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Cultural and genetic evaluation of *Cochliobolus sativus* during successive passages through susceptible barley

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Key words: *Cochliobolus sativus*, *Hordeum vulgare*, phenotypes, spot blotch.

Abstract: The objective of this work was to assess the stability of retrotransposons DNA elements and several key phenotypic traits important for virulence of *Cochliobolus sativus* after serially transferring through susceptible barley plants. A significant increase in virulence was observed in offspring isolates generated from the aggressive isolate Pt4, in contrast to the lack of significant changes in those obtained from the weakly aggressive isolate Pt1 after seven successive passages. No apparent differences in phenotypes, including mycelial growth, conidiation and conidial germination were observed among isolates from the same parent isolate on artificial medium. Based on retrotransposon microsatellite amplified polymorphism (REMAP), parents and their generations were identical during the serial transfers. Taken together, our results suggest that all single-conidials of the parents and their generations were stable genotypically during seven serial transfers with a change in virulence of the aggressive isolate generations.

1. Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.] is an ascomycetous fungus that causes spot blotch (SB) of barley, *Hordeum vulgare* L., a disease responsible for large economic losses in barley-growing areas (Mathre, 1990). Although the production of conidia is expected to produce genetically identical clones, the high rate of appearance of new races with the ability to infect previously resistant varieties of barley suggests that *C. sativus* may have high mutation rates in avirulence genes, which determine race (Kumar *et al*., 2002).

General symptoms of SB include light brown lesions with whitish gray centers and chlorotic margins (Kumar *et al.,* 2002). Most varieties grown around the world are susceptible to *C. sativus*, although partial resistance has been reported (Arabi, 2005; Zhou and Steffenson, 2013). Genetic control of SB resistance is governed by quantitative traits. Two quantitative trait loci (QTLs) have been mapped to chromosomes 1S and 5S (Steffenson *et al.,* 1996). However, in the case of the host-specific toxin produced by *C. sativus,* the fungus which incites SB of barley, the toxin will produce all the symptoms characteristic of the disease; sensitivity to the toxin is correlated with susceptibility to the pathogen and toxin production by the pathogen is directly related to its ability to cause disease (Kumar *et al*., 2002).

Although the most effective control strategy for SB is cultivating resistant varieties, it has often achieved only short-term success due to the frequent breakdown of newly introduced resistance (Poudyal *et al*., 2005; Gontariu and Enea, 2012). This resistance breakdown has been attributed to genetic variability in *C. sativus* (Gilchrist *et al*., 1995). Different mechanisms have been suggested to explain the frequent generation of race variants, including heterokaryosis, parasexuality and mutations (Kumar *et al*., 2002; Arabi and Jawhar, 2007). However, the activity of the retrotransposon microsatellite amplified polymorphism (REMAP) is another possible mechanism that has been suggested which uses 1 LTR primer in combination with a primer designed for annealing at the 3' end of a stretch of a simple sequence repeat (SSR) and detects retrotransposons inserted near SSRs (Chadha and Gopalakrishna, 2005; Biswas *et al*., 2010).

Our overall question was how stable *C. sativus* isolates would be both genotypically and phenotypically during several serial transfers in one growing season. Therefore, the objective of this work was (i) to determine phenotypic variation of the two major pathotypes of *C. sativus* in Syria, Pt1 and Pt4 during seven serial transfers on barely plants and (ii) to investigate genetic variation of the isolates collected in each generation using DNA fingerprinting.

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2. Materials and Methods

Fungal isolates

For the inoculation process, two major pathotypes of *C. sativus* in Syria, Pt1 and Pt4, were used in this study. They were identical in spore morphology and colony colour, but differed widely in DNA patterns and virulence. After extensive greenhouse and laboratory screening over a 10-year period, Pt4 was proven to be the most virulent isolate to all barley genotypes available so far (Arabi and Jawhar, 2003; 2007), therefore it was used in this study. Each isolate was 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 mycelial growth.

Inocula preparation, serial transfer and isolation methods

After culturing Pt1 and Pt4 isolates on PDA medium, spores were collected by flooding each plate with 10 ml sterile water with 200 ppm of Tween 20, filtering through cheesecloth to remove mycelium and adjusting the concentration of the spore suspension to $2x10^4$ conidia/ml using hemacytometer counts of conidia. The universal susceptible control (cv. WI2291) plants from Australia were grown in pots filled with sterilized peatmoss, and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of 10 seedlings. A full replicate consisted of 10 pots inoculated with Pt1 and Pt4 isolates. Pots were placed in a growth chamber at temperatures of 22±1°C (day) and 17±1°C (night) with a daylength of 12 h and a relative humidity (RH) of 80-90%. Plants were inoculated at growth stage (GS) 12 (Zadoks *et al*., 1974) by uniformly spraying each plant with 20 ml of conidial suspension with a hand-held sprayer. Plants were then placed in the dark at 95-100% R.H. for the first 18 h.

Pt1 and Pt4 isolates and single-conidial isolates from each of the three replicates from the 1st to 7th passage generations through barley plants were compared for virulence. Ten days after inoculation, one leaf per plant, for a total of three leaves per replicate, was sampled, surface sterilized and placed on a water agar plate. Three days later, a single conidium from each of the three plates was transferred to PDA for genotypic and phenotypic assays. The infection response based on the measurement of individual lesion size (dimension; mm) for each second leaf was assessed 10 days after inoculation according to Fetch and Steffenson (1999) scale. Each leaf was assessed separately and the assessments were performed by the same person in all experiments.

In vitro *phenotypic assays*

In vitro phenotypic assays were achieved by transferring plugs of mycelim (5 mm diameter) of each parent and generations onto PDA media and incubation at room temperature (22±1°C) in the dark. Conidial germination rate was recorded after 24 h on glass cover slips as described previously (Arabi and Jawhar, 2001). Colony diameter was measured seven days post incubation. Conidia were harvested from 15-day cultures using sterile distilled water and counted with a hemocytometer. All the experiments were repeated three times with five replicates, and a representative set of data is presented. Statistical analysis was performed using the STAT-ITCF program (Anonymous, 1988).

REMAP analysis

DNA extraction from parent and generation isolates was performed according to standard protocols (Leach *et al*., 1986). REMAP analysis and primer sequences were achieved using a standard method described by Kalendar *et al*. (1999). PCR reactions were performed in 25 µL reaction volume containing 1× *Taq* polymerase buffer (10 mmol Tris-HCl/L (pH 8.3), 50 mmol KCl/L, 2.5 mmol MgCl2/L, 0.01% gelatin), 0.5 U *Taq* polymerase (Eppendorf, Germany), 150 µmol of each dNTP/L, 0.4 µmol LTR1 primer/L, 0.6 µmol of ISSR primer/L (Table 1), and 50 ng of template DNA. A PCR was carried out in an Eppendorf DNA thermal master gradient cycler (Eppendorf Netheler-Hinz, Hamburg, Germany). The amplification conditions were as follows: 92°C for 5 min, followed by 40 cycles of 92°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. 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 100V. The gels were stained with ethidium bromide solution and visualized under ultraviolet illumination. Sizes of the amplified products were determined relative to a 100-bp DNA ladder (MBI Fermentas, York, UK).

3. Discussion and Conclusions

Disease symptoms (presence of necrosis and chlorosis) were severe on the susceptible genotype WI2291 that was infected with pathogenic isolates after 10 days of inoculation. Pt1 and their generations induced small round to oblong dark brown necrotic lesions, whereas, Pt4 and their generations induced solid dark brown necrotic lesions with expanding chlorosis (the 'classic' spot blotch lesion) in highly compatible interactions. As offspring isolates from the most diseased plants were serially passaged

Table 1 - REMAP primers used in the study

Primer no.	Sequence
1	(GA)8T+TGTTTCCCATGCGACGTTCCCCAACA
2	(AG)8T+GCATCAAAGGCATTGGAGGTG
3	(GA)8T+ GCATCAAAGGCATTGGAGGTG
4	(GA)8T+CACTAGTGATTCATTATGCTGAGTG
5	(AG)8T+GCATCAAAGGCATTGGAGGTG
6	(AG)8T+ CACTAGTGATTCATTATGCTGAGTG
7	(AG)8G+CCAATGGACTGGACATCCGATGGG
8	(AG)8T+ TGTTTCCCATGCGACGTTCCCCAACA
9	(AG)8G+ TGTTTCCCATGCGACGTTCCCCAACA

on barley, virulence of the aggressive isolate Pt4 was increased during seven transfers on plants, and the ability of weakly aggressive isolate Pt1 to maintain its virulence was observed (Table 2). All Pt4 isolates were highly virulent to cv. WI2291 with a mode of 4 (typifying >90% of the lesions observed on leaves), whereas, all Pt1 isolates were virulent exhibiting a mode of 2 (typifying >10% of the lesions observed on leaves), indicating that virulence was not significantly affected.

Infection responses of WI2291 to *C. sativus* isolates Pt1 and Pt4, and their generations are summarized in Table 2. The pattern of continuous incremental increase in virulence has been reported in *F. oxysporum* f. sp. *ciceris*, on chickpea (Jiménez-Gasco *et al*., 2004), and in *Magnaporthe oryzae* on rice (Park *et al*., 2010). However, the pathosystems involving the genera of *Cochliobolus* and the involvement of host-specific toxins in pathogenicity and virulence are well documented (Olbe *et al*., 1995).

The number of SB lesions (presence of necrosis and chlorosis) were always high in the virulent isolate Pt4 during the serial passages (Fig. 1). This can be attributed to a higher proportion of Pt4 spores being able to establish lesions than Pt1 spores. This indicates that the more virulent Pt4 causes more lesions (per leaf) from a given inoculum dose than Pt1 as presented in figure 1. The existence of pathotypes expressing differential virulence on host genotypes is uncommon in species related to *C. sativus*. Differential reactions, such as those expressed by the pathotypes Pt1 and Pt4 on two-rowed genotypes (WI 2291), usually are a feature of gene-for-gene interactions (Flor, 1956) or an incompatibility system (Briggs and Johal, 1994). A hypothesis that these two differentially virulent *C. sativus* isolates are pathotypes that produce two different types of host-specific toxins may also be valid.

Additionally, no significant differences were observed between the parental isolates and any isolates derived from them through the seven serial transfers for any of the phenotypic characters tested *in vitro* including mycelial growth, conidiation and conidial germination on PDA media (Fig. 2). These results are similar to those of Latterel and Rossi (1986), who reported no changes in cultural characteristics in *Magnaporthe oryzae* isolates after serial transfers of the same isolates from stock cultures over a long period of time.

To evaluate genotypic stability during serial transfers, REMAP fingerprinting technique was used. The REMAP haplotypes of seven generations were identical to their parent isolates (Fig. 3); this might be an indicator of the stability of transposable elements during serial passages. The lack of molecular variation in offspring of Pt4 isolates with increased virulence (Table 2; Fig. 3) is consistent with the fact that mutation rates at virulence loci are higher than those at the molecular loci that define genotypes (Goodwin *et al*., 1995).

The results of this study demonstrate that the virulence of the offspring isolates generated from the aggressive isolate Pt4 significantly increased after seven successive

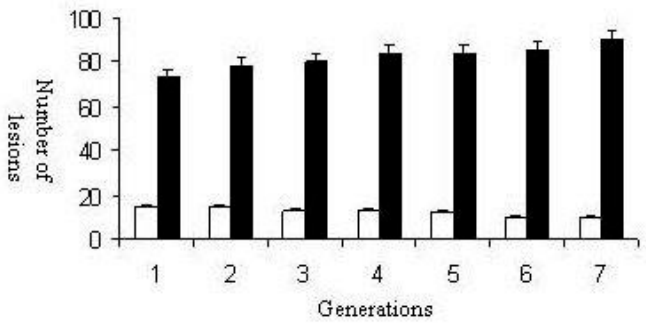

Fig. 1 - Number of lesions caused by Pt1 and Pt4 isolates after seven serial transfers barley cv. WI2291.

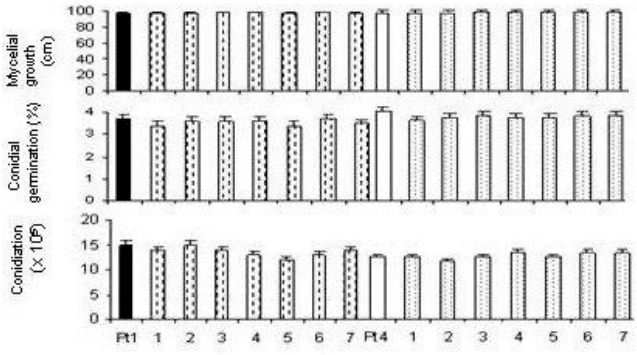

Fig. 2 - Cultural characterization of two *C. sativus* isolates Pt1 and Pt4 after seven passages on PDA medium and through barley plants.

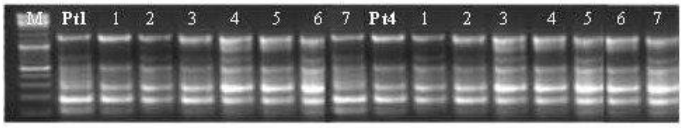

Fig. 3 - Agarose gel electrophoresis of REMAP analysis Pt1 and Pt4 parent isolates after seven passages through barley cv. WI2291 using primer; (GA)8T+GCATCAAAGGCATTGGAGGTG. M–marker ladder 1Kb.

Table 2 - Infection responses of barley cv. WI 2291 infected with parents and progeny isolates of *Cochliobolus sativus* based on the scale of Fetch and Steffenson (1999)

Isolates	Infection response	Parent	Passages						
			1	2	3	4	5	6	7
Pt1	Mode [z]	2	1	2	2	2	2	1	1
	Range [y]	1-2	1-2	2	1-2	1-2	1-2	1-2	1-2
Pt4	Mode	4	4	4	4	4-5	4	4	4-5
	Range	7-9	6	7	7-8	8-9	9	9	9

[z] Mode= The most common infection response observed on the barley cv. WI2291.

[y] Range= The lowest and highest infection responses observed on the barley cv. WI2291.

passages, in contrast to the lack of significant changes in those obtained from the weakly aggressive isolate Pt1. No apparent differences in phenotypes, including mycelial growth, conidiation and conidial germination, were observed among isolates from the same parent isolate on artificial medium. All single-conidial of the parents and their generations were stable genotypically during the serial transfers with a change in virulence of the aggressive isolate generations.

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