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Water potential and time of *Pyrenophora tritici-repentis* inoculation in wheat seeds

Potencial hídrico e duração da inoculação com *Pyrenophora tritici-repentis* em sementes de trigo

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Abstract

Wheat seeds infected with *Pyrenophora tritici-repentis*, the causal agent of tan spot, are partially responsible for outbreaks caused by this pathogen. Seed lots with a high incidence of *P. tritici-repentis* must be rapidly acquired for transmission and pathogen-control studies. Therefore, the aim of this study was to determine whether changes in water potential of culture medium and variations in inoculation time might favor the infection of wheat seeds by *P. tritici-repentis* without compromising seed viability. Colonies of *P. tritici-repentis* were grown on potato-dextrose-agar (PDA) culture medium, adjusted to a water potential of –0.36 MPa, under water stress induced by mannitol at potentials of –0.4, –0.6, –0.8, –1.0, and –1.2 MPa. Analyses were carried out to determine mycelial growth index and seed exposure time to the culture medium to start germination as a function of water potential. Afterwards, wheat seeds were placed in contact with colonies of *P. tritici-repentis* for 24, 48, and 72 hours at potentials of –0.4, –0.6, –0.8, –1.0, and –1.2 MPa. The experiment was arranged in a completely randomized design, in a factorial scheme (water potential × inoculation time). Rates of germination, seedling emergence in soil, and seed infection were assessed. Mycelial growth was stimulated at lower water potentials, which germinated faster. A 24-hour inoculation time and a –0.4 MPa water potential were efficient to infect wheat seeds with *P. tritici-repentis*, without hindering seedling germination and emergence under laboratory conditions.

Key words: *Drechslera tritici-repentis.* Tan spot of wheat. Mannitol. *Triticum aestivum*.

Resumo

Sementes de trigo infectadas com *Pyrenophora tritici-repentis*, agente causal da mancha-amarela da folha, respondem em parte pelas epidemias causadas por este patógeno. A obtenção rápida de lotes de sementes com alta incidência de *P. tritici-repentis* é fundamental para estudos de transmissão e controle. Sendo assim, o objetivo deste trabalho foi verificar se alterações no potencial hídrico do meio de cultura e variações na duração da inoculação podem favorecer a infecção de sementes de trigo por *P. tritici-repentis*, sem comprometer a viabilidade destas sementes. Colônias do patógeno foram cultivadas sobre meio de cultura batata-dextrose-ágar (BDA) básico, no potencial hídrico de –0,36 MPa, e no meio BDA com restrição hídrica, causada pela adição de manitol, nos potenciais hídricos de –0,4, –0,6, –0,8, –1,0 e –1,2 MPa. Foram realizadas análises para determinar o índice de crescimento micelial do fungo e o tempo de exposição das sementes ao meio de cultura para o início do processo germinativo em função

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dos potenciais hídricos. Posteriormente, sementes de trigo foram colocadas em contato com as colônias de *P. tritici-repentis* durante 24, 48 e 72 horas nos potenciais hídricos de –0,4, –0,6, –0,8, –1,0 e –1,2 MPa, em esquema fatorial (potenciais hídricos x duração da inoculação) e delineamento inteiramente casualizado. Foram avaliadas as taxas de germinação, de emergência de plântulas em solo e de infecção de sementes. Houve estímulo ao crescimento micelial nos menores potenciais hídricos, sendo que nesses potenciais, o tempo para início de germinação é maior. A duração da inoculação de 24 horas e o uso de restrição hídrica com potencial de –0,4 MPa foram eficientes para infectar sementes de trigo com *P. tritici-repentis*, sem prejudicar a germinação e a emergência de plântulas em condições de laboratório.
Palavras-chave: *Drechslera tritici*-repentis. Mancha-amarela. Manitol. *Triticum aestivum*.

Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechsler, the anamorphic form of *Drechslera tritici-repentis* (Died.) Shoemaker, is the causal agent of the tan spot (SANTANA et al., 2008), a major foliar blight disease in wheat-growing areas worldwide (PATEL et al., 2011). Seeds and crop residues infected are important epidemiological factors of this disease, serving as a source of primary inoculum (SCHILDER; BERGSTROM, 1995). Studies on factors promoting inoculation of *P. tritici-repentis* in wheat seeds are essential since they enable a rapid acquisition of high-incidence seed lots, for further studies. Knowing the specific relationships between pathogens and hosts is important to encourage research and information regarding transmission and epidemiological behavior of the diseases, pathogen control, and cultivar resistance.

Water stress is used to control or inhibit seed germination under different circumstances such as in health tests on paper substrate (CELANO et al., 2012) and potato dextrose agar medium (PDA) (GARCIA JÚNIOR et al., 2008), and in fungal inoculation tests (FARIAS et al., 2010), besides simulating water deficit (GIROTTO et al., 2012), osmotic and matric conditioning (QUEIROGA et al., 2011) for crops of interest. A water restriction condition can be obtained by adjusting water potential with osmotically active solutes, with the most common being mannitol, polyethylene glycol, maltose (GIROTTO et al., 2012), KCl, NaCl, sucrose (GARCIA JÚNIOR et al., 2008), or $CaCl_2$ (MACHADO NETO et al., 2006). Thus, bipolar water molecules are attracted and retained by these solutes, inducing a decrease in water activity (FERREIRA, 1988).

Water restriction techniques for fungal inoculation of seeds are based on seed-inoculum using a culture medium with known water potential. This stage should last long enough to favor infection, without seed germination midway through the inoculation. Therefore, the water potential of medium must be adjusted so that all germination preparatory processes can occur without inducing cell elongation and radicle extension (HEYDECKER et al., 1975). In this sense, several studies have succeeded in using water restriction for inoculation of phytopathogenic fungi in seeds such as *F. oxysporum* f. sp. *vasinfectum* (SOUZA et al., 2008), *Colletotrichum gossypii*, *C. gossypii* var. *cephalosporioides*, *Botryodiplodia theobromae* (MACHADO et al., 2004) in cotton seeds, *Diplodia maydis*, *Cephalosporium acremonium*, and *Fusarium moniliforme* in corn seeds (MACHADO et al., 2001a).

Selection of a proper water potential by means of solutes, as well as the time required for seed exposure to inoculum, are essential for inoculation to succeed. Therefore, this study aimed to determine whether changes in water potential of culture medium and variations in inoculation time might favor infection of wheat seeds by *P. tritici-repentis* without compromising seed viability for further transmission studies on the wheat-*P. tritici-repentis* pathosystem.

Material and Methods

Basic seeds of wheat cultivar TBIO Sintonia (Biotrigo Genética) were used in the experiment. They were obtained from the propagation of genetic seeds to guarantee genetic identity and varietal purity of the cultivar (FAEP, 2006). The seeds were harvested in the 2013 crop-growing season, being selected after preliminary tests of health, germination, and vigor. Initially, these seeds were subjected to surface disinfestation with 1% sodium hypochlorite for two minutes. *P. tritici-repentis* inoculum was kindly provided by the mycology collection of the Laboratory of Phytopathology, University of Passo Fundo – RS, Brazil. The inoculum was isolated from wheat leaves of cultivar BRS Guamirim, which was developed by the Brazilian Agricultural Research Corporation (EMBRAPA), being collected in the city of Coxilha – RS (Brazil). The inocula were refrigerated at nearly 7 °C on potato dextrose agar (PDA) culture medium, after monosporic isolation.

Preparation of culture medium at different water potentials

Mannitol was added to the PDA basic culture medium (39 g PDA Merck L^{-1} distilled water) to obtain five water potentials: –0.4, –0.6, –0.8, –1.0, and –1.2 MPa. An additional water potential of –0.36 MPa, known to be the water potential of the basic PDA medium, i.e. without adding mannitol (WEARING; BURGUESS, 1979), was used exclusively for tests of mycelial growth index (MGI) and time to start seed germination process. Amounts of 1.8, 8.8, 15.7, 22.5, and 29.1 g mannitol were added in 500 mL PDA medium before autoclaving to reach water potentials of –0.4, –0.6, –0.8, –1.0, and –1.2 MPa, respectively; these values were calculated by the software SPPM (MICHEL; RADCLIFFE, 1995). The temperature used in the calculations was 25 °C. After sterilization, 15 mL of basic PDA medium and those osmotically modified with mannitol were poured into 9-cm diameter plastic Petri dishes in a laminar flow chamber, according to the treatment.

Mycelial growth of P. tritici-repentis at different water potentials

Discs of 4.2 mm diameter were taken from the medial portion of 10-day incubation pure culture of *P. tritici-repentis*, being transferred to the center of each Petri dish with PDA. Ten replications were used for each water potential, being the dishes randomly distributed in a growth chamber with 12-hour photoperiod at 25 °C. Assessments were performed daily by measuring the colony diameter with a digital caliper, until the mycelium reached the edge of the dish. The MGI was determined by the formula: $MGI = C_1 + C_2 + \ldots + C_n / N_1 + N_2 + \ldots + N_n$ (OLIVEIRA, 1991), where C is the mycelial growth of colonies at each assessment and N is the number of running days at each assessment, after transferring the mycelium discs.

Exposure time of wheat seeds to P. tritici-repentis at different water potentials before the start of germination

Ten grams of wheat seeds was added per Petri dish with grown colony of *P. tritici-repentis* on culture medium with different water potentials, in five replications. Assessments were carried out every 12 hours. The time (in hours), within which a 4-mm length radicle appeared in the first seed, was counted at each water potential.

Seed-inoculum contact

Ten grams of wheat seeds were added per Petri dish with grown colony of *P. tritici-repentis* on culture medium with different water potentials, being gently pressed against the medium to increase seed-inoculum contact surface. After 24, 48, and 72 hours, seeds were removed from the medium and placed on filter papers to dry under laboratory

conditions for 24 hours. Afterwards, these seeds were assessed for paper substrate germination, soil emergence, and seed health on PDA medium.

Germination of wheat seeds inoculated with P. tritici-repentis

Four replications with 50 seeds were used per treatment (five water potentials × three inoculation lag times), in addition to a control treatment (non-inoculated seeds). The seeds were conditioned in discs of filter paper moistened with distilled water, using an amount of water 2.5 times the paperweight; these discs were then taken to a germinator at 20 °C. On the seventh day of incubation, the percentage of normally developed seedlings was computed.

Emergence of wheat seedlings inoculated with P. tritici-repentis

Inoculated seeds, submitted to different treatments (five water potentials × three inoculation lag times), were sown in plastic trays filled with a mixture of soil and sand mixed (2:1) at a depth of 1.5 cm. Four replicates with 50 seeds were used per treatment, in addition to a control treatment (non-inoculated seeds). Trays were randomly distributed in a growth chamber at 18 ± 2 °C and 12-hour photoperiod. The substrate was watered every two days with the same water volume for each tray. Fourteen days after sowing, the percentage of normal emerged seedlings was counted.

Health of wheat seeds inoculated with P. tritici-repentis

Seed health was assessed by using the plating method on PDA (BRASIL, 2009) using four replications with 50 seeds per treatment (five water potentials × three inoculation lag times), in addition to a control treatment (non-inoculated seeds). The seeds were disinfested with 1% sodium hypochlorite solution for two minutes and washed with sterile distilled water. The dishes were maintained in an incubation chamber at 25 °C and 12-hour photoperiod. Seven days after plating, colony general characteristics were assessed by the observation of white aerial mycelium and dark-olive creeping mycelium (MEHTA, 1993; WIESE, 1987). In addition, when present, conidia, and conidiophores of *P. tritici-repentis* were observed through a stereoscopic microscope, comparing them with those described in the literature (BARNETT; HUNTER, 1972). Results were given as a percentage of infected seeds in relation to the total number inoculated.

Statistical analysis

All the experiments were completely random designs. Data were analyzed using Assistat software (SILVA; AZEVEDO, 2002). The MGI data and seed germination starting time underwent analysis of variance (ANOVA) and regression analysis. Percentage data were arcsine square root transformed prior to ANOVA, performing a two-factorial analysis of variance (five water potentials × three inoculation times), in addition to a control treatment. Tukey's test was used as a means comparison test for each factor, and a Dunnett's test was performed to compare the treatments with the control.

Results and Discussion

Mycelial growth of P. tritici-repentis

A significant linear effect was observed for MGI as a function of the studied water potentials ($P<0.01$) (Figure 1). Decreasing water potentials of PDA medium with mannitol had no inhibitory effect on pathogen growth. Comparatively, Carvalho et al. (2001) observed that colonies of *C. lindemuthianum* presented a higher average diameter in an osmotically modified PDA medium with mannitol up to a water restriction level of –0.61 MPa. Likewise, Alam et al. (1996) observed that culture media with

osmotic concentrations between –0.3 and –1.0 MPa stimulated the mycelial growth of *Botrytis cinerea* Pers. and *Alternaria alternata* (Fr) Keissler. It might have occurred due to a higher solute absorption and better osmotic adjustment of fungal cells, providing a greater turgor for cell expansion and, therefore, a better stimulus to mycelial growth (DUNIWAY, 1979).

Figure 1. Mycelial growth index (MGI) of *Pyrenophora tritici-repentis* as a function of different water potentials of potato dextrose agar (PDA) culture medium. UPF, Passo Fundo, RS, Brazil, 2015.

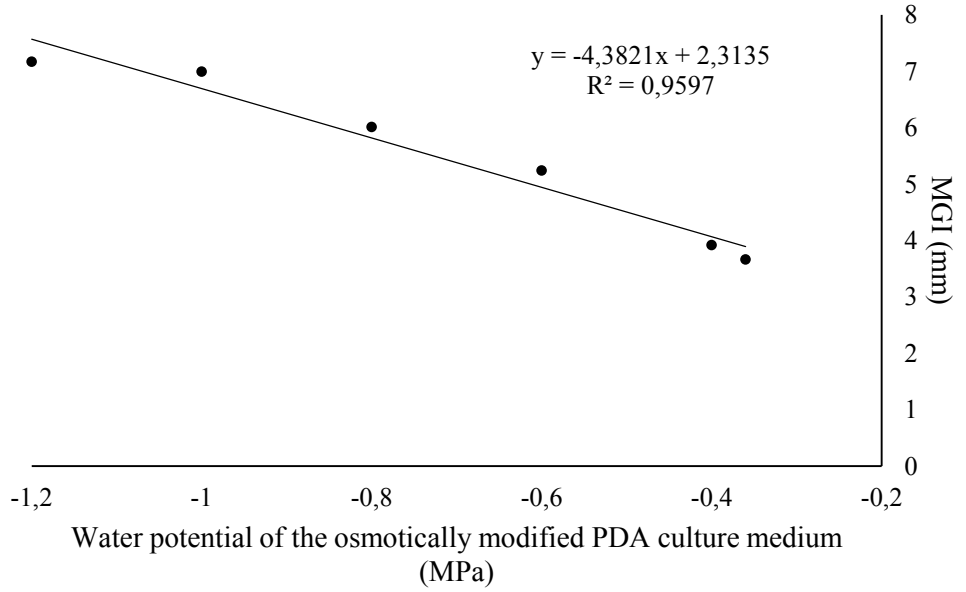
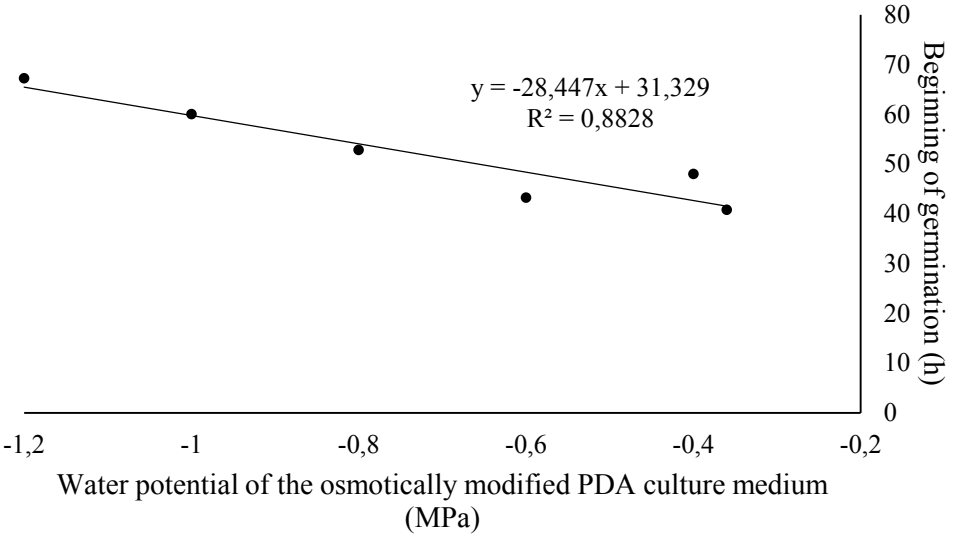


Figure 2. Germination starting time of wheat seeds (cultivar TBIO Sintonia) as a function of different water potentials of potato dextrose agar (PDA) culture medium. UPF, Passo Fundo, RS, Brazil, 2015.



Exposure time of wheat seeds to P. tritici-repentis at different water potentials

The time to start seed germination presented a significant linear response to the studied water potentials (P<0.01) (Figure 2). Lower water potentials promoted longer times to germination and hence a prolonged exposure to the pathogen. The minimum and maximum estimated time lags for the first seed germination were 41.6 and 65.5 h, both at the lowest and highest assessed water potential (-0.36 and –1.2 MPa, respectively). Although the decreasing potentials delayed the radicle emission, it occurred in all the tests. Solutes such as mannitol reduce the free energy of water since they cause disorder in the system (TAIZ; ZEIGER, 2009). Thus, the higher the amount of solute in the medium is, the lower the availability of water for seed germination. This statement justifies the delay in radicle emission at lower water potentials.

Germination of wheat seeds inoculated with P. tritici-repentis

Table 1 shows a significant interaction between water potentials and inoculation times. Germination had no effect of any water potential or inoculation time. At 24 hours of inoculation, germination percentage at –1.2 MPa was statistically the lowest, but not differing from –1.0 MPa. This might have occurred because, at this potential, seeds were deprived of water from the medium. At –1.2 MPa, germination was favored at an inoculation time of 48 hours, but not differing from that of 72 hours. At 48 hours of incubation, there was no statistical difference between the studied water potentials. On the other side, at 72 hours of incubation, germination at –0.4 MPa was statistically lower if compared to the others. Such an outcome might have been a consequence either of the higher water content absorbed by seeds or of the earlier germination start, as observed in this study, leading to a major loss in seed reserves.

Table 1. Germination percentage of wheat seeds (cultivar TBIO Sintonia) on filter paper after inoculation with *Pyrenophora tritici-repentis*, at different water potentials of culture medium and contact time with the pathogen. UPF, Passo Fundo, RS, Brazil, 2015.

Water potential (MPa)	Inoculation time (h)		
	24	48	72
-0.4	92 aA[1]	81 aA	39* bB
-0.6	95 aA	93 aA	85 aA
-0.8	95 aA	94 aA	77* aB
-1.0	80 abA	93 aA	88 aA
-1.2	77* bB	91 aA	85 aAB
CV (%)	8.33		

[1]Means followed by the same letter, lowercase on the column and uppercase on the row, do not differ from each other by the Tukey's test at 5% probability. *Value differs from the control treatment (non-inoculated) by the Dunnett's test at 5% probability, with 93% germination.

Emergence of wheat seedlings inoculated with P. tritici-repentis

Table 2 displays a significant interaction between water potentials and inoculation times for the emergence of normal seedlings. After 24 hours, all studied water potentials provided an emergence of normal seedlings statistically higher when compared to 48 and 72 hours, except for –0.8 MPa that was statistically equal at both 24 and 48 hours, and –1.2 MPa that was statistically higher at 48 hours. Similarly, Barrocas et al. (2014) demonstrated that longer times of contact between seeds and media with low water restriction, even in the absence of pathogens, compromised cotton

seedling emergence. In this sense, the reduced number of normal seedlings emerged after 72 hours of incubation might have been related to the inability of water potential in inhibiting the beginning of germination after 65 hours.

At the inoculation time of 24 hours, a similar behavior to the germination test was observed for seedling emergence in soil with water potential of –1.2 MPa, statistically lower when compared to the other water potentials. At 48 and 72 hours of incubation, the water potential of –0.4 MPa provided an emergence of normal seedlings statistically lower when compared to the other water potentials.

Table 2. Emergence percentage of wheat seedlings (cultivar TBIO Sintonia) in soil substrate and growth chamber after inoculation with *Pyrenophora tritici-repentis*, at different water potentials of culture medium and contact time with the pathogen. UPF, Passo Fundo, RS, Brazil, 2015.

Water potential (MPa)	Inoculation time (h)		
	24	48	72
-0.4	89 aA[1]	49* cB	19* bC
-0.6	88 aA	74* bB	54* aC
-0.8	84 aA	89 aA	54* aB
-1.0	82 aA	68* bB	59* aB
-1.2	71* bB	85 aA	53* aC
CV (%)	6.40		

[1]Means followed by the same letter, lowercase on the column and uppercase on the row, do not differ from each other by the Tukey's test at 5% probability. *Value differs from the control treatment (non-inoculated) by the Dunnett's test at 5% probability, with 90% germination.

Health of wheat seeds inoculated with P. tritici-repentis

Table 3 demonstrates a significant interaction between water potentials and inoculation time. All treatments differed statistically from the non-inoculated (10% infected seeds). It shows that the water restriction technique for infection studies with *P. tritici-repentis* is efficient at all the tested water potentials and inoculation times, varying from 49 to 82%. Fungus infection was higher after 24 hours of inoculation and at –0.4 MPa, after 48 hours at –0.8 MPa, and after 72 hours at –0.6 MPa. In short, 24 hours proved to be long enough for *P. tritici-repentis* infection, penetrating easily through the protective tissues of wheat seeds.

Farias et al. (2010) found similar results studying wheat seeds at different water potentials and inoculation times, noting that infection by *B. sorokiniana* was induced with an average efficiency of 53 to 92%. Several other studies have shown that infection efficiency depends on the pathogen species. In this sense, *Diplodia maydis* required 120 hours in contact with corn seeds to cause infection, contrasting with *F. moniliforme*, which presented a high incidence from 48 hours of exposure (MACHADO et al., 2001b), as well as *Phomopsis sojae*, *Sclerotinia sclerotiorum*, and *C. truncatum* in soybean (MACHADO et al., 2001a).

Table 3. Percentage of infected wheat seeds (cultivar TBIO Sintonia) on potato dextrose agar (PDA) medium after inoculation with *Pyrenophora tritici-repentis*, at different water potentials of culture medium and contact time with the pathogen. UPF, Passo Fundo, RS, Brazil, 2015.

Water potential (MPa)	Inoculation time (h)		
	24	48	72
-0.4	82* aA[1]	67* abAB	59* bB
-0.6	73* abA	71* abA	80* aA
-0.8	58* bAB	74* aA	56* bB
-1.0	59* bA	60* abA	53* bA
-1.2	64* abA	49* bA	55* bA
CV (%)	12.97		

[1]Means followed by the same letter, lowercase on the column and uppercase on the row, do not differ from each other by the Tukey's test at 5% probability. *Value differs from the control treatment (non-inoculated) by the Dunnett's test at 5% probability, with 10% infected seeds.

In summary, a culture medium with a water potential of –0.4 MPa and a inoculation time of 24 hours are efficient to infect wheat seeds with *P. tritici-repentis*, maintaining the percentage of germination and emergence of seedlings statistically equal to a non-inoculated treatment (control), justifying the use of water restriction for inoculating wheat seeds with *P. tritici-repentis*.

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