Detection of *Rhynchosporium secalis* in barley seeds from Argentina through polymerase chain reaction technique

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ABSTRACT

Leaf scald of barley caused by *Rhynchosporium secalis* is an important disease in Argentina. The fungus is a necrotrophic pathogen which survives in stubble, seeds and weeds. Isolation of *R. secalis* from seeds on artificial media usually has not been successful due to the slow growth rate of the pathogen and strong inhibition by contaminants. The objective in this work was to detect *R. secalis* in different genotypes of barley seeds in Argentina using the polymerase chain reaction (PCR)-based diagnostic assay. Four barley genotypes were tested in 2004: Quilmes Ayelén, Quilmes Alfa, Barke and Maltería Pampa 1004. The previously described RS8 and RS9 primers were used for the detection of *R. secalis* in barley seeds. A 264-bp single band was obtained for each cultivar showing the presence of *R. secalis*. The use of specific primers was efficient in the detection of *R. secalis* in barley seeds in Argentina and could be used for routine diagnosis, epidemiology and seed transmission studies. This is the first report on the detection of *R. secalis* in barley seeds in Argentina.

Additional keywords: *Hordeum vulgare*, diagnosis, PCR.

RESUMEN

Detección de *Rhynchosporium secalis* en semillas de cebada de Argentina através de la técnica de la reacción en cadena de la polimerasa

La escaldadura de la cebada causada por *Rhynchosporium secalis* es una importante enfermedad de la cebada en Argentina. El patógeno es necrotrófico y sobrevive en rastrojos, semillas y malezas. La detección de este patógeno por los análisis sanitarios de semilla tradicionales no son exitosos debido principalmente a su lento crecimiento y a la alta contaminación de otros microorganismos. El objetivo de este trabajo fue detectar *R. secalis* en diferentes genotipos de cebada usando la técnica de la reacción en cadena de la polimerasa (PCR). Cuatro genotipos de cebada fueron evaluados en 2004: Quilmes Ayelén, Quilmes Alfa, Barke y Maltería Pampa 1004. Se utilizaron los oligonucleótidos previamente descriptos RS8 y RS9. Como resultado se obtuvo una banda única de 264-pb en cada cultivar indicando la presencia de *R. secalis*. Esta técnica de detección provee más beneficios que los métodos rutinarios de detección y podría ser una herramienta útil para estudios de diagnóstico epidemiológicos y de transmisión del patógeno Este es el primer reporte sobre la detección de *R. secalis* en semillas de cebada en Argentina.

Palabras-clave adicionales: *Hordeum vulgare*, diagnóstico, PCR.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most important crop plants in Argentina due to the uppermost spread in the last few years as a result of malting exports to Brazil, integration into the Mercosur and the increase in domestic beer consumption. Barley scald caused by *Rhynchosporium secalis* (Oudem) J.J. Davis has been recognized as one of the most important diseases of barley due to its high level of prevalence, incidence and severity (Carmona & Barreto, 1995) (Figure 1). In the last surveys carried out in 2003, scald was confirmed as the most important disease for the southern pampeana region of Argentina (Carmona & Barreto, 2003). The main sources of *R. secalis* inoculum are seeds, infected crop residues and weed grasses, where the parasite remains viable in the saprophytic stage between the crop cycles (Caldwell, 1937; Skoropad, 1959). Seeds are the most efficient mean of fungus spread for both short and long distance. Although the microflora of barley grains has been investigated in Argentina, no seed infection by *R. secalis* was observed (Barreto & Carmona, 1993). The recovering of *R. secalis* from barley seeds in an artificial medium generally has not been successful due to the natural slow growth of the pathogen and the presence of contaminants (Kay & Owen, 1973). This is probably the reason why it has not been detected yet on seeds in Argentina (Barreto & Carmona, 1993). An assay carried out using polymerase chain reaction (PCR) allowed the detection of small concentrations of a specific DNA in a complex environment (Henson & French, 1993). PCR is highly sensitive

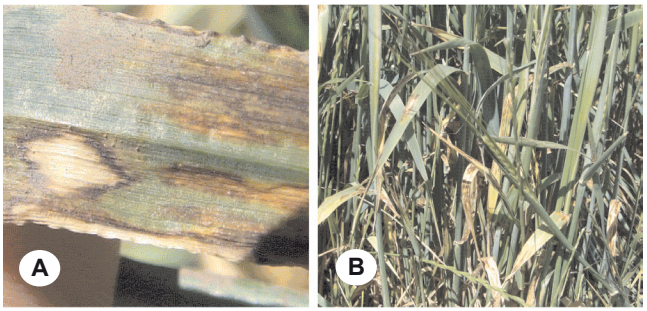

FIG. 1 - Typical scald lesion on leaf (**A**) and barley field (**B**).

and reproducible for amplification of diagnostic molecular markers and is used in detection of several pathogens. Lee *et al.* (2001) developed a PCR assay that successfully detected *R. secalis* in barley seeds, but barley samples from Argentina were not assessed. Among the primers designed, the RS8 and RS9 were the most efficient for the detection and discrimination of *R. secalis*. The objective in this work was to evaluate the presence of *R. secalis* in seeds of different genotypes of barley from Argentina using the polymerase chain reaction (PCR)-based diagnostic assay.

MATERIALS AND METHODS

Seed sampling

Four barley seeds genotypes were analyzed: Quilmes Ayelén, Quilmes Alfa, Barke and Maltería Pampa 1004, during the 2001-2002 season. The first three genotypes were collected in Tres Arroyos (Buenos Aires province) and the fourth one in an experimental field at Maltería Pampa (a malt producer company) in Coronel Suárez (Buenos Aires Province).

DNA extraction and PCR

Seed samples were ground to a powder in liquid nitrogen with mortar and pestle. DNA extraction was performed using CTAB based buffer from pools of 3-4 barley seeds and analyzed in a 1.5% agarose gel, then quantified in a GeneQuant pro spectrophotometer (Amersham Pharmacia, UK) in order to estimate the DNA concentration and purity (Sambrook *et al.,* 1989).

Primers RS8 and RS9, previously described by Lee *et al.* (2001) were used for *R. secalis* detection in barley seeds. These primers amplify a specific fragment of 264 bp from *R. secalis* genome. We amplified actine as a positive housekeeping gene control (upper actine: 5'GTGTTGGACTCTGGTGATGG3' (20 nucleotides, Tm=55ºC; designed upon 552 to 571 bp actine sequence: lower actine: 5'GGAAGCTCGTAGCTCTTCTC3'Tm = 55ºC, 20 nucleotides). In all cases we performed dilutions (1:10, 1:20 and 1:50) in order to reduce inhibitor effects of PCR.

Amplifications were performed on a PT-100 DNA thermocycler (MJ Research, USA). PCR master mix contained 10x PCR reaction buffer, 3.5 mM $MgCl^2$, 10 mM dNTPs (Invitrogen USA) and 0.5U Taq DNA polymerase (Invitrogen USA). PCR was performed under the following conditions: 94ºC (30 s) denaturing step followed by 27 cycles each consisting of a denaturation step at 94ºC (30 s), an annealing step at 66ºC (30 s) and extension at 72ºC for 2 min, and a final extension step at 72ºC (10 min).

RESULTS AND DISCUSSION

According to Lee *et al*. (2001) PCR products from undiluted DNA templates of each cultivar with RS8 and RS9 primers have not been observed by electrophoresis. However, when DNA dilutions were carried out, a single band was observed in each of them. Inhibitor effects of PCR reaction, commonly found in DNA extracts of vegetal material were overcome by DNA dilutions from 10-to 100-fold. The band size detected (Figure 2) was 264 in size, thus showing that primers amplified fungal DNA and that such fungus might be found in seeds. Likewise, we observed some differences in the band intensity among cultivars for each dilution, possibly due to differences in seed infection or different inoculum quantities present in seeds among cultivars. These differences in band intensities between cultivars could be explained by different infection percentages of the seeds with *R. secalis* (Parry & Nicholson, 1996; Lee *et al*., 2001; Lee *et al*., 2002). Differences in band intensities for the same cultivar were also observed, as expected, among the three dilutions carried out (Figure 2).

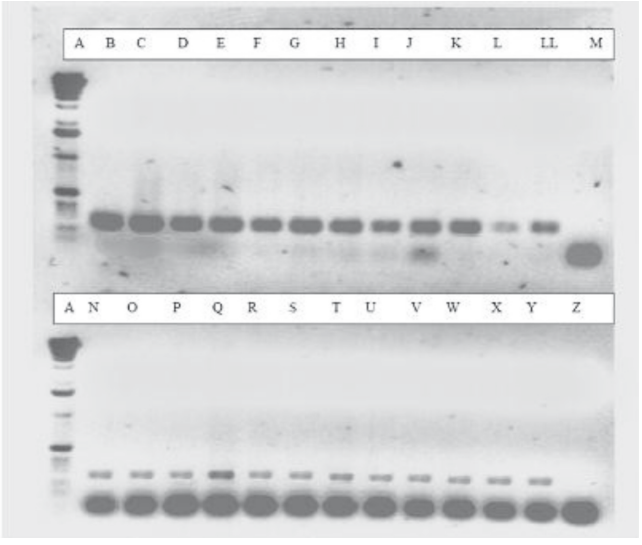

FIG. 2 - Detection of R. secalis in barley seeds from Argentina. **A.** 1Kb molecular marke); **B**., **C** and **D**. Ayelén cultivar dilutions 1:10, 1:20 and 1:50; E, F and G - Barke cultivar dilutions 1:10, 1:20 and 1:50); **H**., **I** and **J.** Alfa cultivar dilutions 1:10, 1:20 and 1:50; **K**., **L** and **LL**. MP1004 cultivar dilutions 1:10, 1:20 and 1:50; **M.** RS8 and RS9 primers negative control; **N**., **O** and **P.** Ayelén; **Q**., **R** and **S**. Barke; **T**., **U** and **V**. Alfa; **W**., **X** and **Y.** MP1004; **Z.** negative housekeeping gene control.

According to Barreto & Carmona 1993, *R. secalis* is not detected in seeds in Argentina by routine seed testing. However, Carmona *et al*. (1999) have not detected foliar scald infection either in fields without barley stubble nor secondary hosts showing seeds as a representative inoculum source. In this work, PCR technique applied to seed pathogen detection evidenced that infected seeds could be important for *R. secalis* survival and spread starting primary *foci* in epidemics of scald in Argentina. In addition, it may be responsible for the introduction of new *R. secalis* strains into new areas (Salamanti *et al*., 2000).

Even though *R. secalis* external symptoms observations were reported (Lee *et al.,* 2001) scald symptoms are confusing and symptomless seeds can be overlooked (Kay & Owen, 1973; Lee *et al.,* 1999b). In Argentina, similar symptoms of scald were observed in seed, but the causal agent was *Fusarium poae* (Peck) Wollenw and not *R. secalis* as expected (Barreto *et al.,* 2004).

R. secalis barley seeds infection commonly occurs when grain filling is exceeded in the floral bracts (Skoropad, 1959). This often happens in the lemma, immediately below the awn base. Other few incidences were detected in the palea. Typical lesions of scald represent a light centre and dark brown edges about 6-10 days after symptom appearance although infections do not always take place during the barley milky development stage to show scald symptoms in the lemma or palea external surface. However, the mycelium is present in the inner part of the floral bract surface as well as in the pericarp external surface and serves as a hidden source of primary inoculums. According to Doohan *et al*. (1998) and Lee *et al*. (2001) PCR assay is able to detect a pathogen before symptoms arise even in symptomless seeds.

Lee *et al.* (1999a) reported fungal isolation in lima bean agar in Canada as another diagnosis alternative. Scald symptoms was distributed over the lemmas, paleas, awns, glumes and rachises of the lima bean. By this method, the authors published the first report on successful isolation of the scald fungus from infected barley seed.

This is why the isolation of *R. secalis* through traditional method is recommended as an alternative one when no other methodology is available, taking into consideration the natural slow-growth rate of the pathogen. Polymerase chain reaction has been vastly used as a diagnosis method to make the detection of extremely small quantities of DNA. Due to the wide amplifying efficiency, PCR has been applied for the detection and quantification of *R. secalis* in barley(Lee *et al.,* 2002).

The use of specific primers was efficient in the detection of *R. secalis* in barley seeds in Argentina and could be used for routine diagnosis, epidemiology and seed transmission studies. The use of this technique for *R. secalis* detection could be used as a fast and reliable method to certify barley seeds in Argentina, thus avoiding the dissemination of the pathogen to new production areas in the country. This is the first report on the detection of *R. secalis* in barley seeds in Argentina.

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