TITLE: Nohutta Ascochyta Yanikliklik Etmeni Ascochyta Rabiei`nin TÜRK Izolatlarinin Toksin Üretimi ve DNA Sekans Analizleri

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PAGES: 31-38

J. Turk. Phytopath., Vol. 39 No. 1-3, 31-37, 2010 ISSN 0378 - 8024

Toxin Production and DNA Sequence Analysis of Turkish Isolates of *Ascochyta rabiei*, the Causual Agent of Ascochyta Blight in Chickpea

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Accepted for publication March 10, 2013

ABSTRACT

In this study, twenty isolates of *Ascochyta rabiei* were isolated from disease-enfecteol chickpea plants which were collected from chickpea growing areas in Turkey. In order to determine solanapyrone production of these isolates, the fungus was grown on Czapek Dox liquid culture medium (CDLCM) for 12 day at two different temperatures. Quantitation of solanapyrones was determined with HPLC analyses. The results demonstrated that all isolates produced solanapyrone A in CDLCM at 20^0C but not at 30^0C.

Confirmation of the identity of the pathogen was sought by sequence analysis of rDNA. These experiments showed that the sequences of the internal transcribed spacers (ITS) and 5.8 S gene of the seven isolates, which were identical to each other, were also identical to that of a Pakistan isolate of *A.rabiei*. rDNA sequences of the PCR products of isolates of *A.rabiei* which were produced different amount of toxin were same.

Keywords: Solanapyrone, *Ascochyta rabiei*, Chickpea, DNA Sequence

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important legume crop in several parts of the world, such as West Asia, North Africa, Central and South America (Nene 1982; Singh and Reddy 1990). It is the most produced legume crop of Turkey (Anonymous 2008). The most important disease of chickpea is Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. Serious crop losses occur when environmental conditions, especially cool, wet weather, favour disease development and spread (Singh and Reddy 1990). This fungus, which is known to be seed-borne, causes characteristic dark necrotic lesions on the stems, leaves and pods of the host, and severe infection can kill the plants (Maden et al. 1975; Nene 1982).

The taxonomy of *Ascochyta* species have been based on morphology and host plant association. Classification systems based upon data from morphological studies have been mostly successful. However, in some cases, morphology has been unsuccessful in characterizing fungi. Recently, significant advances in fungal taxonomy and identification have come about through DNA analysis. A wide range of molecular techniques are available for the identification of fungi, including Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD). Fungi of the genus *Ascochyta* have not been extensively studied by molecular tools. Some publications describe DNA fingerprinting of *A. rabiei*, by means of RFLPs (Weising et al. 1991), RAPD (Fischer et al. 1995) and the RAPD-like PCR technique (DAF)-DNA amplification fingerprinting (Kaemmer et al. 1992). RFLP has been used to solve systematic problems in the genus *Phytophthora* (Förster et al., 1990), to characterize *Pythium* species (Wang and White, 1997) and to determine genetic polymorphism among isolates of *A. rabiei (*Morjane et al., 1994). A large group of *A. rabiei* isolates were analysed by RAPD but analysis of the combined data failed to reveal any correlation between amplification patterns and pathotype clasification (Fisher et al. 1995). Fatehi and Bridge (1998) detected multiple rRNA-ITS regions within nine cultures of *Ascochyta* and Khan et al.(1999) distinguished *A.rabiei* from *Phoma medicaginis* var *pinodella* both of which were found in lesions on chickpea in Australia.

Sequence data of an appropriate part of the fungal genome provides unequivocal identification of fungal species. Analysis of DNA sequences, particularly those of the ribosomal repeat unit, has proved to be a definitive and rapid method for the identification and taxonomic studies of fungi as well as for the studies of evolution and speciation (White et al. 1990). This technique has been used to identify fungi and to delineate species (Sherriff et al. 1995: Kusaba and Tsuga 1995).

An organism may damage plants by secreting one or more toxins. Early symptoms of Ascochyta blight include epinasty, loss of turgor and cellular disintegration. It has been suggested that such symptoms could result from toxin production by the pathogen (Höhl et al. 1991, Hamid and Strange 2000). In liquid culture, isolate of *A.rabiei* have been reported to synthesizses a total of four toxic compounds; solanapyrone A, B, C and cytochalasin D (Alam et al. 1989; Höhl et al. 1991; Latif et al. 1993) which are released in the culture medium. Solanapyrones A, B and C were first found in culture filtrates of *Alternaria solani*, the causal agent of early blight of tomato and potato (Matern et al. 1978). The chemical structure of these toxins were elucidated by NMR-spectroscopy and mass- spectrometry (Ichihara et al.,1983). *Phoma exiqua* var. *heteromorph*a, which was formerly known as *Ascochyta heteromorpha*, produced cytochalasins A, B, U and V when grown on a semi-synthetic medium (Capasso et al. 1991)

The objective of this research was to determine production of toxin by Turkish isolates of *A.rabiei* and demonstrate DNA sequence *of A.rabiei* isolates which produced different amount of toxin or not.

MATERIALS and METHODS

Ascochyta rabiei Isolates

Isolates of *Ascochyta rabiei*, designated T 1-22, were isolated from diseased leaves, stems and pods of chickpea collected from chickpea growing areas in different regions (Central Anatolia, South Eastern Anatolia and Aegean) of Turkey. These cultures were cultivated on the CSMDA medium (Chickpea Seed Meal Dextrose Agar: chickpea meal 40 g, dextrose 20 g, agar 20 g, distilled water 1 l) at 20±2^{0}C with a 12 h period of near UV light for 10 days. From these cultures, single spore isolates were obtained. Inoculum was further multiplied on chickpea seeds according to Alam et al. (1987). After incubation at 20^{0}C for 7-10 days the inoculated seeds were agitated with sterile distilled water and the spores suspended in 10% glycerol (10^7 spore ml^{-1}). The suspension was distributed to 1.8 ml Nunc tubes in 1 ml aliquots and were stored in liquid nitrogen.

Toxin Production and HPLC Analysis

The fungus was grown on Czapek Dox liquid culture medium, consisting of Czapek Dox nutrients supplemented with zinc sulphate (50 mg/l), manganese chloride (20mg/l), calcium chloride (100mg/l), cobalt chloride (20mg/l), cupric chloride (20mg/l) per litre (CDLCM; Chen and Strange, 1991). After distribution to 250 ml Erlenmeyer flasks (30 ml per flask) and autoclaving, each flask was inoculated with 30µl spore suspension (10^7 spores ml^{-1}) of *A.rabiei* and incubated without shaking at 20^{0}C and 30^{0}C in continuous light for 12 days. The fungus was removed by filtration through 6 layer of muslin and filtered using Whatman No.1 filter paper. Mycelial mats and the relatively few spores were discharged and dried at 80^{0}C for 48 h to give a measure of fungal growth. Samples of the filtrates (30 ml) were passed through an Isolute 1g C18 cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK) and after washing with water (5.0 ml), toxin was eluted with acetonitrile (100%:2.0ml). Samples of the acetonitrile eluate (20µl) were injected onto a Philips HPLC consisting of a PU4100 quaternary pump, PU4021 diode array detector and computer equipped with PU6003 diode array software for data handling. The stationary phase was a Jones Chromatography C18 column ODS (5µm particle size: 4.6 x 150 mm: Jones Chromatography Ltd., Mid Glamorgan, UK). The column was developed with a mobile phase consisting of tetrahydrofuran 20.6%, methanol 23.1% and bidistilled water 56.3% at a flow rate 1.0 ml/min. The solanapyrones were recognized by their retention times and their characteristic UV spectra, which were compared with authentic samples. Chromatograms were abstracted from the chromascans at λ=327nm and solanapyrone A quantified by reference to an external standard of the compound.

Growth of *Ascochyta rabiei* and DNA Extraction

Isolates (T-4, 5, 9, 10, 14, 15 from Turkey and P-8 from Pakistan) were grown in a medium consisting of Czapek-Dox Nutrients (45.5 g: Oxoid, Unipath Ltd., UK), bacto peptone (1g), yeast extract (1g), casein hydrolysate (1g), dissolved in 1 L water and supplemented with 200 ml of clarified V-8 juice (Campbell Grocery Products Ltd., UK) prepared by filtering the juice through four layers of muslin and centrifuging at 2000^9g for 5 min. The medium was distributed to 250 ml Erlenmeyer flasks (100 ml per flask) and autoclaved at 121^0C for 20 minutes. After cooling to room temperature, 0.01g of Streptomycin sulphate was added to each flask. Spore suspension (100 µl: 1×10^7 spore/ml) of isolates, which were stored in liquid nitrogen, were used to inoculate the medium. After incubation for 3 days at 25^0C on an orbital shaker the mycelia were harvested. DNA was extracted using a commercial kit (Nucleon Phytopure Plant DNA Extraction Kit, Scotlab, UK) according to the manufacturer`s instructions. Precipitated DNA was resuspended in 180 µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 20µl RNase (1mg/ml) was added. After incubation at 37^0C for 1 hour on a shaker, DNA concentration and purity was ascertained by monitoring UV absorption at 260 and 280 nm and by electrophoresis in 1% agarose gel containing ethidium bromide (0.2 µg ml^{-1} gel).

DNA Amplification

The two internal transcribed spacers (ITS1 and ITS2) and the 5.8S rDNA were amplified with the primer pairs ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Each DNA sample (50ng) and the two primers (10pmol of each) were added to a “Ready to Go” PCR bead (Amersham Pharmacia Biotech Inc., Sweden) which contained 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50mM KCl, 1.5mM $MgCl_2$ and 200 µM of each dNTP when brought to a final volume of 25 µl. The PCR conditions were 40 cycles of 1 min at 94^0C, 45 s at 50^0C, 2 min at 72^0C, followed by a final extension step of 5 min at 72^0C (Fatehi and Bridge 1998) PCR products were electrophoresed in 1.7 % agarose gel.

DNA Sequencing and Analysis

DNA sequencing of PCR products were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Protocol number 4303152) with Ampli*Taq* DNA polymerase (Perkin Elmer Corporation) using an ABI PRISM 377 DNA Sequencer according to the manufacturer`s instructions. Primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al. 1990) were used to sequence the PCR products.

The sequences of the PCR products were aligned by the clustal method using the programme MAGI (Multiple Alignment General Interface) at the HGMP-RC (Human Genome Mapping Project Resource Centre; www.hgmp.mrc.ac.uk) according to the service providers instructions.

A reference *A.rabiei* sequence was obtained from CABI and compared with sequences from the Turkish isolates using the cluster method of MAGI.

RESULTS AND DISCUSSION

Production of Solanapyrone A by *Ascochyta rabiei* Isolates

The variation in solanapyrone A concentrations of the culture filtrates from 21 isolates of *A.rabiei* is shown in Fig.1. Toxin production varied from 0.89 to 11.0 µg/g dry weight of mycelium after 12 days on Czapek-Dox

Liquid Cation Medium (CDLCM) at 20^{0}C. Isolate T-21 produced the highest concentrations of the solanapyrone A with values of 11.0 µg/g dry weight of mycelium. T-17, T-15 and T-12 produced 8.79, 8.04 and 7.73 solanapyrone A µg/g dry weight of mycelium on the same medium, respectively. Three isolates (T-1, T-22 and T-11) produced small amounts of sol A. All of 20 Turkish isolates produced solanapyrone A on CDLCM but not sol B and sol C.

No solanapyrones were detected in culture filtrates of any isolates incubated at 30^{0}C.

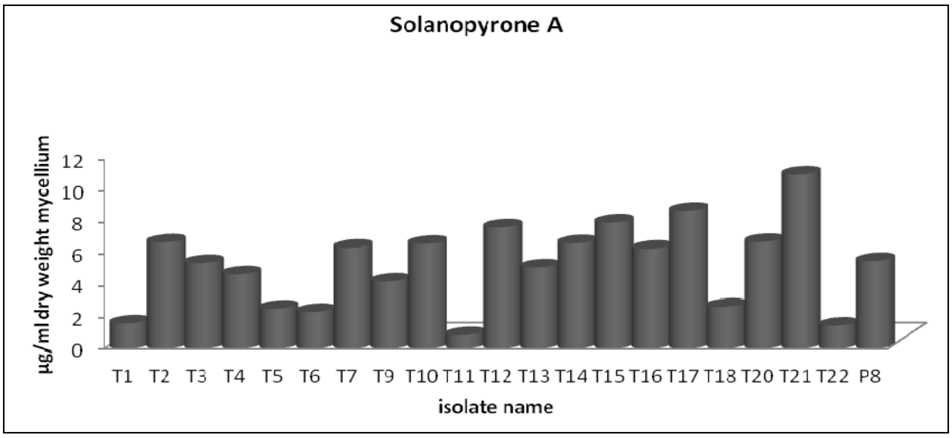

Figure 1. Production of solanapyrone A (µg/g dry weight mycelium) of isolates *Ascochyta rabiei* grown on Czapek-Dox Liquid Cation Medium

The solanapyrones were first described as products of *Alternaria solani*, the causal agent of early blight of potato and tomato (Ichihara et al. 1983). Alam et al. (1989) isolated two toxins from culture filtrates of *Ascochyta rabiei* and identified them as solanapyrones A and C. Further work in which the fungus was grown on chickpea seed extract with glucose or Richard's medium (Höhl et al 1991) or a defined medium (Chen and Strange 1991) allowed additionally the production of solanapyrone B. Latif et al. (1993) found one of nine strains of the *A.rabiei* produced a cytochasin which was identified as cytochalasin D. The results reported in this paper demonstrate the capacity of twenty one pathogenic isolates of the fungus to synthesize phytotoxic compounds *in vitro*. All of the isolates produced solanapyrone A in CDLCM at 20^{0}C but not at 30^{0}C.

It is known that many toxins are responsible for pathogenicity of fungus (Wheeler and Luke 1955, Nadel and Spiegel-Roy 1988, Vidhyasekaran et al. 1990). Whereas it is not difficult to show the relevance of host-selective toxins to pathogenicity since isolates that lose their ability to produce toxin are non-pathogenic, it is more difficult to demonstrate the role of non-selective toxins in disease (Strange 1998). Solanapyrone compounds by the fungus *A.rabiei* are not selectively toxic but the symptoms caused by the solanopyrone A, epinasty, chlorosis and necrosis, are consistent with the disease (Strange 1997). Furthermore cuttings allowed to take up solanopyrone A lodged, a symptom typical of the disease (Hamid and Strange 2000). Solanapyrone produced by *A.rabiei* may therefore be related to the virulence of the pathogen.

Sequence Data

DNA was successfully extracted from six Turkish isolates of *A.rabiei* which produced different amounts solA and a Pakistan isolate of fungus using the commercial kit .An amplicon of about 600 bp was obtained with the primers ITS1F and ITS4 from all isolates. Sequencing of the amplicons which contained the ITS1 region (139bp), the 5,8 S gene (158bp) and ITS2 region (143bp) showed that they were identical (Fig2).

Confirmation of the identity of the pathogen was sought by sequence analysis of rDNA. These experiments showed that the sequences of the internal transcribed spacers and 5.8 S gene of the six Turkish isolates, which were identical to each other and, were also identical to that of a Pakistan isolate of *A.rabiei*. Thus, the causal agent of blight of chickpea in Turkey was additionally confirmed to be *A.rabiei* at the molecular level. Two interpretations of the perfect match of the DNA sequences of the Turkish and the Pakistan isolates are that either these regions are particularly conserved within in *A.rabiei* or that the Pakistan and Turkish isolates are of common origin. *A.rabiei* isolates obtained from Paul Bridge (CABI) contained one more base pair in ITS1 region (140bp) than Turkish and Pakistan isolates of *A.rabiei*.

ITS

1CCTAGAGTTTGTGGGCTTTGCCCGCTACCTCTTACCCATGTCTTTTGAGTACTTACGTTTCCTCGGCGGGTCCGCCCG
CCGATTGGACAAAATCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAACATAATAGTTA

5.8 S

CAACTTTCAACAACG GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTT

ITS 2

GTACCTTCAAGCTTTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCG
TATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTA

Figure 2. Sequences of the internal transcribed spacer (ITS) 1, the 5.8 S and the ITS2 regions (440bp in total) of the Turkish isolates of *Ascochyta rabiei*.

Ribosomal DNA (rDNA) sequences have been aligned and compared in a number of living organism. Studies of rDNA sequences have been used to infer phylogenetic history across a very broad spectrum, from studies among the basal lineages of life to relationships among closely related species and populations (Hillis and Dixon 1991).

In this study, all isolates were confirmed as *A.rabiei* by rDNA sequencing and they all produced solanapyrone A although the amounts were variable. These data provide additional evidence for the importance of solanopyrone A to the pathogen since if its production were gratuitous some isolates would be expected not to produce the compound, particularly as the fungus has a sexual stage, *Didymella rabiei.*

ACKNOWLEDGEMENTS

I am grateful to Dr. Richards Strange for his great help and supervision. I also wish to acknowledge Miss Laura Winskill for sequencing the PCR products. and Dr. Paul Bridge (CABI)) for providing *A.rabiei* isolate. This project was funded by British Council.

ÖZET

NOHUTTA ASCOCHYTA YANIKLIKLIK ETMENİ *ASCOCHYTA RABIEI*’NIN TÜRK İZOLATLARININ TOKSİN ÜRETİMİ VE DNA SEKANS ANALİZLERİ

Bu çalışmada 20 adet *Ascochyta rabiei* izolatı Türkiye’nin nohut üretimi yapılan alanlarından toplanan hastalıklı nohut bitkilerinden izole edilmiştir. İzolatlar solanapyrone üretimlerinin belirlenmesi için 12 gün süreyle iki farklı sıcaklıkta Czapek Dox sıvı kültür ortamında (CDLCM) geliştirilmişlerdir. Solanapyronların kantitatif ölçümleri HPLC analizi ile yapılmıştır. Tüm izolatların sıvı ortamda (CDLCM) 20^0C de solanapyrone A ürettikleri buna karşın 30^0C de üretmedikleri tespit edilmiştir.

Patojenin teşhisinin doğrulanması rDNA sekans analizi ile yapılmıştır. Bu çalışmada yedi izolatın ITS ve 5.8 S gen bölgelerinin sekanslarının hem birbirleri ile hem de *A.rabiei*’nin Pakistan izolatı ile aynı olduğu görülmüştür. Farklı miktarlarda toksin üreten *A.rabiei* izolatlarının PCR ürünlerinin rDNA sekansları aynı bulunmuştur.

Anahtar kelimeler: Solanapyrone, *Ascochyta rabiei*, Nohut, DNA Sekans.

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