Heterogeneity in the ITS region of ribosomal DNA of *Cochliobolus sativus* isolates differing in virulence patterns

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Spot blotch of barley (*Hordeum vulgare*) caused by the fungus *Cochliobolus sativus* has been the major yield-reducing factor for crop production during the last decade. In this study, the nucleotide sequences of internal transcribed spacer (ITS) regions of rRNA genes of *C. sativus* isolates differing in virulence patterns were examined. The results indicate that the sequences of ITS regions in different isolates are not identical. Sequence variations were found at nine positions in the 600 bp that were sequenced. A neighbour-joining diagram, based on Nei's genetic distances, showed that isolates split into two phylogenetic groups and did not result in any clusters/clades specific to virulence pattern or lesion shape. Hence, the results presented here suggest that identification of *C. sativus* isolates by their morphological and biological characteristics should be appropriately supported by molecular analysis.

Keywords: *Bipolaris sorokiniana*, virulence, internal transcribed spacer, rRNA.

Cochliobolus sativus (Ito & Kuribayashi) Drechs. ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok) Shoem.], the causal agent of spot blotch (SB), is a common foliar pathogen of barley (*Hordeum vulgare* L.), a disease responsible for heavy crop losses (Mathre 1990). Several works on morphological, physiological and biochemical aspects have already been undertaken (Kumar *et al.* 2002, Jawhar & Arabi 2009).

Previous studies of *C. sativus* isolates from North America, Syria and Australia have tested SB virulence on a range of barley genotypes, typically assigning pathotypes using coded triplet nomenclature (Valjavec-Gratian & Steffenson 1997, Arabi & Jawhar 2003, Meldrum *et al.* 2004). However, the major weakness with this system of classifying pathotypes is the arbitrary distinction of resistant versus susceptible responses based on dividing a nine point scale into two classes, as a difference of only one rating point or a shift in the arbitrary line, could easily change the designated pathotype. However,

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the expression of lesion types on barley plants infected with *C. sativus* can be influenced by a number of factors such as choice of leaves for inoculation, inoculum densities and position of infection site (Fetch & Steffenson 1999), thus limiting their value for genetic variations studies. The internal transcribed spacers (ITS) region of the nuclear ribosomal DNA (nrDNA) is now perhaps the most widely sequenced DNA region in fungi (Peay *et al.* 2008). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races).

Because of its higher degree of variation than other genetic regions of rDNA (for small-and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1 and ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Lee *et al.* 1998).

In the case of *C. sativus* the ITS-RFLP resulted in heterogeneous restriction patterns by the majority of endonuclease digestion (Arabi & Jawhar 2007). However, despite its substantial importance in plant pathology, there is a lack of appropriate and adequate information regarding ITS structure of *C. sativus* isolates. The primary objective of this current research was to investigate the variability in the ITS region of ribosomal DNA of *C. sativus* isolates collected from different regions of Syria.

Materials and methods

Fungal isolates

During the period 1999–2002, more than 117 isolates of *C. sativus* were obtained from barley and wheat leaves showing SB symptoms in different regions of Syria. Leaf tissues with necrotic lesions were cut into pieces (5×10 mm) and sterilized in 5 % sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, the pieces were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) with 13 mg/l kanamycin sulphate added after autoclaving and incubated for 10 days, at 21±1 °C in the dark to allow mycelial growth and sporulation. After incubation, the spores were examined under microscope and confirmed to be *C. sativus* pathogen. In previous studies (Arabi & Jawhar 2003, 2007), different barley genotypes were inoculated with 117 isolates and evaluated for host-pathogen reaction and lesion formation. Subsequently, 12 monosporic isolates were selected for this study (Tab.1).

Lesion shape and virulence test

Lesion shapes and virulence test of the 12 isolates were determined on susceptible barley cultivar 'WI2291' from Australia, using both the size and type (chlorosis/necrosis) of lesions (Fetch & Steffenson 1999). Seeds were planted in plastic flats (60×40×8 cm) filled with sterilized peatmoss and

Tab. 1. *Cochliobolus sativus* isolates used in this study.

Isolate no.	Origin	Year	Lesion shape[A]	Virulence[B]
C41	Aleppo (north)	1999	1	8.33a
C13	"	2001	4	1.33e
C93	"	1999	1	4.33c
C6	Daraa (south)	2000	1	6.67b
C92	"	2000	3	4.33c
C30	ICARDA[C]	2002	2	1.33e
C9	"	1999	1	7.67a
C74	"	2002	3	4.67c
C49	Hasake (north-east)	1999	4	3.67d
C15	"	1999	2	5.17b
C10	"	1999	2	3.67d
C17	"	1999	4	2.33e

[A] 1: Solid dark brown necrotic with expanding chlorosis
2: Elongated light brown necrotic with expanding chlorosis
3: Light brown necrotic with whitish gray centers and chlorotic margins
4: Small round to oblong dark brown necrotic
[B] Values followed by different letters are significantly different at P <0.001.
[C] ICARDA: International Center for Agricultural Research in Dry Areas, Syria.

placed in a growth chamber at temperatures 22±1 °C (day) and 17±1 °C (night) with a daylength of 12 h and a relative humidity of 80-90 %. Seedlings were irrigated by Knop nutrient solution (1g $NaNO_3$; 0.25g KNO_3; 0.25g $MgSO_4$ $7H_20$; 0.25g KH_2PO_4; and 10 mg $FeCl_3$ per 1000 ml water). Infections were initiated by spraying each plant with 2 ml of conidial suspension of 2×10^4 conidia/ml in pure water. Tween 20 (polyoxyethylene-sorbitan monolaurate) was added as a surfactant (100 µl per liter) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surface. Leaves were covered for one night with plastic bags to increase humidity and plants were kept in the same greenhouse at 20 °C with a 16-h photoperiod. The mean of SB severity was calculated using Student-Newman-Keuls test (Anonymous 1988). The experiment was repeated twice.

DNA isolation

Aliquots of the cultures were used to inoculate flasks containing 50 ml of potato-dextrose broth, which were incubated as described above. Twenty grams of mycelium were used for DNA isolation according to Leach *et al.* (1986), with some modifications. The mycelia were collected by filtration and the samples ground to a fine powder in liquid nitrogen. The mycelial powder was suspended in one volume (w/v) of lysis buffer (0.1 M LiCl, 10 mM Tris-HCl, pH 8.0; 10 mM EDTA, 0.5 % SDS), vortexed and then incubated at 65 °C for 15 min. After incubation, samples were centrifuged for 10 min at 12000 *g*.

The supernatant was collected and two extractions with phenol-chloroform-iso-mylalcohol (25:24:1) were performed, followed by one extraction with chloroform-iso-mylalcohol (24:1). Fifty micrograms RNAseA (Euroclone, Italy) were added to the samples and incubated at 37 °C for 15 min. The DNA was precipitated with 0.1 vol. of sodium acetate 3 M and 2.5 vol. of isopropanol. The DNA was collected carefully and washed with 70 % ethanol, dried and then resuspended in TE (10 mM Tris-HCl and 1 mM EDTA).

ITS amplification and sequencing of fungal ITS

The ITS regions were amplified by the polymerase chain reaction (PCR) with the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) as described by White *et al.* (1990). Amplification procedure was carried out as previously described by Arabi & Jawhar (2007).

When bands of an appropriate size for the ITS region were observed, the remaining PCR product was electrophoresed through 1.0 % agarose gels in a modified TAE buffer (40mM Tris-acetate, pH 8.0, 0.1mM Na2EDTA), and the target bands excised. DNA from excised bands was purified using an Ultrafree-DA DNA extraction kit (Millipore, Bedford, MA, USA) and sequenced by the dye terminator method on an Analyzer (ABI 310, Perkin-Elmer, Applied Biosystems, USA) using the primers ITS1 and ITS4 separately. If sequences were ambiguous after alignment of the forward and reverse sequences, new extracts of DNA were made and sequenced as above.

DNA sequence assembly and alignment

Sequence similarity searches were performed for each of the 12 representative C. sativus sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). The whole ITS region sequences of C. sativus isolates were aligned with the program Vector NTI (Lu & Moriyama 2004) using default parameters, and the alignment was inspected and adjusted manually where necessary. Neighbour-Joining diagrams and bootstrap analysis were constructed on genetic distances among populations using the Nei's distance (Saitou & Nei 1987) by the PHYLIP package ver 3.5c (Felsenstein 1993). Bootstrapping was used to resample the data (1000 resamplings) and the proportion of neighbour-joining tree possessing internal branches was used to assess their support.

Results and discussion

The different virulence patterns and lesion form of the 12 isolates are presented in Tab. 1. However, four lesion forms were observed and numbered from 1 to 4. These are in full agreement with findings of Arabi & Jawhar (2003). The variation can be attributed to the genotype interactions and that

several genes for virulence are operating in the pathosystem. The specificity of pathosystems and the different types of resistance have been defined by Ghazvini & Tekauz (2008).

PCR amplification with the specific primers ITS1 and ITS4 yielded single DNA fragments present in all isolates with 600 bp, which is in agreement with the results obtained by a previous study (Arabi & Jawhar 2007). The ITS region nrDNA was completely sequenced in both directions. A total alignment of 600 bases including gaps was obtained and used in the comparisons among isolates (accession numbers in NCBI databases are given in Tab. 2). The results indicate that the sequences of ITS regions in different isolates are not identical. However, the sequences showed high similarity (98 %) to the *Cochliobolus sativus* isolate CSMMA2 (Genebank accession no. AB774149.1) and sequence variations were found at nine positions in the 600 bp that were sequenced (Tab. 2).

Tab. 2. Sequence differences in ITS region among *Cochliobolus sativus* isolates (with accession numbers in NCBI databases) examined in this study in reference to comparison with the ITS region of *C. sativus*-isolate CSMMA2 having GenBank accession number AB774149.1.

Isolate no.	Accession number	Base position								
		312	357	411	485	530	573	582	586	590
C41	KC616331	G	T	T	G	G	A	A	T	G
C13	KC616332	G	T	T	G	G	A	A	T	G
C93	KC616333	G	T	C	A	G	A	A	T	T
C6	KC616334	G	T	T	A	G	A	A	T	T
C92	KC616335	G	T	T	A	G	A	A	T	T
C30	KC616336	G	T	C	C	G	A	A	T	G
C9	KC616337	G	T	T	A	G	A	A	T	T
C74	KC616338	-	A	T	A	G	C	T	G	T
C49	KC616339	-	A	-	A	-	C	T	G	A
C15	KC616340	-	A	T	A	-	C	T	G	A
C10	KC616341	-	A	T	A	G	C	T	G	T
C17	KC616342	G	T	C	C	G	A	A	T	G

Only positions where differences occurred are shown; all other positions are identical. Dashes represent alignment gaps

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 degree of genetic diversity we observed. However, many researchers have investigated intraspecific variability of the nucleotides sequences in the nrDNA ITS regions for various fungal species.

Neighbour-joining analysis showed that *C. sativus* isolates were separated into two groups with high bootstrap support. (Fig.1). This molecular data in conjunction with the high level of morphological and physiological

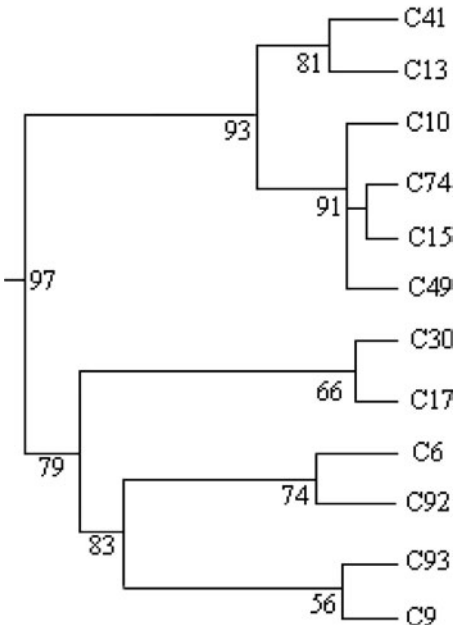

Fig. 1. Neighbour-joining dendrogram of 12 *Cochliobolus sativus* pathotypes showing the agreement obtained by the ITS sequence data. Percentages from 100 bootstrap replications are given on tree branches.

similarity (Arabi & Jawhar 2007) indicates their close genetic relatedness. No single nucleotide base substitution at a specific position in the ITS was sufficient to design effective isolates specific PCR primers. However, our results which utilized sequence variability within ITS region among *C. sativus* agree with results of similar studies conducted with other fungi such *Ampelomyces quisqualis* (Kiss & Nakasone 1998), *Pythium* (Martin 1990) and *Pneumocystis carinii* (Lee *et al*. 1998).

The results of this study can be supported by preliminary data from random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism of PCR-amplified internal transcribed spacers of the rDNA (ITS-RFLP) analyses showing that genome-wide polymorphisms are common (Arabi & Jawhar 2007). However, this variation points to the need of monitoring ITS sequences of *C. sativus*, for determining sources of infection, predicting the spread of disease across locations, and studying local spreading and recolonization. The present study emphasize that ITS marker can be successfully employed in, and may give comparable results for, assays of genetic differentiation among *C. sativus* isolates. However, the results presented here suggest that identification of *C. sativus* isolates by their morphological and biological characteristics should be appropriately supported by molecular analysis.

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