion with the presence of numerous HMCs per infection site. Secondary infection hyphae were also observed in some of the infection sites. When observed at 5 dpi, secondary infection with HMCs accompanied by host cell necrosis (HCN) was very frequently recorded in *T. monococcum*. Growing hyphae in *T. boeoticum* and RIL101 did not exhibit any hypersensitive response at this stage. Two days later (7 dpi) HCN was also present at some sites in *T. boeoticum* and RIL101, but the growth of hyphal colonies was not much affected. Compared to this, HCN had spread restricting the growth of fungal structures in *T. monococcum*. There was complete cell death at the infection site and thus no mycelial colonies were developed. This difference was more significantly noticed at 10 dpi. The high hypersensitive response was observed at the site of infection in three genotypes (*T. monococcum*, *T. boeoticum* and RIL101) at this stage. Fully established mycelial colonies were recorded in *T. boeoticum* and RIL101 which were rather more pronounced in *T. boeoticum* at 12 dpi (data not given). No fungal growth occurred beyond the necrotic areas in *T. monococcum* at 12 dpi (data not given). The susceptible check, WL711, had numerous HMCs at third day post-infection which fully developed into mycelial colonies at further stages post-infection despite some necrosis at the fifth- and seventh-day post-infection.

Mechanism of infection against P. triticina *against pathotype* Pt *97512-19*

Infection with pathotypes *Pt* 97512-19 also showed a similar pattern of fungal growth in the leaf samples as observed in TCB/TD (Fig. 3). The hyphal growth progressed at a very slow rate in *T. monococcum*. *T. monococcum* developed 1-3 HMCs 3 dpi which increased at the second sampling stage. Although, the HMC counts increased in some infection sites in *T. monococcum* but no further growth of the fungus was observed in the leaf segments sampled until 10 dpi. The infection sites at 7, 10 and 12 dpi were accompanied by necrosis of the cells. On the other hand, in *T. boeoticum* HMC count were more than six at the first sampling stage followed by complete hyphal network formation on the fifth day of infection. The fungal growth increased in *T. boeoticum* forming colonies of hyphae

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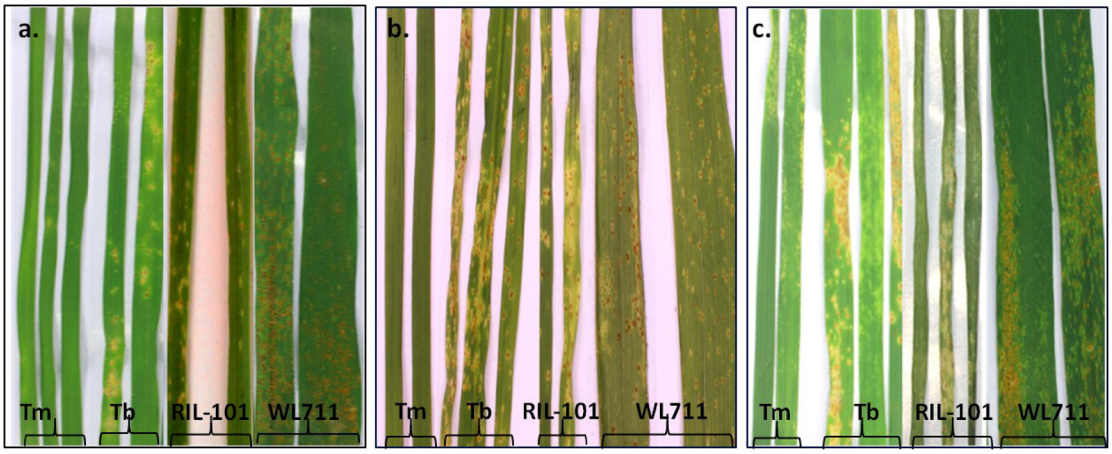

Fig. 1. Adult plant leaf rust reaction (left to right) of *T. monococcum* (Tm), *T. boeoticum* (Tb), RIL101 and WL711 against (a) *Pt* pathotype TCB/TD; (b) *Pt* pathotype 97512-19 and (c) *Pt* pathotype 97512-11.

Table 1. Leaf rust severity in *T. monococcum*, *T. boeoticum*, RIL101 and WL711 against three *Puccinia triticina* isolates used in the study

Genotypes	Pathotype TCB/TD	Pathotype 97512-19	Pathotype 97512-11
T. monococcum	0	0	5 MR
T. boeoticum	10–20 MR	40 MR	10–20 MR
RIL101	10–20 MR	40 MR	10–20 MR
WL711	40 MS	60 MS	60 MS

Disease severity recorded as infection type viz. 0 = immune, TR = traces of resistance, MR = moderately resistant, MS = moderately susceptible and S = susceptible (as modified Cobb's scale).

without any HCN. The infection process in RIL101 was similar to *T. monococcum* with 1–3 HMCs at the first sampling stage. But it behaved more like *T. boeoticum* in the later stages with complete hyphal formation at 7 dpi. However, some HCN was observed at 10 dpi. The susceptible check, WL711 showed the presence of numerous HMCs with infection hyphae at third day post-infection which developed into mycelial colonies in later stages. Some HCN was also observed at 5 and 7 dpi.

Discussion

Diploid wild wheat parental accessions (*T. monococcum* and *T. boeoticum*), RIL101 and WL711 were examined phenotypically and microscopically for resistance or susceptibility, and classified based on phases of development of the infection units. Disease reaction of these lines indicated that *T. monococcum* and *T. boeoticum* have different levels of resistance against Swiss and Mexican pathotypes. *T. monococcum* and *T. boeoticum* are closely related diploid wild wheat species but their host-status for the wheat leaf rust fungus is different (Anker *et al.*, 2001). The present study results also suggests that *T. monococcum* and *T. boeoticum* accessions have different genes conferring resistance to leaf rust. The gene (s) from *T. monococcum* is/are conferring a high level of resistance against both the pathotypes tested. *T. boeoticum* and RIL101, on the other hand, has the gene which is showing moderate resistance to these pathotypes. The low level of resistance in RIL101 could be due to the fact that this RIL carries leaf rust resistance gene from *T. boeoticum*. However, it was not feasible to determine how many leaf rust resistance genes are present in *T. monococcum* and *T. boeoticum* as the RIL population from this cross is completely resistant against all of the pathotypes that were tested (data not published).

Different fungal structures were observed when the development process of two rust pathotypes TCB/TD and 97512-19 were studied in *T. monococcum*, *T. boeoticum*, RIL101 and WL711. All the fungal growth stages including spore germination to sporulation can occur within a 7–10 days period at optimum and constant temperatures. The uredospore germinates forming a germ tube that continues to grow along the leaf surface until a stoma is reached or the endogenous spore reserves are depleted (Dickinson,

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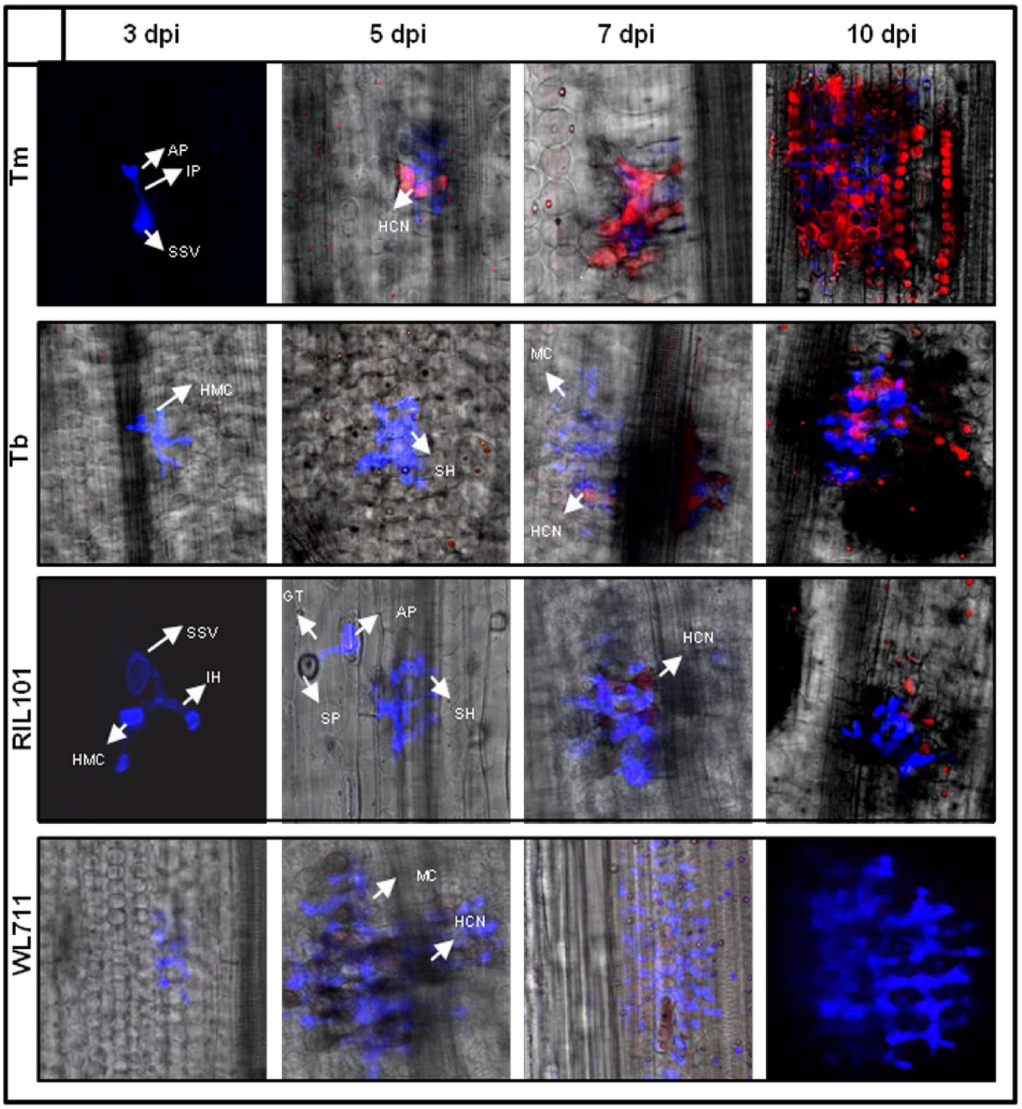

Fig. 2. Development of infection units of *Puccinia triticina* pathotype TCB-TD in *T. monococcum* (Tm), *T. boeoticum* (Tb), RIL101 and WL711 at 3, 5, 7, 10 days post-infection under Nikon Labophot fluorescence microscope. The growing fungal structures (SP = spore, GT = germ tube, AP = appressorium, IP = infection peg, SSV = sub stomatal vesicle, IH = infection hypha, HMC = haustorial mother cell, SH = secondary hyphae, MC = mycelial colonies) are depicted in blue (excitation 351/364 nm, filter 400–500 nm) and the necrotic mesophyll cells (HCN = host cell necrosis) are depicted in red (laser excitation 514/543 nm, filter 560–680 nm).

1969). On reaching the stoma, the appressorium is formed within 24 h of infection. The penetration peg from the appressorium enters the stoma and a sub stomatal vesicle is formed in the intercellular space within-host leaf. Thereafter, infection hyphae begin to grow from this substomal vesicle towards the mesophyll cells (Allen, 1926). The haustorial mother cell (HMC) develops against the mesophyll cell and subsequently, haustoria is formed in the host cell. Secondary infection hyphae are produced, which come in contact with other host cells resulting in additional HMC and haustoria, thus forming a branched network of fungal mycelium (Allen, 1926).

T. monococcum showed a post-haustorial type of resistance to the Mexican pathotype TCB-TD and pre-haustorial resistance against the Swiss pathotype 97512-19. The first sampling was carried out at 3 dpi and hence the initial fungal penetration in the plant material was not studied. The resistance mechanisms operating in hosts and non-host genotypes do not affect the spore germination, appressorium formation, stomata penetration and sub stomatal vesicle formation (Jacobs, 1989a). Lee and Shaner (1984) also observed no significant difference in pre-penetration stages between the susceptible and slow leaf rusting wheat genotypes.

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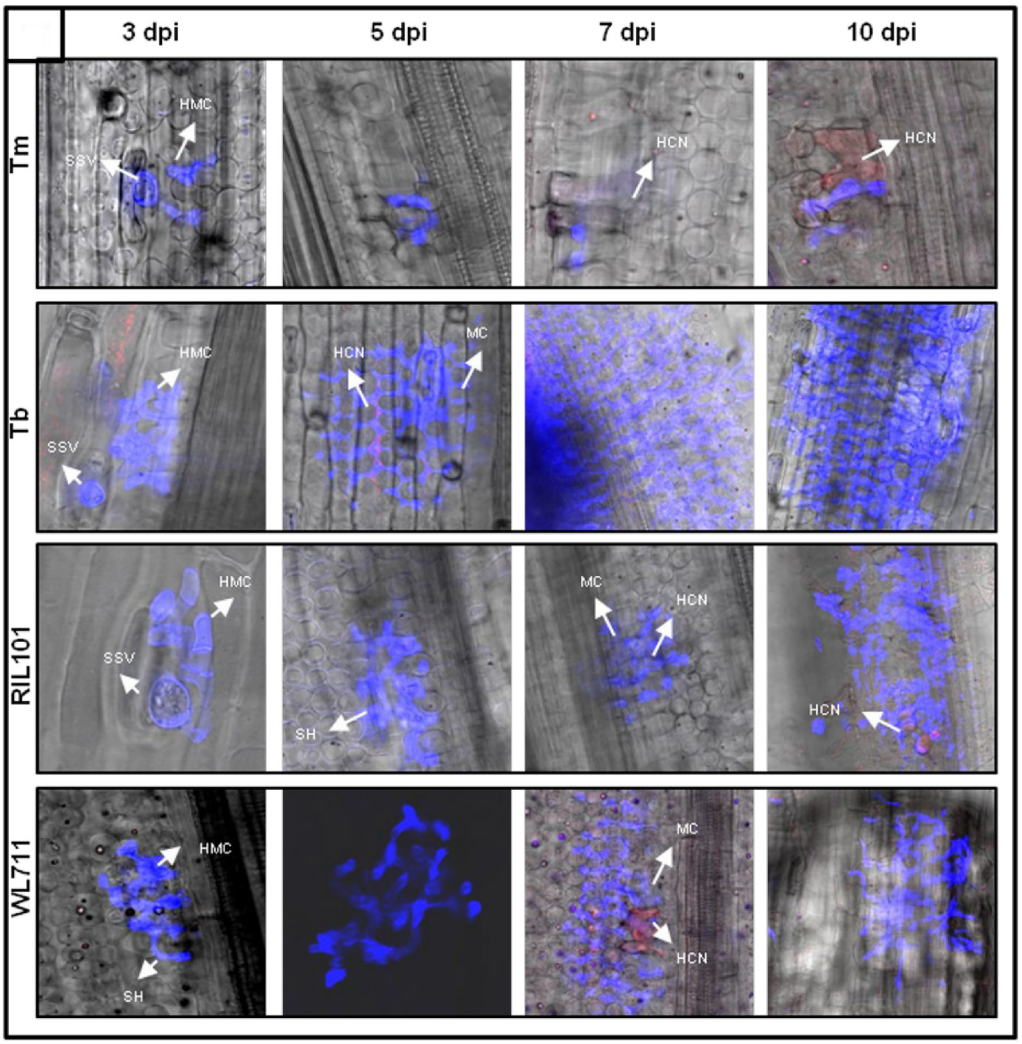

Fig. 3. Development of infection units of *Puccinia triticina* pathotype 97512-19 in *T. monococcum* (Tm), *T. boeoticum* (Tb), RIL101 and WL711 at 3, 5, 7, 10 days post-infection under Nikon Labophot fluorescence microscope. The growing fungal structures (SP = spore, GT = germ tube, AP = appressorium, IP = infection peg, SSV = sub stomatal vesicle, IH = infection hypha, HMC = haustorial mother cell, SH = secondary hyphae, MC = mycelial colonies) are depicted in blue (excitation 351/364 nm, filter 400–500 nm) and the necrotic mesophyll cells (HCN = host cell necrosis) are depicted in red (laser excitation 514/543 nm, filter 560–680 nm).

Histological observations revealed a significant difference in the development of the two *P. triticina* pathotypes (TCB/TD and 97512-19) in *T. monococcum*. The development of pathotype TCB/TD in *T. monococcum* was initially slow but haustoria mother cells soon developed into hyphae. However, further fungal growth was completely restricted by necrosis of the host cell, thus suggesting that the gene (s) in this accession confer a hypersensitive type of resistance to leaf rust. On the other hand, the resistance to 97512-19 in *T. monococcum* appeared to be pre-haustorial as the HMCs did not form any haustoria or primary hyphae till 10 dpi which are essential for effective infection. Moreover, necrotic areas surrounding HMCs often restricted the further growth of fungus, thus inhibiting colonization. This differential development of two pathotypes in *T. monococcum* could be due to different genes conferring specific resistance to these pathotypes.

The mechanism of resistance in *T. boeoticum* against both TCB/TD and 97512-19 was post-haustorial, i.e. a hypersensitive response elicited after the formation of a haustorium. Although the resistance response in *T. boeoticum* was quite similar for the two pathotypes, the level of resistance varied against these pathotypes. The HCN was observed more frequently in the infection sites tested with TCB-TD. Despite HCN, the mycelial colonies were

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successfully formed in *T. boeoticum* at 7 dpi in TCB-TD and at 5 dpi in 97512-19. Against 97512-19, only small necrotic areas relative to large colonies were measured in *T. boeoticum*. These results confirm phenotypically observed moderate resistance to *P. triticina* pathotypes TCB-TD and 97512-19 in *T. boeoticum*. Serfling *et al.* (2016) also observed weak post haustorial resistance reaction with weak signs of autofluorescence 3 dpi in the phenotypically susceptible *T. boeoticum* accession 36554 against the isolate under study. The dissimilarity in host–pathotype interaction could be the reason for the higher proportion of infection sites with HCN in TCB-TD than in 97512-19. Kloppers and Pretorius (1995) also observed a significant difference in the number of colonies for South African pathotypes tested against *Lr37* in RL6081. Similarly, in a study by Bender *et al.* (2000), infection sites with HCN were more frequent in the lines with *Lr12* and *Lr13* against pathotype UVPrt2 compared to UVPrt13.

As in *T. boeoticum*, the infection sites with established colonies were observed in *Tb*5088/*Tm*14087 derived RIL101 but a hypersensitive response was more frequent, thus indicating a post-haustorial mechanism of resistance. *T. aestivum* cv WL711, susceptible to most Indian pathotypes, showed some hypersensitive response despite of complete colonization at very early stages of infection. Terminal disease severity recorded in WL711 against TCB/TD (virulent to *Lr13*) was 40 MS (Table 1, Fig. 1a). HCN was observed in WL711 at fifth- and seventh-day post-infection, which further showed fully developed colonies at tenth-day post-infection (Fig. 2).

A few necrotic cells in WL711 could be attributed to stress due to different environmental conditions or host–pathogen interaction or due to the presence of *Lr*13 gene (Agarwal *et al.*, 2003). As suggested by Samborski *et al.* (1977), HCN in the compatible host–pathogen interactions may result from a general stress reaction. Susceptibility is not necessarily always associated with the absence of necrotic cells at infection sites (Bender *et al.*, 2000).

The mechanism of rust resistance in diploid wild wheat ranges from pre-haustorial resistance without necrosis to post-haustorial resistance with frequent necrosis. Niks and Dekens (1991) reported high levels of pre-haustorial resistance in two out of three accessions of *T. monococcum* and post-haustorial resistance in a *T. boeoticum* accession. Among the 152 resistant *T. monococcum* accessions reported by Anker and Niks (2001), only three accessions were found to have a high percentage of pre-haustorial resistance to *P. triticina* pathotype Felix. Lind (2005) screened 348 accessions of *T. monococcum*, obtained from different gene banks, for leaf rust resistance and detected 13 accessions (3.7%) with pre-haustorial resistance. Serfling *et al.* (2016) reported pre-haustorial resistance in *T. monococcum* accession with complete inhibition of generation of haustoria and weak post-haustorial resistance in *T. boeoticum* accession. Our histology study reported both pre-haustorial and post-haustorial resistance in *T. monococcum* accession pau14087 and post-haustorial resistance in *T. boeoticum* accession pau5088 against both the pathotypes, thereby justifying the differential defence response to leaf rust pathogen in the two wild wheat accessions.

T. monococcum acc. pau14087 conferring a pre-haustorial kind of resistance to one of the two pathotypes tested could prove a durable source of resistance in rust resistance breeding. Pre-haustorial resistance in *T. monococcum* can be used to replace the currently widely used single gene and race-specific resistance against leaf rust in wheat which is at constant risk of being overcome by the emergence of new virulent races. The present study describing the development of fungal structure and host–pathogen response may assist in understanding the molecular genetic aspects of the genes transferred from wheat wild relatives (*T. monococcum* and *T. boeoticum*) into the hexaploid wheat background. Thus, the leaf rust resistance genes in *T. monococcum* and *T. boeoticum* will be different with different mechanisms of resistance. We have already initiated the independent transfer of leaf rust resistance from *T. monococcum* and *T. boeoticum* into hexaploid wheat (*T. aestivum*) using *T. durum* as a bridging species. Once transferred independently, a pyramiding of the two leaf rust resistance genes through marker-assisted selection may result in durable resistance, because of their different mechanisms of infection.

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