Reaction of wheat cultivars and differential lines to *Puccinia triticina* races in detached leaves

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ABSTRACT

Turra, C., M. Reis, E.M.; Barcellos, A.L. Reaction of wheat cultivars and differential lines to *Puccinia triticina* races in detached leaves. ***Summa Phytopathologica,*** v.40, n.4, p.353-357, 2014.

The method of preserving detached wheat leaves in Petri dish was used for the inoculation and development of the fungus *Puccinia triticina*, the causal agent of wheat leaf rust. The reaction of 26 wheat cultivars was compared by using seedlings cultivated in pots (*in vivo*) and detached leaves (*in vitro*) inoculated with four physiological races of the pathogen. After inoculation, the material was kept in a growth chamber for 15 days. The reaction was evaluated on the 15th day after inoculation. Results for each race in the evaluated genotypes confirmed the efficiency of the detached leaf method in assessing the reaction of wheat cultivars.

Additional keywords: Leaf rust, physiologic races, *Triticum aestivum*.

RESUMO

Turra, C.; Reis, E.M.; Barcellos, A.L. Reação de cultivares de trigo e de linhagens da série diferencial à raças de *Puccinia triticina,* em folhas destacadas. ***Summa Phytopathologica,*** v.40, n.4, p.353-357, 2014.

O **método de preservação de folhas de trigo** destacadas , em placa de Petri, foi usado para inoculação e desenvolvimento do fungo *Puccinia triticina*, agente causal da ferrugem da folha do trigo. Foram comparadas as reações de 26 cultivares de trigo em plântulas cultivadas em vasos (*in vivo*) e em folhas destacadas (*in vitro*) inoculadas com quatro raças fisiológicas do patógeno. Inoculou-se também 24 linhas da série diferenciadora de raças do fungo nas mesmas condições. Após a inoculação, o material foi mantido em **câmara climatizada por 15 dias**. A avaliação da reação foi realizada no 15º dia após a inoculação. Os resultados obtidos para cada raça nos genótipos avaliados confirmaram a eficiência do método de folha destacada na avaliação da reação de cultivares de trigo.

Palavras-chave adicionais: Ferrugem da folha, raças fisiológicas, *Triticum aestivum.*

Leaf rust caused by *Puccinia triticina* (*Pt*) Eriks. is one of the major leaf diseases affecting wheat in Brazil. Damage caused to wheat by leaf rust can be assessed by using the normalized function Y = 1,000 - 6.4 I (Y = yield kg/ha and I = leaf incidence) (16).

The main strategies for leaf rust control have been the development and the cultivation of genetically resistant cultivars, as well as the application of fungicides to aboveground plant parts (16). Genetic resistance of wheat to *Pt* is not durable due to the high fungal variability, resulting in occurrence of at least one new virulent race at every wheat growing season (3).

Resistant cultivars are developed by inoculating *Pt* in new lines of wheat. For wheat leaf rust, resistance is race-specific; thus, dominant races are individually inoculated into the material to be selected (3). This requires a lot of material, facilities to grow the plants, and facilities to maintain the pure races. Each *Pt* race should be maintained, multiplied and kept pure, preventing mixtures with other races.

The technique of detached leaves can be an important tool to rationalize the evaluation of the reaction of cultivars in the determination of *Pt* races.

This technique has been used in studies of host-pathogen interaction with obligate parasites, such as fungi that cause rusts. Grass leaves or leaf pieces (wheat, oats, barley, rice and others) have been allowed to float on water or sucrose-based solutions, with or without benzimidazole, cytokine or kinetin (4, 7, 10, 11, 12, 17, 18).

Mignucci (14), Felsenstein (8) and Felsenstein (9) mention that adaptation of the detached leaf method was implemented to monitor the sensitivity of *Pt* to different fungicides in Europe.

Considering *Pt* new races, race determination in every season and resistance break in some cultivars, the search for new resistant cultivars requires a lot of material and work.

The aim of this study was to compare the reaction of wheat cultivars *in vivo* and in *vitro* to four *Pt* races.

MATERIAL AND METHODS

The experiments were conducted at the Laboratory of Plant Pathology/ Mycology, Faculty of Agronomy and Veterinary Medicine, University of Passo Fundo, Passo Fundo/RS, in 2010 and 2011.

The twenty-six wheat cultivars most commonly cultivated from 2004 to 2006 seasons and 24 breeding lines of the race differential set were sown in polyethylene pots containing 200 ml of soil added of poultry litter. Twenty seeds of each genotype were sown per pot, including two replicates. The seedlings were grown in a growth chamber at 20ºC ± 2ºC and 12-hour photoperiod for 10 days. Kristalon compound fertilizer, diluted 100 g/5 L water, was applied at 5 ml per pot on the seventh day after emergence.

Purified isolates of MFT-MT 4002S, TFP-HT, TPT-HT and MFT-HT, the most prevalent *Pt* races in the 2009 season, were provided by the Laboratory of Wheat Leaf Rust, OR Seed Improvement Ltd. The inoculum of each race was increased by spraying a uredospore suspension in mineral oil (Soltrol) on susceptible Morocco seedlings, grown in plastic pots (200 mL volume). Following such inoculation, seedlings were kept for 18 to 20 hours in the dark at 20ºC ± 2 ºC and 100% relative humidity. After incubation, plants were transferred to a greenhouse at 20 ºC ± 2 ºC, where they were kept for 14 days until uredospore collection with a vacuum pump.

The detached leaf assay was performed as described by Browder (4).

The medium used to prevent leaf senescence and maintain the viability of wheat leaf segments was composed of agar, distilled water, streptomycin and benzimidazole salt, as described by Browder (4), Hooker & Yarwood, (11), Felsenstein (8), and Felsenstein et al. (9).

The main limitations were the source of benzimidazole and the best concentration for preserving leaf segments for a long time. The fungicides Carbendazim (Portero 50% SC and Derosal 50 % SC), thiophanate methyl (Cercobin 80% SC) and benzimidazole salt (Sigma-98%) were used as benzimidazole sources, and the tested concentrations were 0, 500, 1000, 1500, 2000, 2500 µL/L a. i. concentrations in distilled water.

Benzimidazole salt stock solution consisted of 10.2 g/100 mL distilled water prepared in a 100-mL volumetric flask kept in refrigerator at 4°C.

Three leaf segments of each plastic Petri dish (9 cm diameter) were deposited on aluminum foil and a spatula was employed to introduce its ends into the culture medium [8 g agar, 0.3 g streptomycin sulfate, benzimidazole salt (98%, diluted 10.2 g/100 ml water), 1,500 µg/L distilled water]. The medium was poured into plastic Petri dishes of 60 mm diameter and stored in refrigerator at 5°C.

For all combinations, 7-8cm long segments were obtained from the first leaves of 14-day-old seedlings. Segments were surface disinfected, placed on agar surface, and had their ends introduced with a spatula into the medium; three segments per dish were kept on aluminum foil to prevent the direct contact of the leaf with the medium.

Uredospore density in three drops of 0.01 mL was determined by counting the spores under an optical microscope (100 x magnification), followed by inoculum concentration adjustment. Inoculation of races consisted in spraying an inoculum density of 8×10^4 uredospores/mL distilled water.

To improve inoculum distribution and leaf wetting, 120 µL/L adjuvant (Tween 20) in distilled water was added to the inoculum suspension.

Inoculation was performed by spraying the spore suspension both on the leaves of wheat seedlings (whole seedlings), i.e., *in vivo,* and on the leaf segments, *in vitro*, for each genotype kept in Petri dishes.

After inoculation, the potted-seedlings and the leaves in Petri dishes remained in a growth chamber, covered by dark plastic for 24 hours, which provided favorable environment for spore germination and penetration. At the end of the incubation period, the plastic coverage was removed and the plants were atomized with distilled water plus Tween 20 adjuvant (120 µL/L water), to keep leaf wetness. Seedlings and Petri dishes were kept in a growth chamber at 20 ºC ± 2 ºC and 12-hour photoperiod for 13 days.

Plant reaction, both *in vivo* and *in vitro*, was assessed on the 15th day after inoculation, according to the disease rating scale described below:

Table 1. Description of type of infection and symptoms caused by *Puccinia triticina* to inoculated wheat leaves

Infection type	Avirulence/ virulence	Symptoms
0	avirulent	No uredinia or another macroscopic sign of infection
0;	avirulent	Few faint flecks
;	avirulent	No uredinia but presence of hypersensitive necrotic or chlorotic flecks
1	avirulent	Small uredinia often surrounded by necrosis
2	avirulent	Small-to-medium uredinia often surrounded by chlorosis
Y	avirulent	Ordered distribution of variable-sized uredinia, larger at the leaf tip
X	avirulent	Random distribution of variable-sized uredinia
3	virulent	Medium-sized uredinia without chlorosis or necrosis
4	virulent	Large uredinia without chlorosis or necrosis
The infection types are often refined by modifying characters as follows		
(=)	uredinia at the lower size limit for the infection type	
(-)	uredinia somewhat smaller than normal for the infection type	
(+)	uredinia somewhat larger than normal for the infection type	
(++)	uredinia at the upper size limit for the infection type	
C	more chlorosis than normal for the infection type	
N	more necrosis than normal for the infection type	

* Adapted from Roelfs & Martens (17)

RESULTS AND DISCUSSION

At OR Lab, the reaction of about 400 hundred new wheat lines and race populations was assessed by using 168 samples in the 2013 season (each rust sample is inoculated in 24 *Lr* genes).

The limitations had to be overcome to maintain the viability of leaf segments, either healthy or infected. In general, segments were yellowed and decayed, resulting from high humidity in the Petri dishes. Such difficulty was also pointed out by Yarwood (18) and other authors, who have dealt with failure in several studies using rapid senescence of leaf segments soon after detachment from the plants.

This problem was solved by: a) placing aluminum foil on the substrate in the Petri dish to prevent the direct contact of the leaf with the medium; b) identifying the leaf portion and the section type; c) after inoculation, protecting dishes with transparent plastic to keep moisture during the period required for the disease development, eliminating the need of opening dishes to water the leaf segments; and d) maintaining leaf segments in Petri dishes which promote the formation of water droplets in the dishes; water excess should be dried with paper towel to prevent rot of the segments.

Benzimidazole sources such as carbendazim (Portero 50% SC and Derosal 50% SC) and thiophanate methyl (Cercobin 80% SC) were not satisfactory for chlorophyll preservation in the leaf segments. These commercial products are similar and have an antifungal effect; thus, they are not recommended for the preservation of detached leaves. The best method to preserve chlorophyll in wheat leaf segments was the use of pure benzimidazole such as salt (Sigma 98% a.i.) in agreement with *Boydom* et al. (5).

The most suitable agar medium (8 g agar; after autoclaving 0.3 g streptomycin sulfate diluted in 50 mL; and the benzimidazole salt 1,500 µg/L salt) allowed leaf segments to prevent longer senescence and did not affect leaf rust development. The optimal benzimidazole salt concentration to preserve leaf segments in Petri dishes was 1,500 µL/L distilled water, in which the segments remained viable for 18 days. Concentrations of 2,000 and 2,500 µL/L changed the leaf segment reaction, not expressing resistance/susceptibility to specific races. The induced resistance of wheat leaf segments can be explained by the change caused by benzimidazole salt to the chemical composition of plant tissues, hindering fungal development after penetration.

The wheat is a species of Liliopsida, Poaceae, which does not produce roots in detached leaf segments, hindering its maintenance and the assessment of *P. triticina* reactions in inoculated genotypes under controlled environment. However, detached leaves of soybean infected

Table 2. *In vivo* and *in vitro* reaction of wheat cultivars inoculated at seedling stage with four *Puccinia triticina* races

Cultivar	MFT-MT 4002S		TFP-HP		TPT-HT		TFT-HT	
	in vivo	*in vitro*	*in vivo*	in vitro	in vivo	in vitro	in vivo	in vitro
Abalone	3	3	3	3	3	3	3	3
Atlax	-	3	3	-	-	-	-	-
BRS 220	0	3	3	3	3	0;	;	;
BRS Guabiju	-	-	-	0;	0	3	3	3
BRS Guamirim	;	3, 32;(2 P)	3	0; (6P), 3(3P)	0;	0;	0;	0;
BRS Pardela	;	0	0;	0;	0;	0; 2(2P)	21;	21;
BRS Tangara		;2 3(Pust.)	2;	0; (3Pust.)	;	0;, ;2(3P)	2;	2;
Campeiro	3	3=3	3	3	3	3	3	3
CD 104	-	-	-	;	;1	3	3	3
CD 105	-	-	-	3	3	2	2	2
CD 111	3	;1	12	-	-	-	-	-
Cronox	3	;	;1	0; 3(Pust.)	; 3(2Pust.)	3	3	3
Fundacep 30		;	;	0;	0;	;1	;1	;1
Fundacep Cerejinha	0;	3	3 (1Pust.)	3	3	3	3	3
Fundacep Cristalino	;1	;2 3(Pust.)	0; 3(Pust.)	;-2 (10P), 3(2P)	;(2P) 3(1P)	;12	21	21
Fundacep Horizonte	;	0; 3(Pust.)	;	;1	;1	;(11P), 3(1P)	;(2P), 3(1P)	;(2P), 3(1P)
Fundacep Nova Era	-	-	-	3	3	;2	2	2
Fundacep Raízes	;1	0;	0;	0;	0;	3	0(2P) 3(1P)	0(2P) 3(1P)
Marfim	;1	3	3	3	3	3	3	3
Mirante	3	;	0;	;	;1	3	3	3
Morocco	3	3	3	3	3	3	3	3
Pampeano	3	32;	3	3	3	3	3	3
Quartzo	3	3 3-	3	3	3	3	3	3
Safira	3	3	3	3	3	3	3	3
Supera	3	;	;	;1	;1	;	;	;
Vaqueano	1+2	;	;	0;	0;	3	3	3

Table 3. *In vivo* and *in vitro* reaction of wheat differential lines to four *Puccinia triticina* races

	MFT-MT 4002S		TFP-HP		TPT-HT		TFT-HT	
	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro
Lr 1	3	3	3	3	3	3	3	3
Lr 2[a]	0;	0;	3	3	3	3	3	3
Lr 2c	0;	0;	3	3	3	3	3	3
Lr 3	3	3	3	3	3	3	3	3
Lr 9	0;	-	0;	0;	3	3	0;	0;?
Lr 16	1	1	1	1	1	1	1	11^{+}
Lr 24	3	3	3	3	3	3	3	3
Lr 26	3	3	3	3	3	3	3	3
Lr 3ka	3	3	3	3	3	3	3	3
Lr 11	3	3	2(3P)	-	3	3	3	3
Lr 17	3	3	3	3	3	3	3	3
Lr 30	3	3	3	3	3	3	3	3
Lr 10	3	3	;1	;1	;1	;1	;1	;1
Lr 18	;1	;1	3	3	3	3	3	3
Lr 21	2	2	2	2	2	2	2	2
Lr 23	3	3	3	3	3	3	3	3
Lr 14a	3	3	3	3	3	3	3	3
Lr 14b	3	3	3	3	3	3	3	3
Alondra	-	-	-	-	-	-	-	-
Lr 20	3	3	3	3	3	3	3	3
Lr 3bg	32	3	3	3	3	$33^{=}$	32	$32^{=}$
Lr 19	0;	0	;	0	;	;	0	0
Lr 27+31	32	33=	3	3	3	3	32	3
ORL 04002	3, ;1(1P)	3	;1	;1	;-2	$;12^{=}$	;12	$;11^{+}$

by *Phakopsora pachyrhizi* Sydow can be maintained for long periods without the need for specific substrate, only requiring moistening with water and aluminum foil on styrofoam layer, on which roots are easily produced (6, 14, 15).

During the adaptation process of the wheat detached leaf method, preliminary tests were conducted to maintain the inoculation, the incubation method and the disease development similar to that used for artificial inoculation in greenhouse under controlled light and temperature for the best onset of symptoms. The temperature in our study was 20ºC ± 2ºC and 12-hour photoperiod. Lower temperatures delayed the disease onset but allowed longer preservation of leaf segments since wheat is a cold-demanding plant.

In vivo and *in vitro* tests inoculating urediniospores suspended in mineral oil (Soltrol) caused phytotoxicity, preventing infection. However, inoculation with uredospores suspended in distilled water added of 240 µL/L adjuvant (Tween 20) showed positive results in the infection implementation, in both *in vivo* and *in vitro* tests.

The light played an essential role in the rust infectious process. In our study, Petri dishes containing wheat leaf segments had to be removed from the presence of light since direct light accelerated chlorophyll degradation and senescence of leaf segments (2, 5, 13). In dishes kept away from light sources, leaf segments remained green for a longer time, allowing the normal process of photosynthesis and thereby promoting the development of the fungus.

The first symptoms were observed from the 6th day after inoculation *in vivo* and *in vitro* on the 8th day at 20°C. The evolution of symptoms suggests the assessment of the reaction on the 15th day; shorter periods may hinder the assessment of the infection type (Table 1).

Our results (Tables 2 and 3) showed the reciprocal reactions of avirulence /virulence between *in vivo* and *in vitro* tests, leading to the conclusion that the detached leaf method can be used in studies with *Pt*.

After the 17th day, pycnidia of *Darluca filum* (Biv.) Castagne were found in some uredia on wheat leaf segments, probably due to high humidity inside the dishes.

Similarly, detached wheat leaf segments can be used to study *P. graminis* f. sp. *tritici* Eriks. & E. Henn for wheat stem rust, determining the reaction of cultivars/lines and the pathogen races (17).

The main advantages of the detached leaf technique are space and host material saving to evaluate genotype collections, economy of inocula of the pathogens to be studied, ease and accuracy of observations, lowest risk of contamination, uniformity of the experimental unit, ease of control and manipulation of environmental conditions, and possibility of keeping the detached leaves, in some cases, for a longer period than the normal life of plant leaves, as well as keeping the most prominent features and biochemical functions of plant leaves, including perspiration, respiration, photosynthesis and protein synthesis (13).

This method can be useful to evaluate the reaction of wheat genotypes to different races of the pathogen, preventing risk of spreading to other regions. The advantages are (i) best environmental

control of the experiments; (ii) smallest space for experiments; (iii) prevention of contamination among fungal isolates; (iv) reduction in plant material loss; (v) greater reliability; and (vi) possibility of use to assess the sensitivity of *Pt* races to the fungicide (1).

The detached wheat leaf method was efficient for assessing the reaction of the wheat genotypes most frequently grown in Brazil to the prevalent races *Pt* MFT-MT4002S, TFT-HT, TPT-HT and TFT-HT, similarly to the results obtained by Boydom et al. (5).

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