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EXPERIMENTAL ARTICLES

Molecular and Xylanolytic Variation Identified among Strains of *Pyrenophora Graminea*[1]

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Abstract—The inter-retrotransposon amplified polymorphism (IRAP) was used to confirm the genetic variation among 22 strains of *Pyrenophora graminea* differing in their xylanase production. A total of 162 bands were scored of which 151 (93.21%) were polymorphic. The molecular parameter used showed that *P. graminea* strains reside in four phylogenetic groups. There was observed the resolution between clustering strains and their xylanase production. Hence, the described approach presented here constitutes no prior assumption about the characterization of *P. graminea* strains differing in xylanase production.

Keywords: *Pyrenophora graminea*, genetic diversity, IRAP, xylanase, submerged culture.

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Xylanase has attracted considerable research interest because of their biotechnology applications [1, 2]. Although xylanase from eubacteria and archaebacteria have considerable higher temperature optima and stability than those of fungi, the amount of enzyme produced by these bacteria is comparatively lower than that produced by fungi [3, 4].

Filamentous fungi, particularly those representing *Pyrenophora* genus, are a useful producer of xylanases from an industrial point of view. The reasons are that they are capable of producing high levels of extra cellular enzymes and can be cultivated very easily. However, very little is known about the genetic variation among *P. graminea* strains 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. Inter-Retrotransposon Amplified Polymorphism (IRAP) is a PCR based technique which detects a high level of polymorphism which does not need DNA digestion, ligations or probe hybridization to generate marker data, thus increasing the reliability and robustness of the assay [5, 6].

IRAP method has recently been exploited to study genetic diversity and phylogenetic in plant species [7, 8] and fungal pathogens [6]. The objectives of the present study were (i) to investigate, on artificial growth media, the xylanase production by *P. graminea* strains collected from different regions of Syria, and (ii) to study the relationship between xylanase production and IRAP profiles.

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MATERILAS AND METHODS

Microorganism. Over several years, more than 93 isolations of *P. graminea* were obtained from barley leaves showing leaf stripe symptoms. Each isolate was grown separately in 9 cm Petri-dish containing potato dextrose agar (PDA, Difco, Detroit, MI. USA) and incubated for 10 days, at 21 ± 1°C in the dark to allow mycelia growth. During a preliminary study, 22 strains of *P. graminae* selected on the basis of cultural morphology and virulence [9, 10] were selected for this study.

Enzyme production. Spore suspensions were made from ten-day-old cultures that had been grown on PDA slopes at 22 ± 1°C. Sterile distilled water was aseptically added to each slope and a suspension of the spores was made by gently brushing the mycelium with a sterile wire loop. The suspension of each strain, where necessary, was diluted with sterile distilled water to give a final spore account of 10^6 spores / ml. The strains were screened for xylanase production in Erlenmeyer flasks (100 ml) containing 25 ml of basal culture medium (g/l); 10.0, wheat bran, 5.0, yeast extract, 10.0, $Na_2HPO_4 \cdot 2H_2O$, 0.5, KCl, 0.15, $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.5 before sterilization. The flasks were sterilized at 12°C for 20 min. Fresh fungal spores have been used as inoculums and the flasks were incubated at 30°C for 5 days in a rotary shaker (120 rpm).

Enzyme assays. Xylanase activity was assayed by the optimized method described by [11], using 1% birchwood xylan as substrate; The solution of xylan and the enzyme at an appropriate dilution were incubated at 55°C for 5 minutes and the reducing sugars were determined by the dinitrosalicylic acid procedure

Table 1. Primer names, orientation, sequences and polymorphism in 22 strains of *P. graminea*

Name	Orientation		Retrotransposons/Sequence	Total number of bands	Polymorphic bands	Percentage polymorphism
D12	TDK2	→	GAA GTT AGT GGG AGC AAA AGA TGT			
D13	TDK2	←	TAC CAA TGT CGG GAG GCT TGT GTC A	47	42	89.39
D14	TDK14	→	GTT TGT GAT AGA ACT TGG GTT TGC T			
D15	TDK15	←	AGA CTT GGT CCA TCC TTC CTT TAG A	15	13	86.6
D16	TDK16	→	AGG TAT GGT TTC AAG ATG ATG GAT G			
D17	TDK17	←	ACC CGC TGG TTG TGT CAG ATA GAT T	25	24	96
D18	TDK18	→	ATA CAA CAG ACT CAA TGC CGA CCC T			
D19	TDK19	←	ACC TGC CAA CCA ACT TCT TTT CCT C	22	21	95.45
D20	TDK13	→	TCC TGA TGG GAA CTT CGT TGC TCG T			
D21	TDK13	←	CCT GAC ACC TCA AAA CCT TCT GGC T	53	51	96.23
Total				162	151	93.21

[12], with xylose as a 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 per minute under the described assay conditions. The experiments were repeated twice. Statistical analyses were performed using the STAT-ITCF program [13]. A Newman-keuls test was used to test the differences in xylanase activity among strains.

DNA extraction. Twenty two strains 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 [14], resuspended in TE buffer (10 mM *Tris*-HCl, pH 8.0; 1 mM EDTA) and stored at –20°C.

Z-IRAP analysis. The IRAP method was carried out as previously described by [5, 7]. The primer sequences, retrotransposon type, and orientation are shown in Table 1. The reactions of 20 μl contained: 0.075 M *Tris*-HCl pH 8.8, 0.02 M $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM dNTPs, 0.2 μM each primer, 1.5 U *Taq* polymerase, and 10 ng DNA template. The primer sequences and orientation are shown in Table 1. PCR was performed in a Thermocycler (BIO-RAD system, USA). Initial denaturation of 95°C for 2 min was followed by 35 cycles [1 cycle consists of denaturation for 1 min at 95°C, annealing for 1 min at 45°C and extension for 2 min at 72°C]. A final extension of 72°C for 5 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples.

Amplified products were electrophoresed in a 2% agarose gel using 1 × *Tris*-borate-EDTA buffer (100 mmol Tris-HCl/L, pH 8.3, 83 mmol boric acid/L, 1 mmol EDTA/L) at 100 V. The gels were stained with ethidium bromide (0.5 μg/mL) solution and visualized under ultraviolet illumination. Sizes of the amplified products were determined relative to a 100-bp DNA ladder (Q.BIOgene, Heidelberg, Germany).

The reproducibility of DNA profiles was tested by repeating the PCR amplification with each of the selected primers. Each amplified fragment was treated as a unit character and scored as a binary code 1 and 0 for presence or absence, respectively, using 1Dscan eX 3.1 software (Scanalytics, Inc). The software was set up to score only sharp and prominent bands. The data were converted to a distance matrix using Nei and Li's [15] coefficient, which was used to construct a dendrogram by the UPGMA (un-weighted pair-group method with arithmetic averages) provided on the computer package [16].

RESULTS AND DISCUSSION

IRAP primer combinations produced many distinctively amplified fragments ranging in size from 100 to 1800 bp. Based on the electrophoretic pattern, a polymorphism of 93.21% was observed. A total of 162

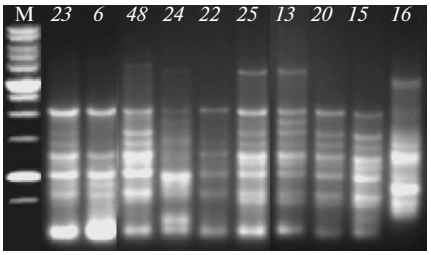

Fig. 1. Agarose gel electrophoresis of the IRAP (primers D12 and D13) for 10 *P. graminea* strains. M - Marker ladder 1 Kb.

bands were amplified using 1RAP assay of which 151 were polymorphic (Table 1). The maximum amplification was observed from D20 and D21 and the lowest was from D14 and D15 primer combinations (Table 1).

Significant differences ($P < 0.05$) in the mean yield values of xylanase were detected among strains, with high values being consistently higher in the strain *Pg*16 with mean value 14.26 U/ml (Fig. 2). Xylanase activity by different fungi is shown in Table 2. Here, xylanase activity was produced which was comparable to or higher than that reported for most fungal species in submerged cultivations (Table 2).

Dendrogram analysis of the IRAP profiles showed that the strains of *P. graminea* clustered into four groups (Fig. 2). Significant correlation ($r = 0.61$; $P < 0.01$) was existed between the differentiation of the strains according to the IRAP analysis and their capacity to produce xylanase enzyme. However, despite the high variation observed, IRAP profiles exhibited some bands that were common to strains yielded high xylanase, and absent to that produced less xylanase (Fig. 1).

On the other hand, the results show that among all tested strains; *Pg*16, *Pg*13 and *Pg*24 strains were the highest xylanase producers with a high genetic distance value (Fig. 2), which might be attributed to the genetic differentiation (DNA fragments) between species. However, these strains could be a good candidate for biotechnological applications.

IRAP analysis produced a large number of bands that is in agreement with the previous reports suggesting the presence of the retrotransposon element in high copy number in fungi [6, 19]. In contrast, in other studies using RAPD [20] or polypeptide patterns [10] as genetic markers revealed only a low level of poly-

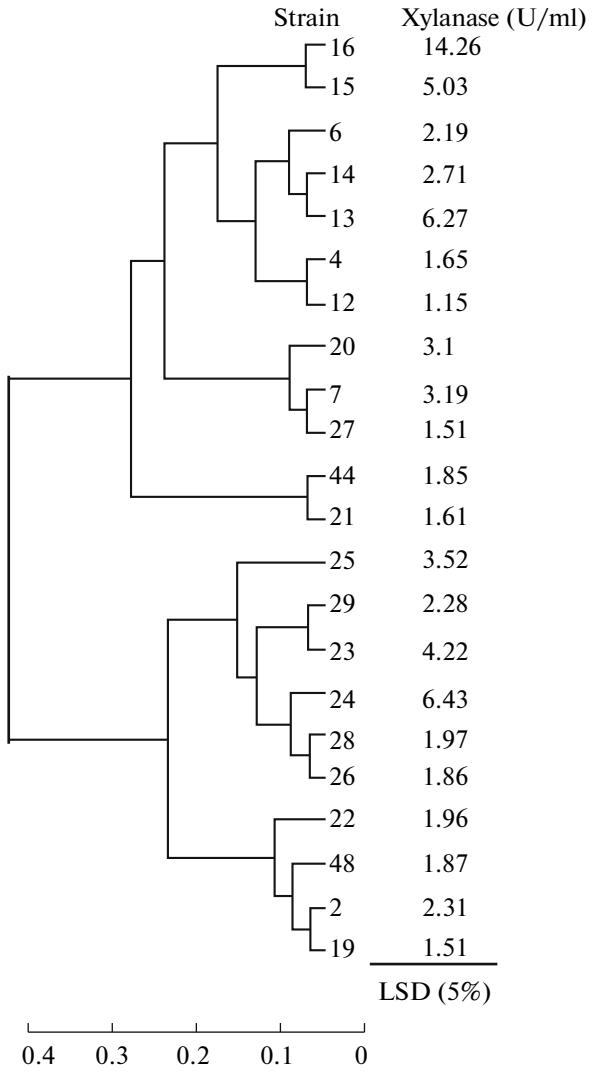

Fig. 2. UPGMA tree of 22 *Pyrenophora graminea* strains based on 1RAP data, and their xylanase production under submerged culture. LSD (5%): Least Significant Differences at $P < 0.05$.

Table 2. Comparison of xylanase activities produsedby various filamentous fungi grown under submerged culture

Microorganism	Substrate	Cultivation	Activity (IU/g of substrate)	Referense
Aspergillus sp.	Xylan	30°C, 7 d	10.6	[2]
Penicillium Kloecheri	Xylan	30°C, 7 d	12.2	[17]
Pyrenophora graminea	Wheat bran	30°C, 5 d	14.26	This work
Penicillium janthinellum	Sugar-cane bagasse	30°C, 5 d	11.4	[18]
Trichoderma harzianum Rifai	Wheat bran	30°C, 5 d	1.21	[19]

morphism, and thus lower intraspecific diversity in *P. graminea*.

This report describes the first large-scale investigation into the possibility of employing IRAP for genome analysis in *P. graminea* strains differing in xylanase activity. The technique is simple, rapid, and reproducible as well as does not involve time-consuming DNA hybridization. The results provide a vast amount of information that can guide hypothesis-driven research to elucidate the molecular mechanisms involved in xylanase activity and fungal plant pathogens.

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