Polish Journal of Microbiology
2008, Vol. 57, No 1, 77–79

SHORT COMMUNICATION

Polymorphism in the ITS Region of Ribosomal DNA of *Cochliobolus sativus* Isolates Differing in Xylanase Production

YASSER BAKRI, MOHAMMED JAWHAR and MOHAMMED IMAD EDDIN ARABI*

Department of Molecular Biology and Biotechnology, AECS, Damascus, Syria

Received 26 October 2007, revised 25 November 2007, accepted 30 November 2007

Abstract

The restriction of PCR-amplified internal transcribed spacers (ITS) of ribosomal DNA was used to confirm the genetic variation among 22 isolates of *Cochliobolus sativus* differing in their xylanase production. Results show a high level of diversity of ITS-RFLP markers among the isolates. The molecular parameter used showed that *C. sativus* isolates reside in three phylogenetic groups. There was observed the resolution between clustering of isolates and their xylanase production level.

Key words: *Cochliobolus sativus*, genetic diversity, ITS-RFLP, xylanase

Xylanase have gained increasing attention because of their various biotechnology applications (Buchert *et al.*, 1994; Wong *et al.*, 2000). Although xylanases from bacteria and archeons have considerable higher temperature optima and stability than those of fungi, the amount of enzyme produced by these microorganisms is comparatively lower than that produced by fungi (Singh *et al.*, 2003). Filamentous fungi, particularly those representing *Cochliobolus* genus, are a useful producers of xylanase from the industrial point of view. The reasons is that they are capable of producing high levels of extra cellular enzymes and can be cultivated very easily. Several enzymatic activities were identified in different isolates of the fungus *Cochliobolus sativus*, the causal agent of barley spot blotch disease, such as cellulose-hydrolysing enzymes, endo-1, 4-β-xylanase and endopolygalacturonase (Peltonen, 1995; Geimba *et al.*, 1999). Very little is known about the genetic variation among *C. sativus* isolates in relation to their xylanase production ability.

Molecular methods involving the use of the polymerase chain reaction (PCR) have recently been proposed to resolve genetic variation in various organisms. The internal transcribed spacer (ITS) region of the nuclear rRNA repeat units, evolve faster, and may vary among species within a genus or among populations (Redecker *et al*., 1997; Hsiang and Wu, 2000; Martin and Rygiewicz, 2005).

The objectives of the present study were (i) to investigate, on artificial growth media, the xylanase production by *C. sativus* isolates collected from different regions of Syria, and (ii) to study the relationship between xylanase production and ITS-RFLP profiles.

Over several years, more than 117 isolations of *C. sativus* were obtained from leaves and subcrown internodes of barley and wheat showing spot blotch and common root rot symptoms. Each isolate was grown separately in 9 cm Petri-dish containing potato dextrose agar (PDA, Difco) and incubated for 10 days, at 22±1°C in the dark to allow mycelia growth. During a preliminary study, 22 isolates of *C. sativus* selected on the basis of cultur morphology and virulence (Arabi and Jawhar, 2003; 2004) were selected for this study.

Xylanase was assayed by the optimized method described by Bailey *et al.* (1992), using 1% birchwood xylan as substrate; The solution of xylan and the enzyme at appropriate dilution were incubated at 55°C for 5 min and the reducing sugars were determined by the dinitrosalicylic acid procedure (Miller, 1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol xylose/ml/minute under the described assay conditions. All experiments were repeated twice. Statistical analyses were performed using the Statview program (Abacus concepts,

* Corresponding author: M.I.E. Arabi, Department of Molecular Biology and Biotechnology, AECS, P.O. Box 6091, Damascus, Syria; fax: (965) 11 6112289; **e-mail: miaraabi@aec.org.sy**

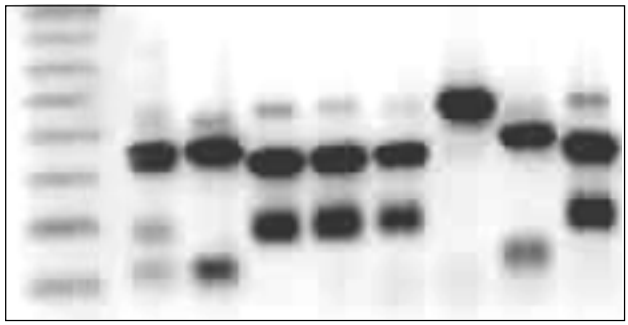

Fig 1. Agarose gel electrophoresis of the ITS-RFLP for 8 *C. sativus* isolates digested by the restriction enzyme XbaI. M – marker ladder 1 Kb.

1996) to test for differences in xylanase production among isolates.

Twenty-two isolates were grown on PDA medium for 2 weeks at 21 ± 1°C and stored at 4°C for further study. Mycelium was harvested and DNA was extracted according to standard protocols (Leach *et al.*, 1986), resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at –20°C.

The ITS regions and the 5.8S rDNA were amplified for all the isolates using the primers ITS1 (5'TCCGTA GGTGAACCTGCGG3') and ITS4 (5'TCCTCCGC TTATTGATATGC3') designed by White *et al.* (1990). Amplification reactions (25 µl) contained 1×PCR buffer, 1 U *Taq* polymerase (MBI Fermentas), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 10 ng of genomic DNA per reaction mixture. PCR was performed in a Gene Amp 9700 Thermocycler (Applied Biosystems, USA). Initial denaturation of 95°C for 2 min was followed by 36 cycles of 94°C for 1 min, 57°C for 1 min (primer annealing), 72°C for 1 min (primer extension). A final extension of 72°C for 10 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples. PCR products were visualized using UV light and separated in 1.5% agarose gel following staining with ethidium bromide.

In separate reactions, 10 µl of PCR reaction was digested for 3 h with six different endonucleases (AluI, EcoR1, HpaII, XbaI, RsaI and HaeIII) following manufacturer's recommendations (MBI Fermentas). The DNA fragments were size-fractionated by electrophoresis through 1.5% agarose gels. The sizes of amplification products were determined by comparison with their molecular weight relative to a DNA ladder (Q.BIOgene). Amplicons were electrophoresed in 1.5% agarose gels that were photographed under UV light (302 nm) after staining with ethidium bromide, and presence (1) or absence (0) of bands was recorded. The experiments were repeated twice for each isolate to confirm the repeatability and the monomorphic bands were removed from the analysis. The data were converted to a distance matrix using Nei and Li's (1979) coefficient, which was used to construct a dendrogram by the UPGMA (unweighted pair-group method with arithmetic averages) provided on the computer package (Statsoft, 2003).

PCR amplification with specific primers ITS1 and ITS4 yielded single DNA fragments, present in all isolates with ~600 bp in size. The fingerprints generated from the six restriction digestion of nrDNA ITS region demonstrated high levels of intraspecific variability within the *C. sativus* population (Fig. 1). For the enzymes AluI, HaeIII and HpaII, six distinct patterns were recognized, for XbaI there were three and for EcoR1 and for RsaI there were two. The differentiation among isolates could be due to selection occurring in these isolates. However ITS-RFLP analysis can reveal variations within a small region but the banding patterns are very stable and consistent (Farmer and Sylvia, 1998). In contrast, while RAPD banding patterns are much more influenced by components and conditions of the PCR. ITS-RFLP markers have been suggested by several authors for investigating genetic variation in different fungal populations (Hsiang and Wu, 2000; Geln *et al.*, 2001).

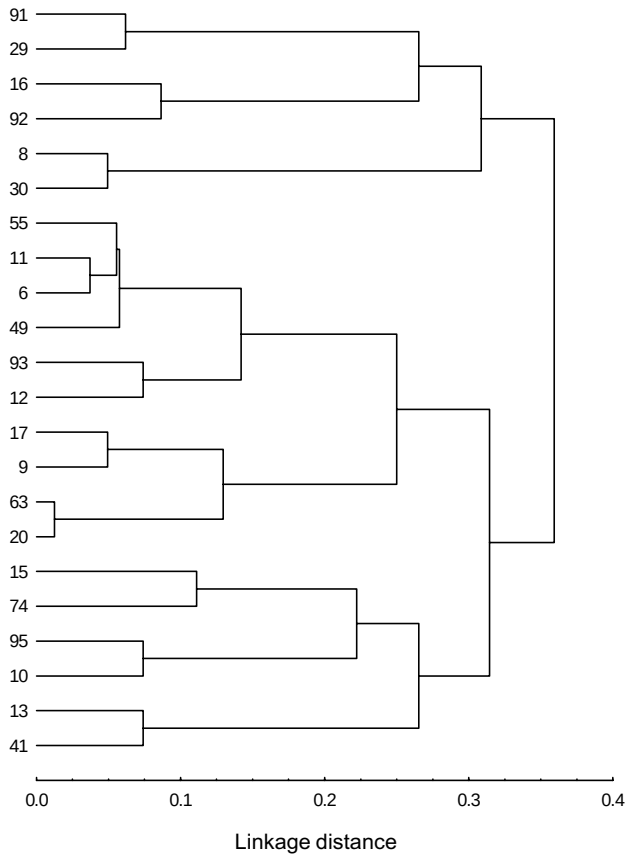

Fig. 2. UPGMA dendrogram generated from restriction fragment length polymorphisms of the ITS region using enzymes EcoRI, AluI, HpaII, XbaI, Rsa and HaeIII, for 22 isolates of *C. sativus* from Syria.

LSD (5%): Least Significant Differences at $P<0.05$.

Significant differences ($P<0.05$) in the mean yield values were detected among isolates, with high values being consistently higher in the isolate C29 with mean value 32.53 U/ml (Fig. 2). Dendrogram analysis of the ITS-FRLP profiles (Fig. 2) showed that the isolates of *C. sativus* clustered into three groups. Good correlation existed between the differentiation of the isolates according to the ITS-RFLP analysis and their capacity to produce xylanase enzyme. However, despite the high variation observed, ITS-RFLP profiles exhibited some bands that were common to strains yielded high xylanase, and absent to that produced less xylanase.

On the other hand, the results show that among all tested isolates; C29, C91 and C8 isolates were the highest xylanase producers with a small genetic distance value (Fig. 2), which might be attributed to the genetic differentiation (DNA fragments) between species. However, these isolates could be a good candidate for biotechnological applications.

The patterns generated from ITS-RFLPs demonstrated variability among *C. sativus* isolates. While this variability may have arisen through point mutations, gene flow and/or recombination (Parry *et al.*, 1995), we were not able to determine which, if any, of these particular mechanisms was responsible for the high degree of genetic diversity observed.

Numerical analysis of profiles obtained with the selected ITS-RFLP showed genetic diversity among the isolates and allowed clear differentiation of *C. sativus*. There was resolution between clustering of isolates and their xylanase production. However, PCR-RFLP analysis of the ITS is a simple, rapid and reproducible technique that doe not involve time-consuming DNA hybridization. The potential discrimination ability described in this paper suggests that this method is suitable for large-scale characterization of *C. sativus* isolates differing in xylanase production.

Literature

Abacus concepts, 1996. Statview 4.5 statistical program. Abacus Concepts Corporation, Berkeley, CA, USA.

Arabi M. I. E. and M. Jawhar. 2003. Pathotypes of *Cochliobolus sativus* (spot blotch) on barley in Syria. *J. Plant Pathol.* 85: 193–196.

Arabi M.I.E. and M. Jawhar. 2004. Identification of *Cochliobolus sativus* (spot blotch) isolates expressing differential virulence on barley genotypes in Syria. *J. Phytopathol.* 152: 461–464.

Bailey M.J., P. Bailey and R. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotech*. 23: 257–270.

Buchert J., M. Tenkanen, A. Kantelinen and L. Vilkari. 1994. Application of xylanase in the pulp and paper industry. *Bioresource Tech.* 50: 65–72.

Farmer D.J. and D.M. Sylvia. 1998. Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. *Mycol. Res.* 74: 859–865.

Geimba M. P., A. Riffel, V. Agostini and A. Bandelli. 1999. Characterization of cellulose-hydrolysing enzymes from the fungus *Bipolaris sorokiniana*. *J. Sci. Food Agric.* 79: 1849–1854.

Geln M., I.C. Tommerup, N.L. Bougher and P.A. O'Brien. 2001. Interspecific and intraspecific variation of ectomycorrhizal fungi associated with Eucalyptus ecosystems as revealed by ribosomal DNA PCR-RFLP. *Mycol. Res.* 105: 843–858.

Hsiang T. and C. Wu. 2000. Genetic relationships of pathogenic Typhula species assessed by RAPD, ITS-RFLP and ITS sequencing. *Mycol. Res.* 104: 16–22

Leach J., D.B. Finklstein and J.A. Ramosek. 1986. Rapid miniprep of DNA from filamentous fungi. *Fungal Genet. Newsl.* 33: 32–33.

Martin K.J. and P.T. Rygiewicz. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microb.* 5: 21–28.

Miller G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* 31: 426–428.

Nei M. and W.H Li. 1979. Mathematical model for studying genetic variation in terms of restricting endonucleases. *Proc. Natl. Acad. Sci*. USA 76: 5269–5273.

Parry D.W., P. Jenkinsen and L. Mcleod. 1995. Fusarium ear blight (scab) in small grain cereals – a review. *Plant Pathol*. 44: 207–238.

Peltonen S. 1995. Comparison of xylanase production by fungal pathogens of barley with special reference to *Bipolaris sorokiniana*. *Mycol. Res.* 6: 717–723.

Redecker D., H. Thierfelder, C. Walker and D. Werner. 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Appl. Environ. Microb.* 63: 1756–1761.

Singh S., B. Pillay and B.A. Prior. 2003. Thermal stability of β-xylanase produced by different *Thermomyces lanuginosus* strains. *Enzyme Microb. Technol.* 26: 502–208.

Statsoft 2003. STATISTICA, data analysis software system, 6th ver.' Available at www.statsoft.com (verified 25 October 2006).

White T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand D.H., Sninsky J.J. White T.J. (eds), *PCR Protocols; a Quide to Methods and Applications*, pp. 315–322. Academic Press, San Diego, CA, USA.

Wong K.K.Y., C. S. James and S.H. Campion. 2000. Xylanase pre and post-treatments of bleached pulps decrease absorption coefficient. *J. Pulp Pap. Sci.* 26: 377–383.