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EXPERIMENTAL ARTICLES

Heterogeneity in the ITS of the Ribosomal DNA of *Pyrenophora graminea* Isolates Differing in Xylanase and Amylase Production[1]

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Abstract—Xylanase and amylase have gained increasing interest because of their various biotechnology applications. In this research, the restriction of PCR-amplified internal transcribed spacers (ITS) of ribosomal DNA (rDNA) was used to confirm the genetic variation among 22 isolates of *Pyrenophora graminea* differing in their xylanase and amylase production. The fingerprints generated from the six restriction digestions of the rDNA ITS region showed high levels of intraspecific variation within the *P. graminea* population. Neighbour–Joining diagram, based on Nei's genetic distances, showed that isolates formed two phylogenetic groups. No apparent association could be observed between xylanase and amylase production and genetic diversity among the twenty-two isolates.

Keywords: *Pyrenophora* graminea, xylanase, α-amylase, genetic diversity, ITS-RFLP.

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Barley diseases caused by *Pyrenophora* spp. result in severe economic losses for growers in many different countries [1, 2]. As plant pathogens, *Pyrenophora* species primarily direct their attack on the cell wall by producing key enzymes associated with ITS hydrolysis [3, 4]. Hydrolytic enzymes that may be used for this purpose include polygalacturonase, amylase, cellulose, pectin-methylesterase [5] and β-1,4-xylanase [6].

Identification of *P. graminea* isolates requires some expertise in taxonomy, may be complicated by variation in morphological features among isolates, and is time consuming task particularly in such cases when similar species may be present in the same field [7].

Molecular methods involving the use of the polymerase chain reaction (PCR) have recently been proposed to resolve genetic variation in various organisms. The restriction fragment length polymorphisms (RFLP) of PCR amplified of internal transcribed spacers (ITS) region of the nuclear rRNA repeat units, evolve faster, and may vary among species within a genus or among populations [ITS-RFLP; e.g., 8, 9].

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

MATERIALS AND METHODS

Fungal isolates. The 22 monosporic isolates of *P. graminea* are described by Arabi et al. [10]. They were isolated from infected barley leaves showing leaf stripe symptoms, and screened among 93 isolates for their host-pathogen reaction and lesion formation. The isolates were grown separately in 9 cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) and incubated for 10 days, at 22 ± 1°C in the dark to allow mycelia growth.

Liquid culture. Enzymes were produced by inoculating 1-ml spore suspension (10^6 spores/ml) of each *P. graminea* isolate in Erlenmeyer flasks (250 mL) containing 50 ml of culture medium [(wheat bran (1% w/v)] as carbon source and yeast extract (0.5% w/v) in numeral salt medium ($Na_2HPO_4 \times 2H_2O$ (1% w/v), KCl (0.05% w/v), $MgSO_4 \times 7H_2O$ (0.015% w/v)]. The pH was adjusted to 6.5 before sterilization. After inoculation, the flasks were incubated at 30°C in a rotatory shaker at 120 rpm for 5 days. At each sampling time, the culture medium was vacuum filtered using Whatman n° 41 filter paper (fast-flow rate), and the filtrate was used for further enzymatic assays.

Enzyme assay. Xylanase was assayed by the optimized method described by Bailey et al. [11], 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 [12], 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

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releasing 1 μmol xylose/ml per minute under the described assay conditions.

α-Amylase activity was measured as described by Okolo et al. [13]; the reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid method of Miller [12], the blank contained 0.5 ml of 0.1 M acetate buffer (pH 5.0), 125 ml of 1% starch solution and 0.25 ml of distilled water. One unit (*U*) of amylase activity is defined as the amount of enzyme releasing 1 μmol glucose/ml per minute under the described assay conditions.

The experiments were repeated twice, and all the results represent mean values. Statistical analyses were performed using the STAT-*I*TCF program [14] to test for differences in xylanase and amylase production among different isolates tested.

DNA extraction. Twenty-two isolates were grown on PDA medium for 2 weeks at 20 ± 1°C and stored at 4°C for further study. Mycelium was harvested and DNA was extracted according to standard protocols [15], resuspended in TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) and stored at –20°C.

ITS-RFLP analysis. The ITS regions and the 5.8S rDNA were amplified for all isolates using the primers ITS 4R (5' TCCGTAGGTGAACCTGCGG 3') and ITS 5F (5'TCCTCCGCTTATTGATATGC 3') designed by White et al. [16]. Amplification reactions (25 μl) contained 1× PCR buffer, 1 U *Taq* polymerase (MBI Fermentas, York, UK), 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, 72°C for 1 min. A final extension of 72°C for 10 min was, followed by cooling to 4°C until recovery of the samples. PCR products were separated on a 1.5% agarose gel and visualized using UV light (302 nm) after staining with ethidium bromide.

In separate reactions, 10 μl of PCR product was digested for 3 h with six different endonucleases (*Alu*I, *EcoR*1, *Bsur*I, *Bam*HI, *Rsa*I and *Hind*III) following the manufacturer's recommendations (MBI Fermentas, York, UK). The DNA fragments were size-fractionated by electrophoresis through 1.5% agarose gels. The sizes were estimated by comparison with a DNA ladder (Q.BIOgene, Heidelberg, Germany).

RFLP banding profiles for each restriction enzyme were scored for the presence (1) or absence (0) of bands. The experiments were repeated twice for each isolate to confirm the repeatability and the monomorphic bands were removed from the analysis). Neighbour–Joining diagrams and bootstrap analysis were constructed on genetic distances among populations using the Nei's distance [17] by the PHYLIP package ver 3.5c [18].

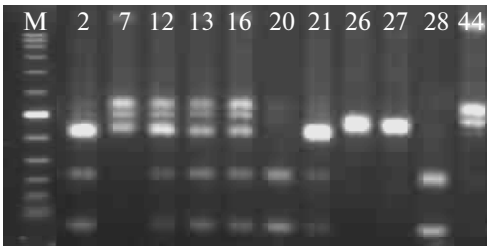

Fig. 1. Agarose gel electrophoresis of restriction fragments of the ITS amplification products of 11 *P. graminea* isolates digested by the restriction enzyme *Hind*III, M represents the 100-bp DNA marker (*Hinf*I; MBI Fermentas, York, UK).

RESULTS AND DISCUSSION

PCR consistently yielded single amplification product of approximately 650 bp, present in all isolates. A total of 594 bands were scored of which 453 (76.3%) were polymorphic and the number of DNA bands varied between 5 and 17. The fingerprints generated from the six restriction digestions of the nrDNA ITS region demonstrated high levels of intraspecific variation within the *P. graminea* population. For *Rsa*I and *Bsur*I, three distinct patterns were recognized, one for each of *EcoR*1, *Bam*HI and *Alu*I, and five for *Hind*III (Fig. 1).

Significant differences ($P < 0.05$) in the mean yield values of xylanase and amylase were detected among isolates, with high values being consistently higher in the isolate *Pg*16 with mean value 14.26 U/ml for xylanse, whereas the two isolates *Pg*24 and *Pg*14 produced high mean values of amylase 2.56 and 2.61 U/ml, respectively (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.

Dendrogram analysis of the ITS profiles showed that the isolates of *P. graminea* clustered into two groups (Fig. 3). No apparent correlation was existed between the differentiation of the isolates according to the ITS-RFLP analysis and their capacity to produce both xylanase and amylase enzymes, similar results were obtained by Zhou and Stanosz [19] on *Botryosphaeria* isolates. Saldanha et al. [20] also found that no correlation was present between cell wall-lytic enzymes production and genetic diversity of *Botryosphaeria* isolates using ITS region. In contrast, Bakri *et al.* [21] observed a resolution between clustering of *Cochliobolus sativus* isolates and their xylanase production level.

The patterns generated from ITS-RFLPs demonstrated variability among *P. graminea* isolates. While this variability may have arisen through point mutations, gene flow and/or recombination [22], we were not able to determine which, if any, of these particular

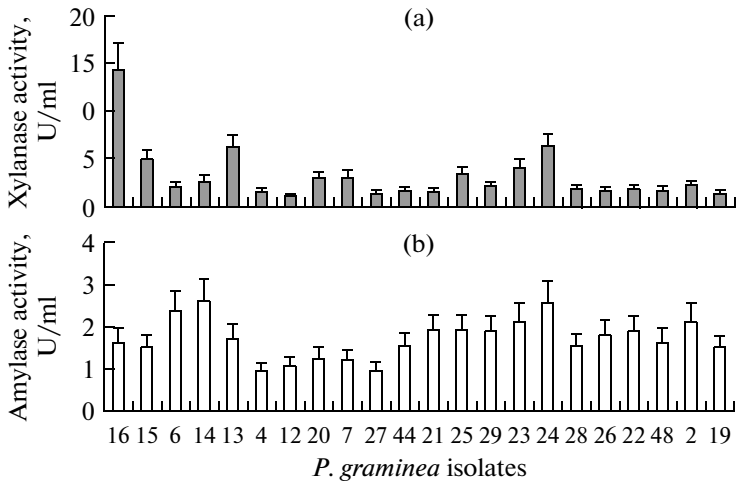

Fig. 2. Xylanse (a) and amylase (b) production by *Pyrenophora graminea* isolates grown in submerged liquid using 1% wheat bran as substrate.

mechanisms was responsible for the high degree of genetic diversity observed.

The present results confirm the findings of Arabi et al. [10] on the genetic diversity of Syrian *P. graminea*. Although different polypeptide patterns were observed among the fungal isolates, the authors suggested the use of other markers to better clarify genetic diversity in Syrian *P. graminea* populations. In this study, ITS-RFLP was applied and new types of polymorphism were identified. This method reveals variation within a small region of the genome but the banding patterns obtained are very stable and consistent [8].

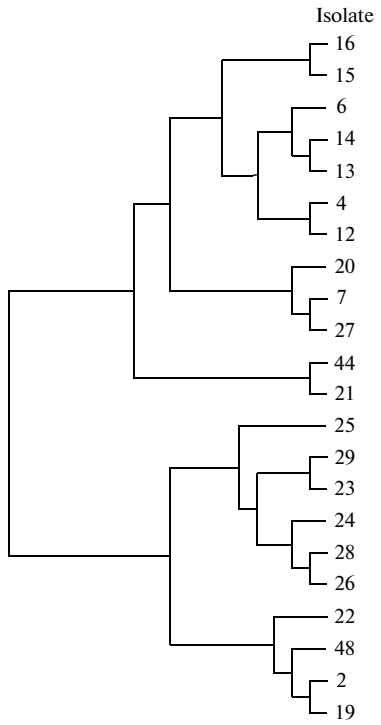

Fig. 3. Neighbour–Joining dendrogram generated from restriction fragment length polymorphisms of the ITS region using enzymes *Alu*I, *Eco*R1, *Bsur*I, *Bam*HI, *Rsa*I and *Hind*III, for 22 isolates of *Pyrenophora graminea* from Syria.

Numerical analysis of profiles obtained with the selected ITS-RFLP showed genetic diversity among the isolates and allowed clear differentiation of *P. graminea*. No apparent association could be observed between xylanase and amylase production and genetic diversity among the twenty-two isolates. 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 *P. graminea* isolates differing in xylanase and amylase production.

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