Turkish Journal of Agriculture and Forestry

Volume 31 | Number 1 Article 5

1-1-2007

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Turk J Agric For
31 (2007) 41-46

Mating Type Groups of *Ascochyta rabiei* (Teleomorph: *Didymella rabiei*), the Causal Agent of Chickpea Blight in Central Anatolia

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Received: 16.05.2006

Abstract: The distribution of mating types of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) was studied on isolates collected from chickpea growing areas of Central Anatolia. Mating type assessments of 45 isolates from 6 different provinces were conducted under laboratory conditions. After keeping for 5-6 weeks, the pseudothecial development and ascospore production were observed on chickpea stem pieces that were incubated in 8 ± 1 °C and 85% relative humidity. Both mating types were found in all the provinces, except for Kayseri and Sivas. The majority of the isolates belonged to Mat 1-1 (57.8%) and the others (42.2%) to MAT 1-2. Additionally, the mating types of *A. rabiei* were differentiated from each other using the multiplex PCR method with the primers Com1, Tail 5, and SP21. MAT 1-1 isolates were amplified to approximately a 460 bp fragment and MAT 1-2 isolates to a 700 bp fragment, in accordance with the discrimination of mating type groups with conventional methods.

Key Words: Chickpea, *Ascochyta rabiei*, multiplex PCR, mating type

Nohutlarda Yanıklık Etmeni *Ascochyta rabiei*'nin (Teleomorph: *Didymella rabiei*) Orta Anadolu Bölgesindeki Mating Type Grupları

Özet: Orta Anadolu bölgesindeki 6 farklı nohut ekim alanından toplanan 45 *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) izolatının mating type gruplarının dağılımı laboratuar koşullarında araştırılmıştır. Nohut gövde parçaları üzerinde pseudothecium gelişimi ve ascospor üretimi 8 ± 1 °C sıcaklık ve % 85 nispi nem içeren koşullarda 5-6 haftalık inkübasyon periyodundan sonra gözlenmiştir. Kayseri ve Sivas illeri hariç diğer tüm illerde her iki mating type grubu da bulunmuştur. Test edilen izolatların büyük bir kısmı Mat 1-1 (% 57.8) içerisinde yer alır iken % 42.2'si ise MAT 1-2 grubu içinde yer almıştır. Ayrıca multiplex PCR (primer: Com1, Tail 5 ve SP21) metodu kullanılarak *A. rabiei*'nin mating type grupları birbirinden hızlı olarak ayırt edilmiştir. Mating type gruplarının klasik yöntemler kullanılarak yapılan ayırımı ile uyumlu olarak MAT 1-1 izolatlarından 460 bp, MAT 1-2 izolatlarından ise yaklaşık 700 bp büyüklüğünde spesifik fragmentler çoğaltılmıştır.

Anahtar Sözcükler: Nohut, *Ascochyta rabiei*, multiplex PCR, mating type

Introduction

Chickpea blight caused by *Ascochyta rabiei* (Pass.) Labr. (teleomorph, *Didymella rabiei* (Kov.) v. Arx) is a devastating disease of chickpea plants (*Cicer arietinum* L.) in most of the chickpea growing countries of South Asia, the Middle East, the Mediterranean region, and North Africa (Nene, 1982; Nene and Reddy, 1987; Kaiser and Muehlbauer, 1988; Nene et al., 1989). The fungus infects all aerial parts of the plant and causes necrotic lesions, which are circular on the leaflets and pods, and are elongated and irregular on the stems and petioles. Under favorable environmental conditions and in the presence of susceptible host plants, this pathogen devastates chickpea crop areas and causes heavy yield losses of up to 100% in severely affected fields (Singh and Reddy, 1990).

Didymella rabiei, the sexual stage of *A. rabiei*, forming on the debris of infected chickpea plants was first described occurring in Bulgaria by Kovachevski in 1936 and was later reported from the USSR (Gorlenko and Bushkova, 1958), Greece (Zachos et al., 1963), Hungary (Kövics et al., 1986), the United States (Kaiser and

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Hannan, 1987), Spain (Jimenez Diaz et al., 1987), and Syria (Haware, 1987). The fungus is a bipolar heterothallic and forms the sexual stage when 2 compatible mating types come together (Trapero-Casas and Kaiser, 1992; Wilson and Kaiser, 1995). The amount of genetic variability of *A. rabiei* is enhanced by the presence of the teleomorphic stage (Akem, 1999). This may contribute to the emergence of pathogen populations with higher virulence and fungicide resistance (Kaiser, 1997; Kaiser and Küsmenoğlu, 1997). Moreover, pseudothecia formed on chickpea debris, which overwinter on the soil surface, contributing to the long-term survival of the pathogen (Navas-Cortes et al., 1995). Trapero-Casas and Kaiser (1992) reported that the teleomorphic stage of *A. rabiei* plays an important role as the primary inoculum for infection and long distance spread of the pathogen.

The pathogenic variability of *A. rabiei* isolates is determined based on their aggressiveness on different chickpea cultivars. Reddy and Kabbabeh (1985) divided 50 isolates of *A. rabiei* into 6 physiological races in Syria and Lebanon, based on the reactions of a set of 6 different chickpea cultivars. Grouping *A. rabiei* isolates from 11 different provinces into 8 groups based on morphological and cultural characteristics, Dolar and Gürcan (1992) reported the existence of races 1, 4, and 6, in Turkey.

Many studies have been performed on genetic polymorphisms among *A. rabiei* isolates and molecular markers related to pathogenic groups of this pathogen have been identified (Fischer et al., 1995; Santra et al., 2001). DNA fingerprinting was successfully used to estimate the genetic diversity in *A. rabiei* by many researchers (Weising et al., 1991; Morjane et al., 1994; Geistlinger et al., 1997). Navas-Cortes et al. (1998a) classified *A. rabiei* isolates from different geographical origins into 10 RAPD groups with 7% dissimilarity, but did not find a relationship between RAPD groups and pathogenic variability or mating type groups of *D. rabiei*. Barve et al. (2003), by cloning and characterizing the mating type (MAT) locus of *D. rabiei* with TAIL and inverse PCR methods, developed the MAT-specific multiplex PCR assay. Genetic variability on the high mobility group (HMG) region of MAT 1-2 appeared to be substantially useful for the investigation of genetic relationships among *Ascochyta* spp.

For the last 7 years there have not been any studies about mating type distribution of *A. rabiei* isolates in Turkey; therefore, the objectives of this work were to determine the mating types of *A. rabiei* and their distribution in chickpea production areas of Central Anatolia, and the usefulness of the multiplex PCR method for rapidly determining mating types.

Materials and Methods

Fungal material

A. rabiei isolates used in this study were collected from 6 different provinces of Central Anatolia in 2001 and 2002, and were maintained in the culture collection of the Department of Plant Protection, Faculty of Agriculture, University of Ankara. The mating type tester strains, USDA Ar-483 (MAT 1-1) and USDA Ar-158 (MAT 1-2), were obtained from Dr. S. Banniza (University of Saskatchewan, Dept. of Plant Sciences, Canada). Single conidia of *A. rabiei* were germinated and grown on chickpea seed meal–dextrose agar (CSMDA: 4% chickpea flour, 2% dextrose, and 2% agar in 1 l of distilled water) at 21 °C with a 12 h light-photoperiod. Cultures were deposited in Microbank tubes (Pro-Lab Diagnostics, UK) at –80 °C.

Mating type studies

The mating types of *A. rabiei* were determined using the stem inoculation method described by Trapero-Casas and Kaiser (1992). Spore suspensions of each isolate were prepared at 1×10^6 spores per ml of concentration and mixed separately with an equal volume of spore suspension of mating type testers, either MAT 1-1 or MAT 1-2. The stem pieces of healthy chickpea plants were surface sterilized in 1% NaOCl for 3 min and placed in a mixed spore suspension of each isolate. Stem pieces were soaked in the spore suspension for 1 h, drained, and placed in petri dishes containing 10 filter papers moistened with sterile distilled water. Each isolate was replicated in 3 petri dishes, each containing 5 stem pieces. The dishes were incubated in the dark at 8 ± 1 °C for 5-6 weeks. After incubation, the stems were dried and examined for pseudothecia and discharge of ascospores.

Genomic DNA isolation

Genomic DNA was purified as described by Weising et al. (1991). Mycelial discs were put in 100 ml of potato dextrose broth (PDB, Difco) in 250 ml flasks and then incubated at 21 °C at 140 rpm on an orbital shaker for 7 days. Mycelia were harvested, frozen in liquid nitrogen, and stored –80 °C until use. Ground mycelia were suspended in 2% hexadecyltrimethyl-ammonium bromide (CTAB) extraction buffer (1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 20 mM EDTA, 0.2% β-mercaptoethanol). After phenol-chloroform 25:24 (v/v) extraction, RNAse-A was added to each tube and the tubes were incubated at 37 °C for 30 min. The samples were re-extracted with chloroform-isoamyl alcohol 24:1 (v/v). Nucleic acids were then precipitated with isopropanol, rinsed with ethanol, adjusted to a final concentration of 10 ng/µl in TE (pH: 7.4), and stored at –20 °C.

Multiplex PCR assay

PCR analysis was performed using primers Com1, SP21 and Tail 5, based on Barve et al. (2003). PCR was carried out in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1 mM of $MgCl_2$, 0.4 µM of each primer, 10 ng of DNA, 0.2 µM of dNTPs, and 1 unit of *Taq* DNA polymerase (Promega). Amplifications were performed in a thermal cycler (Biometra) with an initial denaturation step for 2 min at 96 °C, followed by 25 cycles of 15 s at 96 °C, 30 s at 60 °C, and 45 s at 72 °C, with a final extension of 5 min at 72 °C (Peever et al., 2004). The PCR products were separated electrophoretically in 1.7% agarose gels using 1X Tris-acetate-EDTA (TAE) buffer (Sambrook et al., 1989). The gels were stained with ethidium bromide and visualized with a Syngene bio imaging system.

Results and Discussion

A major limitation of yield in most chickpea growing areas of Turkey is the incidence of chickpea blight caused by *A. rabiei*. The development of the teleomorphic stage of *A. rabiei* is an important factor in the pathogen's disease cycle and significantly affects the spread, survival, and genetic structure of the pathogen (Wilson and Kaiser, 1995; Kaiser and Küsmenoğlu, 1997; Peever et al., 2004).

Both mating types were found to occur throughout the chickpea production areas of Central Anatolia in this study, which was conducted to determine the distribution frequencies of mating type groups. From several provinces (Ankara, Eskişehir, Kayseri, Kırşehir, Sivas, and Yozgat), 45 isolates of *A. rabiei* were collected and successfully crossed with one or the other tester of mating types. The formation of mature pseudothecia was observed on the stem pieces of chickpea plants in petri dishes after 5-6 weeks of incubation at 8 ± 1 °C (Figure 1). Mature pseudothecia are erumpent, dark brown or black, subglobose, and 120-270 µm in diameter, with an

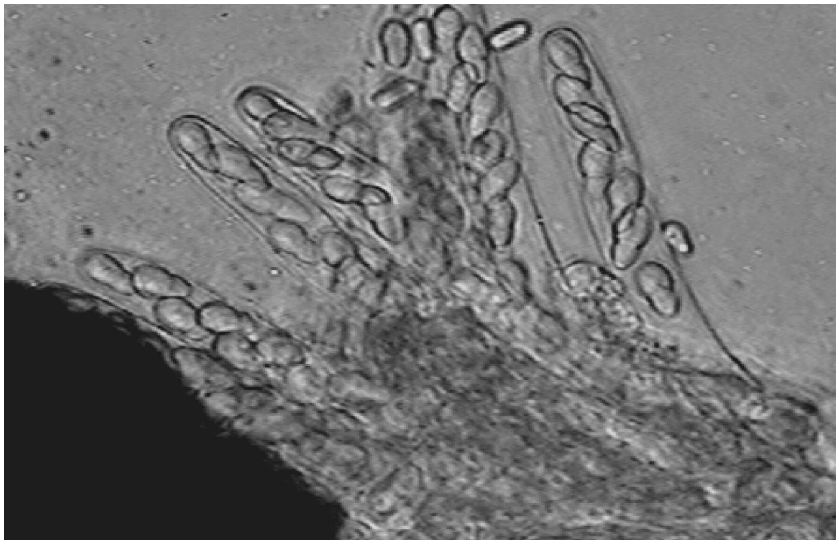

Figure 1. Asci and ascospores of *Didymella rabiei* produced on chickpea stems in laboratory conditions.

inconspicuous ostiole. Asci are bitunicate, 8-spored, cylindrical to subclavate, and measure 50-80 × 10-12 µm. Ascospores are hyaline, 2-celled (with the upper cell broader than the lower cell), ellipsoidal to biconical, irregularly distichous, and 9.5-16 × 4.5-7 µm in size (Trapero-Casas and Kaiser, 1992).

The emergence of pseudothecia and ascospores on chickpea crop residues needs the pairing of 2 compatible isolates, which are referred to as MAT 1-1 and MAT 1-2 (Kaiser, 1997). The development of mating types controlled by 1 gene with 2 alleles may be responsible for the emergence of new races with varying virulence as a result of genetic variation in the pathogen (Wilson and Kaiser, 1995).

The frequencies of mating type distribution of *A. rabiei* isolates were variable by province (Table 1). The isolates of MAT 1-1 were more common than those of MAT 1-2 in chickpea growing areas of Central Anatolia. Of the isolates tested, 57.8% were identified as MAT 1-1 and 42.2% as MAT 1-2. Both mating types of *A. rabiei* were found in Ankara, Eskişehir, Kırşehir, and Yozgat provinces. MAT 1-1 was recovered more frequently than MAT 1-2 in Ankara, Eskişehir, and Kırşehir, but not in Yozgat province. Only one group of mating type was found in 2 provinces, Kayseri and Sivas. Another group could not be determined in these provinces, probably due to the limited samples. Kaiser and Küsmenoğlu (1997) identified mating types of 145 isolates of *A. rabiei* collected from 23 provinces of Turkey between 1984 and 1994. The isolates from 18 of these provinces were crossed with each mating type tester isolate. Of the isolates tested, 59% were identified as MAT 1-1, while 41% were found to pair with MAT 1-2. These researchers stated that of the 42 isolates obtained from Central Anatolia, 23 isolates belonged to MAT 1-1, while 19 isolates belonged to MAT 1-2. However, the higher ratio of MAT 1-2 they detected in Ankara and Kırşehir provinces and the fact that MAT 1-1 was more common in Yozgat province contrast with the results of the present study. Armstrong et al. (2001) reported the existence of both mating types with similar frequencies of distribution across the chickpea growing region of western Canada. Navas-Cortes et al. (1998a) tested 48 isolates of *D. rabiei* from India, Pakistan, Spain, and the USA for mating type and found that 58% were MAT 1-1, while 42% belonged to MAT 1-2.

Table 1. Distribution of mating types of *Ascochyta rabiei* in different provinces of Central Anatolia between 2001 and 2002.

Provinces	No. of isolates tested	Type of isolates	
		MAT 1-1	MAT 1-2
Ankara	26	15	11
Eskişehir	8	5	3
Kayseri	2	2	-
Kırşehir	3	2	1
Sivas	1	-	1
Yozgat	5	2	3
Total	45	26	19

Infected seeds significantly contribute to the introduction of mating types to chickpea production areas that were previously disease-free. Maden (1987) obtained seeds from various places in Turkey and found that more than 50% of the seed samples were infected by the pathogen. This clarified why both mating groups were widespread throughout Central Anatolia. Moreover, ascospores discharged from pseudothecia formed on chickpea debris, which overwinter in soil, make an important contribution to the long-distance spread of the pathogen as a primary inoculum source (Trapero-Casas et al., 1996).

The development and maturation of pseudothecia require approximately 1-2 months in the laboratory, and so this procedure is time consuming and troublesome. Additionally, conditions for sexual recombination are very specific and require cool and humid conditions (Navas-Cortes et al., 1998b). In this respect, the method of multiplex PCR was used to rapidly identify the groups of mating type of *A. rabiei* on chickpea plants. Mating type-specific PCR was performed with primers Com1, SP21, and Tail 5, which is designed to conserve regions of the alpha and high mobility group (HMG) proteins (Barve et al., 2003). Primer Com1 was specific to the 3' flanking region of the MAT genes. Forward primer SP21 was designed to MAT 1-1 idiomorph, while Tail 5 was designed to MAT 1-2 idiomorph. Multiplex MAT-specific PCR with primers Com1, SP21, and Tail 5 was amplified by an approximately 460-bp fragment from mating type 1 isolates, while a 700-bp PCR product was amplified from mating type 2 isolates (Figure 2), according to the original mating type classification of Trapero-Casas and

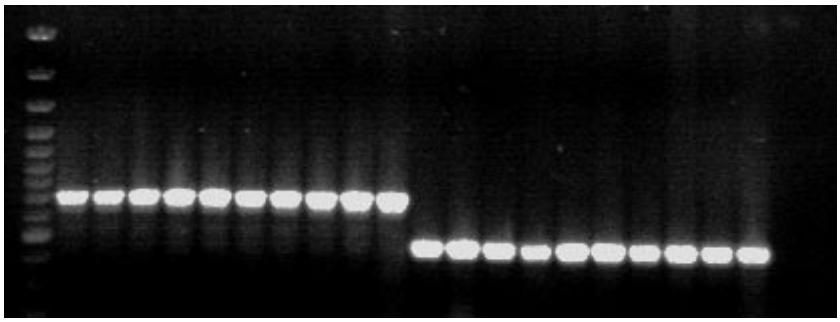

Figure 2. Multiplex PCR analysis of mating types of *Ascochyta rabiei* in Central Anatolia with primers Com1, SP21 and Tail 5. Lane 1 contains the molecular weight size marker (Gene ruler 100 bp DNA ladder plus MBI, Fermentas). Lanes 2-11 contain MAT 1-2 isolates of *A. rabiei* (700 bp). Lanes 12-21 contain MAT 1-1 isolates of *A. rabiei* (460 bp).

Kaiser (1992). All the isolates tested separately grouped around mating type groups according to classical differentiation.

Conclusions

Development of chickpea blight-resistant or blight-tolerant cultivars is the most practical and effective means of disease control (Nene and Reddy, 1987). Some genotypes resistant against chickpea blight have been developed and registered in Turkey; however, these lines have not been accepted by farmers because of their late maturing and small seeds problems. The genetic background of chickpea cultivars is rather restricted and eventually becomes susceptible to ascochyta blight (Dey and Singh, 1993). In this respect, the preservation of the available resistant resources appeared to play an important role in decreasing crop losses caused by this disease. Furthermore, environmental conditions closely correlated with pseudothecial development and ascospore production of *Didymella rabiei* on artificially infested chickpea debris. Hence, the methods of cultural management, including the removal and destruction of chickpea residues, and crop rotation, as well as seed treatment, need to be taken into consideration in order to prevent infection of chickpea plants by primary inoculum in the new growing season.

Acknowledgments

We are grateful to Dr. S. Banniza (University of Saskatchewan, Dept. of Plant Sciences, Canada), who provided us the tester isolates, and the European Union for a grant, Ascorab (Contact no: ICA4-CT-2000-30003).

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