ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Basem ATTAR

GENETICS OF VIRULENCE AND PATHOGENIC DIVERSITY FOR ASCOCHTA BLIGHT AFFECTING CHICKPEA

DEPARTMENT OF BIOTECHNOLOGY

ADANA, 2016

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Master of Science (MSc) by the board of jury on 07/18/2016

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This MSc Thesis is written at the Department of biotecnology of Institute of Natural And Applied Sciences of Çukurova University.
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ABSTRACT

MSc. THESIS

GENETICS OF VIRULENCE AND PATHOGENIC DIVERSITY FOR ASCOCHYTA BLIGHT AFFECTING CHICKPEA

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INSTITUTE OF BASIC AND APPLIED SCIENCES
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Supervisor : Prof. Dr. Mukaddes KAYIM
Year: 2016, Pages: 84
Jury : Prof. Dr. Mukaddes KAYIM
: Prof. Dr. Hakan ÖZKAN
: Assoc. Prof. Sibel DERVİŞ

This research was conducted to study the inheritance of virulence, mating type distribution, pathogen diversity for *Ascochyta rabiei* affecting chickpea, and to screen sub set chickpea accessions against the highly virulent pathotype IV. Inheritance of virulence was studied by doing crosses between pathotypes three crosses were made and 20 progenies per cross were tested on susceptible and resistant chickpea genotypes, chi square test showed that inheritance of virulence was controlled by single gene. Mating type distribution for 78 isolates collected from four countries was investigated by using MAT-specific primers, and the study showed no significant deviation from 1:1 ratio. Pathogen diversity for 96 isolates collected from four countries was studied studied by using eight SSR primers , the study showed highly genetic diversity between the isolates. Finally the reaction of 200 chickpea genotypes against pathotype IV the highly virulent pathotype was studied and just one genotype showed moderately resistant reaction.

Key words: *Ascochyta rabiei*, chickpea, inheritance, mating type, pathotypes.

ÖZ

YÜKSEK LİSANS TEZİ

GENETICS OF VIRULENCE AND PATHOGENIC DIVERSITY FOR ASCOCHYTA BLIGHT AFFECTING CHICKPEA

Basem ATTAR

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BİYOTEKNOLOJİ ANABİLİM DALI

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Bu çalışma, nohutta hastalık etmeni *Ascochyta rabiei*'nin patojen çeşitliliğini, eşeyli üreme tipinin dağılımını, virülensliğinin kalıtımını araştırmak ve kayıtlı nohut hatlarını oldukça virulent pathotype VI (PIV)'e karşı testlemek amacıyla yürütülmüştür. Virulensliğin kalıtımını göstermek amacı ile Pathotype IV ile diğer patotipler arasında 3 melezleme yapılmış, her bir melezleme için 20 yeni nesil duyarlı ve dayanıklı nohut hatları ile testlenmiştir. Ki-kare testi virulensliği tek bir genin kontrol ettiğini göstermiştir. Dört farklı ülkeden toplanan78 izolatın eşeyli üreme tipi dağılımı MAT spesifik primerleri kullanılarak araştırılmış ve 1:1 oranında açılım göstererek önemli bir standart sapmanın olmadığı gösterilmiştir. Dört ülkeden toplanan 96 izolatın patojen çeşitliliği 8 SSR primeri kullanılarak çalışılmış ve izolatlar arasında son derece yüksek genetik çeşitlilik olduğu gösterilmiştir. Sonuçta 200 nohut genotipinin yüksek virulent patotip olan PIV karşı reaksiyonu çalışmış ve sadece bir genotip orta düzeyde dayanıklılık göstermiştir.

Anahtar Kelimeler: *Ascochyta rabiei,* Eşeyli üreme tipi, Kalıtım, Nohut, Primerler

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Basem ATTAR

July 2016

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LIST OF ABBREVIATIONS

AR :*Ascochyta rabiei*

AMOVA :Analysis of Molecular Variance

bp :Base Pair

CDA :Chickpea Dextrous Agar

DNA : Deoxyribonucleic Acid

FIGs :Focused Identification of Germplasm Strategy

ICARDA :International Center for Agricultural Research in the Dry Areas

LARI : Lebanese Agricultural Research Institute

MAT :Mating type

PCR :Polymerase Chain Reaction

PDB :Potato Dextrous Broth

PIC :Polymorphism Information Contents

SSR :Simple Sequence Repeat

TBE :Tris/Borate/EDTA

WA :Water Agar

X2 :Chi-square test

1. INTRODUCTION

1.1. Importance of chickpea

Chickpea is a cool season legume crop grown in several countries worldwide as a food and feed sources. Chickpea probably originated in southeastern Turkey and spread west and south via the Silk Route. There are two types of chickpea, one is the 'Kabuli' white and other is 'Desi' brown. Kabuli type is grown in temperate regions while the 'Desi' type chickpea is grown in the semi-arid tropics (Malhotra et al., 1987; Muehlbauer and Singh, 1987). It is grown and consumed in large quantities in South East Asia, and Middle East and Mediterranean countries. It ranks second in area and third in production among the pulses worldwide (FAO, 2010). During 2006-2009, the global chickpea production area was about 11.3 million ha, with production of 9.6 million tons and average yield of 849 kg ha^{-1}(FAO, 2011). Chickpea is a good source of protein and essential amino acids like isoleucine, leucine, lysine, phenylalanine, and valine (Karim and Fattah, 2006). The productivity of chickpea is affected by much fungal disease where Ascochyta blight caused by *Ascochyta rabiei* (Pass.)Lab. (perfect stage *Didymella rabiei* (Kovachevski) von Arx]. is the key yield limiting factor in Algeria, America, Australia, Bangladesh, Bulgaria, Canada, Cyprus, Ethiopia, France, Greece, India, Iran, Iraq, Palestine, Italy, Jordan, Lebanon, Mexico, Morocco, Pakistan, Romania, Spain, Syria, Tanzania, Tunisia, Turkey and the USSR (Nene, 1982; Kaiser, 1997). The disease can cause up to 100% yield and quality loses under favorable conditions (Singh et al., 1981; Nene, 1984; Reddy and Singh, 1990a ; Singh and Reddy, 1990; Solh et al., 1994; Dusunceli et al., 2007). Economic losses due to Ascochyta blight damage have been substantial in Australia (Ackland et al., 1998; Knights and Siddique, 2002), Canada (Chongo and Gossen, 2001), Latin America (Kaiser et al., 2000), and West Asia (Akem et al., 2000).

Ascochyta blight is caused by *Ascochyta rabiei* (teleomorph *Didymella rabiei) Didymella rabiei* belongs to the class Ascomycota, order Pleosporales, family Didymellaceae and genus *Didymella*. The sexual stage of *A. rabiei* was discovered

by Kovachevski in 1936 on overwintering chickpea debris in southern Bulgaria (Kaiser, 1997).

1.2. Disease symptoms

Air-borne conidia and ascospores infect younger leaves and produce small water-soaked necrotic spots that rapidly enlarge and coalesce. Conidia may also be water-borne and splash dispersed to infect foliage tissue on the same or nearby plants. Development of pycnidia in concentric rings on lesions is the characteristic symptom of *A. rabiei* infection. Lesions that develop on leaves and pods appear circular with brown margins and a grey center that contains pycnidia, whereas lesions developing on petiole, stems, and branches are elongated. Seed-borne infection leads to brown lesions at the stem base of emerged seedlings. Subsequently, symptoms spread rapidly to all aerial parts including leaves, petioles, flowers, pods, branches, and stems, which lead to rapid collapse of tissues and death of the plant. Plants are attacked at any growth stage, depending on the inoculum availability. However, AB is most prominent during the flowering to early pudding growth stages. Diseased pods with visible blight symptoms often fail to develop any seed. Pod infection often leads to seed infection through the testa and cotyledons. Infected seed can be discolored and possess deep, round, or irregular cankers, sometimes bearing pycnidia visible to the naked eye. Infection during the pod maturation stage often results in shriveled and infected seed (Nene, 1982; Singh and Sharma, 1998; Akem, 1999).

1.3. Epidemiology of Ascochyta blight

Ascochyta rabiei survives either on or in seed or plant debris in the form of mycelium, pycnidia, and various teleomorphic stage (Kaiser, 1997). The pathogen can survive in a free state in the soil, and it can survive at a temperatures of 10–35°C, for 8 months in infected chickpea debris (Nene and Reddy, 1987), 20 months on

infected stem (Kaiser and Hannan, 1987), and five months on the surface of chickpea seed (Singh et al., 1995).

Temperature, rainfall and wind are the environmental factors that have the greatest influence on disease development (Weltzien and Kaach,1984; Nene and Reddy, 1987; Trapero-Casas and Kaiser, 1992b). The amount of rain is closely correlated with disease severity as it is crucial for pseudothecia maturation, discharge of ascospores and the infection process (Akem, 1999). The disease can reach epiphytotic proportions when the relative humidity is greater than 60%, leaf temperatures are between 10 and 20°C, combined with more than 150 mm of annual rainfall or a leaf wetness period of at least 7 hours (Reddy and Singh, 1990c; Trapero-Casas and Kaiser, 1992b). Disease severity increases as the duration of wetness periods exceed a minimum of 6 hours, and with increasing temperatures to a maximum of approximately 20°C (Trapero-Casas and Kaiser, 1992b). Lower and upper temperature limits for infection and disease development are about 5°C and 30°C, respectively (Trapero-Casas and Kaiser, 1992b). In order for an epidemic of ascochyta blight to occur a monthly average temperature of at least 8°C and a monthly rainfall of at least 40 mm is needed (Ketelaer et al., 1988).

1.4. Host ranges of *Ascochyta rabiei*

Artificial inoculation of *Vigna unguiculata*, *P. vulgaris* (Kaiser, 1973; Khan et al., 1999a), *Lactuca serriola*, *Lamium amplexicaule*, *Medicago sativa*, *Melilotus alba*, and *Thlapsi arvense* (Kaiser, 1991), *Melilotus alba*, lentil, field pea, vetch, common bean, and cowpea showed infections by *A. rabiei* and can play an important sources of primary inoculum to initiate disease development in chickpea fields (Nene and Reddy, 1987; Khan et al., 1999b). *Ascochyta rabiei* has also been isolated from *Brassica nigra*, *Descurainia sophia*, *Galium apanine*, *Lamium amplexicaule*, and *Triticum aestivum*, grown in fields where infected chickpea debris of the previous season remained on the soil surface during the off-season (Kaiser, 1991).

1.5. Ascochyta blight disease management strategies

The major components of Ascochyta blight management strategies are cultural, chemical, host plant resistance and integrated management.

1.5.1. Cultural methods

Reduction in the source of inoculum is the primary focus in the management of the disease that consists of planting of healthy seed, crop rotation with non-host crop such as cereal, destruction of chickpea stubbles and deep sowing (Pande et al., 2005). Selection of disease-free seed will reduce the risk of seed-borne infection in new plantings (Gan et al., 2006) and the seed lots for planting should be tested for ascochyta blight infection in accredited laboratories (Pearse et al., 2000). Large-sized seeds were more beneficial than small seeds, both in terms of yield and disease severity (Morrall, 2001). Crop rotation with non-host crops is another strategy that helps to reduce the background level of ascocchyta blight inoculum. Effectiveness of crop rotations entirely depends upon the environmental conditions and in tropical regions, a break of 1-2 years between chickpea cultivation reduced the disease severity (Kaiser et al., 2000), whereas in the temperate regions, planting of non-host plants was recommended for four years between successive chickpea crops (Gossen and Miller, 2004). Altering the sowing time, late or early in order to avoid exposure of plants to ascospores, depending upon the time of epidemics, could minimize the damage caused by ascochyta (Gan et al., 2006). Proper and timely application of balanced fertilizer like use of potassium fertilizer in soils with higher contents of nitrogen definitely helped to retard the ascochyta blight (Pande et al., 2005). Burning of source of inoculum is an important measure but not preferred because it adversely affects the soil organic matter and essential nutrients.

1.5.2. Biological control

Different antagonistic micro-organisms can be utilized to control plant pathogens in host pathogen interaction. The experiments with *A. rabiei*, showed that upon burial of inoculum in the sterilized soil, resulted in production of pseudothecia and pycnidia more uniformly and rapidly than in natural soil, indicating that the fungus was affected by other saprophytic microorganisms present in that soil (Navas-Cortez, 1992).

The antagonistic activity of *Trichoderma viridi* greatly influenced the growth and survival of *A. rabiei* (Wang et al., 2003), while in Pakistan a strain of Rhizobium named Thal-8, proved to be very effective by producing an acid that is antifungal in nature and limits the growth of *A. rabiei* in soil (Khokhar et al., 2001). The efficacy of *Aureobasidium pullulans* and *Conostachya rosea* under the laboratory conditions demonstrated that both *A. rabiei* and *D. rabiei* stages were inhibited (Dugan et al., 2005). Botanical extracts with antimicrobial activity are being used to control various insect pests and pathogens. Aqueous extract of onion (*Allium cepa*) has shown antifungal activity against *A. rabiei* (Khan et al., 1998). Biological control is relatively less effective in combating pathogens than insect pests (Butt et al., 2001). However, biological control could be included as one of the components of the integrated disease management strategies for ascochyta blight of chickpea.

1.5.3. Chemical control

Ascochyta blight is seed-borne and infested seed is an important source of primary inoculum in the field (Nene and Reddy, 1987; Dey and Singh, 1993). As a result seedlings emerging from infected seeds showed severe disease development (Maden et al.,1975). Under the condition when disease-free seed is not available, seed treatment is preferred to prevent spread of the disease. Disease transmission was reduced by more than 95% when the seeds were treated with benomyl (Demirci et al., 2003). Application of benomyl, thiram, carbendazim and chlorothalonil reduced

the transmission by more than 90% (Demirici et al., 2003). The performance of seed treatments in the field depended on environmental conditions (Demirci et al., 2003). Research in different part of the world has shown that applications of foliar fungicide are not cost effective when ascochyta blight severity is very low. When there is chance of rain, applications of a foliar fungicide once or twice significantly increase seed yield and quality. Since ascochyta blight is polycyclic disease, one time application of fungicide will control only one of the disease cycles, it will not prevent further infection of the pathogen (Kaiser and Hannan, 1988). Fungicide application strategies depend upon environmental conditions that may alter the level of resistance in the cultivar and the efficacy of the fungicides (Davidson and Kimber, 2007). Under conducive environmental conditions, multiple applications of fungicides are required during the growing season to manage the disease but in some cases, even multiple applications of fungicides were not sufficient to control the disease (Shtienberg et al., 2000). The timing and number of fungicide applications are critical to achieve effective control of the disease and to attain the maximum crop yield (Shtienberg et al., 2000; Chongo et al., 2003). For maximum protection and control against the disease timely fashion ahead of rain is necessary (Shtienberg et al., 2006).

Similarly, the timing and number of applications depends upon the weather conditions. Rainy, windy and humid conditions increase the chances of epidemic outbreak and thus influence the decision to spray and in case of severe infestation under favorable environmental condition, the spread of the disease is very rapid, and it is very difficult to implement a fungicide schedule under such conditions especially in case of susceptible varieties (Nene and Reddy 1987; Shtienberg et al. 2000) and under moderate epidemic, susceptible varieties may require four to six sprays to reduce the disease (Nene, 1982) and only under dry conditions can fungicide applications be reduced (Chongo *et al.*, 2003). Many fungicides have been tested for their efficacy in ascochyta control. The fungicides registered for ascochyta blight control in chickpea differ among countries. Foliar application with protectant fungicides such as Chlorothalonil (BravoR), a contact fungicide was effective against *A. rabiei* (Reddy and Singh, 1984; Bretag et al., 2008,). Also Strobilurin fungicides,

i.e. azoxystrobin and pyraclostrobin have been used in recent years, and azoxystrobin was found effective fungicide against *A. rabiei* (Demirci et al., 2003).

1.5.4. Host plant resistance

The most realistic approach for the management of Ascochyta blight relied on the identification of resistance germplasm through screening in the field and in the controlled environment (Muehlbaur and Singh, 1987; Reddy and Singh, 1990b) and development of resistant varieties through usual procedures. Even though Ascochyta blight has been known for almost 90 years, a little progress has been made on its control through the use of host plant resistance (Reddy and Singh, 1990b; Singh and Reddy, 1991). (Malik et al., 2006) screened 355 chickpea germplasm lines for resistance to Ascochyta blight under greenhouse conditions, ten genotypes were found to be resistant, 32 genotypes were found to be moderately resistant, whereas all the others were susceptible. In search of additional genes for resistance, wild relatives of crops have received increased attention because they often possess genes that confer resistance to biotic stresses (Molhotra, et al., 2000, Tivoli et al., 2006).

1.5.5. Integrated disease management

The conventional approaches for disease control have not stimulated an active holistic approach to disease management. As a result, some potentially useful integration and combination of techniques have been attempted. Integrated disease management includes all the management strategies in compatible manners to keep the losses below economic injury level. It is essential to get benefit from the cultivars with partial resistance. Research during the last few decades have proven that integrated management of ascochyta blight of chickpea depends upon the use a combination of disease free seed (Morrall, 2001), destruction and avoidance of inoculum source (Wallen and Jeun, 1968) and manipulation of sowing dates (Gan et al., 2002; Siddique and Bultynck, 2004). Moderately resistant cultivars sown early produced 15-30% greater grains yield than those sown late (Siddique and Sedgley,

1986; Gan et al., 2002) and also the use of seed and foliar fungicides (MaCleod and Gallway, 2002; Chongo et al., 2003), sowing of cultivars with improved resistance (Malik et al., 2006) help in the integrated management of the disease.

The disease can also be managed as the growers should observe a break of 3-4 years between successive chickpea crops to minimize the risk of disease carry over from previous crops, and isolate crops from infected residues and volunteers (Bretag et al., 2008). Other cultural practices such as reducing plant canopy thickness and planting pathogen free seed help minimize disease epidemics (Akem, 1999). An understanding of pathosystems and inter relationship between the host, pathogen and the environment are essential in making correct decision for disease control (Davidson and Kimber, 2007).

Objectives of study; it is important to do special studies at pathogens so our study was conducted to:

To determine the mating type distribution and genetics of virulence of *A. rabiei*.

To determine the pathogenic diversity of *A. rabiei* isolates from different countries.

And to screen mini-core chickpea accessions against the highly virulent pathotype (P4).

2. REVIEW OF LITERATURE

2.1. Diversity of *Ascochyta rabiei*

The most important method to control ascochyta blight disease is to grow resistant cultivars, but this method is not always effective because of changes in genetic structure of the pathogen causing high rate of pathogen variability that makes the resistant cultivar susceptible to the pathogen. Therefore resistance breakdown is the greatest challenge to breeding for Ascochyta blight resistance in chickpea (Singh and Reddy, 1991). It is not only important to develop cultivars with durable forms of resistance, but also to monitor changes in the population structure to anticipating resistance breakdown in existing chickpea cultivars and to design better strategies for sustainable cultivation of high yielding farmer and consumers preferred cultivars (Pande *et al*., 2005; Vail and Banniza, 2009).

A. rabiei shows high degree of pathogenic and genetic variability and Ascochyta blight resistant chickpea cultivars have become susceptible in some countries. It has been reported that severe epidemics of ascochyta blight has occurred in various chickpea production regions including those regions where ascochyta resistant cultivars have been adopted (Nava cortes et al., 1998; Nene, 1982; Singh et al., 1984; Nene and Reddy,1987). Genetic diversity of *A. rabiei* was reported from different countries using differential chickpea lines and molecular markers. Significant genetic variation was shown in populations of *A. rabiei* in Italy (Fischer et al., 1995), Spain (NavasCortés et al., 1998), India, Syria, Lebanon, Tunisia , and Pakistan (Gowen et al., 1989; Hussain et al., 1991; Jamil et al ., 1997; Morjan et al, 1994; Nene, 1984;Udupa et al., 1998;Santra et al., 2001), Canada, USA, Australia (Phane et al., 2003; Vail and Banniza, 2009), and Turkey (Bayraktar et al., 2007 ; Kaiser and Kusmenoglu, 1997).

2.1.1 Diversity studies using differential chickpea genotypes

Reddy and Kabbabeh (1985), reported the existence of six races in Syria and Lebanon and later Udupa and Weigand (1997), reported the existences of three pathotypes (Pathotype-1, Pathotype-2 and Pathotype-3). None of the pathotypes described by Udupa and Weigand (1997), was virulent on chickpea genotypes ICC-12004 and ICC-3996.

Recently Imtiaz et al.(2011), described a highly virulent Pathyotpe-4 in Syria that was capable of affecting the highly resistant chickpea genotypes (ICC12004 and ICC-3996) known for their resistance to pathotypes 1, 2 and 3, breeding materials at International Center for Agricultural Research In The Dry Areas (ICARDA) are being screened against this new pathotype-4, and so far low levels of resistance have been observed (Bayaa et al., 2004; Imtiaz et al., 2011).
High genetic diversity has also been reported from USA, Tunisia and Canada where popular varieties have become susceptible to new aggressive pathotypes (Peever et al., 2004; Rhaiem et al., 2008; Vail and Banniza, 2009).

In Syria Atik et al. (2013), did phenotyping on isolates randomly selected from the different genetic groups using five chickpea genotypes. The isolates were grouped into four pathotypes, namely, pathotype-1, pathotype-2, pathotype-3, and pathotype-4 with varying degrees of virulence on the chickpea genotypes. their findings showed a clear genetic shift towards more virulence over time and space in the populations of *A. rabiei* in Syria. This showed that the chickpea breeding materials should be tested for the more virulent pathotypes, and serious measures should be taken to send healthy seeds of international chickpea nurseries to avoid any gene flow from country to country.

In Turkey Turkkan and Dolar (2009), studied pathogenic characterization of 64 isolates of *A. rabiei* obtained from 5 different regions from Turkey. The isolates was determined with a set of 7 different chickpea cultivars (ILC 1929, F8, ICC 1903, ILC 249, ILC 482, ILC 3279, and ICC 3996). All the isolates were classified into both 3 pathotypes and 6 physiological races. Of the isolates used in this study, it was determined that 38 (59.4%) belonged to pathotype I, 3 (4.7%) to pathotype II, and 23

(35.9%) to pathotype III. Pathotypes I and III were determined in 5 regions of Turkey including the Mediterranean, Aegean, Southeastern Anatolia, Central Anatolia, and Black Sea regions, but pathotype II was not found in the Mediterranean and Black Sea regions. All 6 races of the *D. rabiei* were determined in Turkey. Race 1, 2, and 3 were established in pathotype I, while race 4 was represented in both pathotypes I and II. Pathotype III included the race 5 and 6, which were aggressive isolates.

In Pakistan Jamil et al. (2000), studied pathogenic and genetic diversity in *A. rabiei* population in Pakistan. Biological pathotyping of 130 isolates was conducted on three chickpea differentials (susceptible, tolerant, resistant), statistical analysis grouped the isolates into three pathotypes classes four isolates belong to pathotype I, 79 isolates to pathotype II, and 47 isolates to pathotype III. In Canada 14 pathotypes of *A.rabiei* was identified among isolates tested based on reaction of chickpea differentials (Chongo et al., 2004).

2.1.2 Diversity studies using molecular markers

In addition to the previous studies that depended on the reaction of chickpea genotypes against the different isolates of *A.rabiei* molecular markers have been widely deployed to detect and identify *A. rabiei* isolates and to examine genetic diversity, genetic structure, and virulence in populations of this fungus (Morjane et al., 1994; Peever et al., 2004; Phan et al., 2003; Santra et al., 2001; Udupa et al., 1998).

In Canada isolates of *A.rabiei* were collected from chickpea plants grown in southern Alberta, and RAPD analysis of genomic DNA extracted from these isolates was conducted using short sequence primers and analyzed to establish the genetic relationship and distance between isolates. A total of 75 bands were polymorphic the isolates were found to be belong to five genotypes , indicating that the *A. rabiei* population of southern Alberta is genetically diverse.(Chang et al., 2008).

Additionally in Canada Vail and Banniza (2009), compared 30 isolates from 1998 and 30 isolates from 2002 by random amplified polymorphic DNA fingerprint Cluster analysis and analysis of molecular variance suggested differentiation of the 1998 and 2002 populations, yet equal amplicon diversity between populations with the majority of the variation occurring within each population was observed.

Geistlinger et al. (1997), studied the allelic variation in *A. rabiei* by screening the genome of the fungi using microsatellite-Pried PCR. A high level of sequence variation was observed suggesting that multiple mutational mechanism have contributed to polymorphism.

In Syria Atik et al. (2013), used simple sequence repeat and mating type markers to estimate the genetic diversity of *A. rabiei* isolates collected from nine provinces of Syria The genetic diversity of *A. rabiei* population was high, inter-population variability accounted 83% of the total variation, whereas the genetic diversity among populations was very low.

In Iran Khoshnood et al. (2011), used Simple sequence repeat (SSR) and mating type (MAT) markers to determine the genetic structure, and estimate genetic diversity and the prevalence of mating types in *A. rabiei* isolates from seven counties in the Ilam and Kermanshah provinces of western Iran (Ilam, Aseman abad, Holaylan, Chardavol, Dareh shahr, Gilangharb, and Sarpul). A high level of genetic variability was observed among *A. rabiei* isolates in the region. Genetic diversity was high (He=0.788) within populations with corresponding high average gene flow and low genetic distances between populations.

In Turkey Bayraktar et al.(2007), studied the genetic diversity within *A. rabiei* in Turkey, genetic diversity among isolates of *A. rabiei* obtained from diseased chickpea plants in different provinces of Turkey was characterized by microsatellite-primer PCR using di-, tri-, and tetra-nucleotide repeats. UPGMA analysis performed with the resulting data of SSR finger print clustered Turkish isolates of *A. Rabiei* into seven groups.

Later in Turkey Ozer et al. (2012), used RAPD analysis on 81 isolates of *A. rabiei* representing different pathotypes and geographic origins with 54 primers. Results of RAPD analysis clustered all isolates into nine groups an arbitrary level of

73% similarity. Total gene diversity (HT=0.221) was mostly attributable to diversity within populations (HS=0.213), Only 4% of the total variability were attributable to variation among populations. The results revealed low genetic differentiation and high gene flow among populations of *A. rabiei* in Turkey.

In India Barve et al. (2004), studied 37 isolates of *A.rabiei* collected from different states and 38 isolates from fifteen other countries in the world, the isolates were examined for their diversity at *ARMS1* microsatellite locus. 26 alleles on the basis of size 228-451 base pair were detected in the world isolates examined, while 15 alleles of size 287-418 base pair were observed in the isolates from India.

In Pakistan Jamil et al. (2000), conduct genetic analysis using RAPD and oligonucleotide fingerprint, dendrograms produced by cluster analysis discriminated 46 genotypes in *A. rabiei* population, genetic distance and relatedness between isolates were calculated, at a genetic distance 0.3 , and genotypes were divided into six distinct genotype groups.

Later Ali et al. (2012), did a comparison between *A. rabiei* isolates collected from United states and Pakistan by studying the genetic variation at six microsatellite locations. Population structure of the isolates was inferred using Bayesian analyses implemented in the structure software which differentiated isolates into three distinct clusters, two clusters of the isolates from Pakistan and one of the US isolates. However, few isolates from the US shared the same genetic background with one cluster of the Pakistani isolates suggesting frequent gene flow of *Ascochyta rabiei* among different locations.

There have also been studies that attempted to correlate molecular data to aggressiveness or pathotypes in order to hasten pathogenicity studies. For example, isolates from Pathotype III (most aggressive) were found to have less genetic diversity than Pathotype I (least aggressive) isolates suggesting that because of adoption of resistant cultivars there was selection pressure on populations of *A. rabiei* (Udupa et al., 1998).

2.2. Mating system in *A. rabiei*

In ascomycetes sexual reproduction is controlled by single regulatory locus referred to as mating type locus, alternate sequences at mating type locus are completely dissimilar and code different regulatory genes (Metzenberg and Glass, 1990).

Fungi capable of sexual reproduction use heterothallic (self-sterile) or homothallic (self-fertile) mating strategies. In most Ascomycetes, a single mating type locus, MAT, with two alternative forms (MAT1-1 and MAT1-2) called idiomorphs, controls mating ability. In heterothallic ascomycetes, these alternative idiomorphs reside in different nuclei. In contrast, most homothallic ascomycetes carry both MAT1-1 and MAT1-2 in a single nucleus, usually closely linked (Lee et al., 2003).

Ascochyta rabiei is heterothallic with a bipolar biallilic mating system (Wilson and Kaiser, 1995), and DNA binding protein containing an ɒ domain is coded by mating type 1 gene or MAT1-1, and DNA binding protein that contains a high mobility group domain is coded by MAT1-2 (Coppin et al., 1997; Turgeon, 1998).

Studying the mating type of ascochyta and the other species can provide an idea and estimate concerning the fungi ability to form other generations with more aggressiveness, thus the presence of the two mating types in a specific area means the ability of sexual reproduction that leads to unexpected changes in the cultivars reaction against new generations of the fungi that might lead to disease outbreaks.

In addition to providing researches with ability to perform segregation analysis and to characterize genes suspected of playing a role in host range avirulence, pathogenesis, and mechanism of generating variation (Kronstad, 1995; Ahmed and Morall, 1996).

Finally teleomorph may play an important role in long-distance dissemination of the pathogen and in increasing genetic diversity in the pathogen population (Kaiser and Kusmenoglu, 1997), it develops only on chickpea debris that overwinters on the soil surface in the presence of both mating types (Antonio et al.,2012).

Studying mating type could be achieved using compatible MAT1-1 and MAT1-2 tester isolates by crossing the tested isolates with tester isolates (Kaiser and Kusmenoglu, 1997), but this method is tedious and time consuming. Thus a MAT-specific, multiplex PCR assay was developed by Barve et al. (2003), Primers Com1 (common flanking primer), SP21 and Tail 5 were designed to amplify different size of PCR fragments from *A. rabiei* MAT1-1 and MAT1-2 isolates. The combination of Com1/SP21 amplifies about 700 bp of the MAT1-1 idiomorph while the primers Com1/Tail 5 amplify about 470 bp of the MAT1-2 idiomorph.

Mating type distribution was reported and studied from different countries around the world. Kaiser (1997), reported world-wide distribution of the two MATs (MAT 1-1 and MAT 1-2) in nature. Navas-Cortes et al. (1998), studied mating type distribution on 48 isolates collected from India, Pakistan, Spain and the USA, of the tested isolates 58% was MAT1-1 , and 42% was MAT1-2. Many research have been done in Canada for mating type occurrence and distribution.

Ahmed et al. (1996), studied the frequency distribution and virulence of two mating types of *Ascochyta faba* f.sp. *lentis* under controlled conditions using crosses of 223 isolates from western Canada and 14 other lentil growing countries of the world were made with mating type 1, mating type 2 at 10° C. Both mating type were identified in samples from local and foreign sources with varying frequencies but MAT1-1 was the more frequent.

Another study in Canada Armstrong et al. (2001), detected the mating type of 42 isolates from 34 fields in Saskatchewan, using tester isolates, both mating types occurred at similar frequencies across the region, and suggested that the occurrence of teleomorph stage in the region may increase field to filed spread of ascochyta blight of chickpea and contribute to increase genetic variability of the pathogen in western Canada.

Later in Canada Vail and Banniza (2009), analyzed the mating type frequencies on 30 isolates each from 1998, 2001, and 2002 populations and the distribution of mating type within these isolates was not significantly depart from a 1:1 ratio suggesting random mating of each population. However, when 121 isolates from 2002 were analyzed for mating type, a significant departure from a 1:1 ratio

was found suggesting smaller sampling sizes for mating-type frequencies may not reveal true differences.

In Turkey isolates of *Ascochyta rabiei* were collected from chickpea in 23 provinces of Turkey, each isolate was tested for mating type with compatible MAT1-1 and MAT1-2 tester isolates and both mating types were found in 18 provinces (Kaiser and Kusmenoglu, 1997). From the isolates tested, 59% was MAT1-1 and 41% was MAT1-2 suggested the increasing of genetic diversity of *A.rabiei* in Turkey.

Additional study in Turkey, Bayraktar et al. (2007), studied mating type distribution on *A. rabiei* isolates collected from chickpea growing areas of Central Anatolia the mating type assessment of 45 isolates from six different provinces were conducted under laboratory conditions, both mating types were found in all the provinces, except for Kayseri and Sivas.

The majority of the isolates belonged to MAT1-1 (57.8%) and the others (42.2%) to MAT 1-2, at the same time they used MAT-specific primers to differentiate mating types from each other using multiplex PCR method with primers com1, tial5 and sp21.

Moreover in Turkey Ozer et al. (2012), studied mating type distribution within five populations of *A. rabiei* , of 122 isolates tested for mating type, 52.5% was identified as MAT 1-2 and 47.5% as MAT 1-1. Both mating types were observed in all populations, supporting the hypothesis of a randomly mating population.

In Iran, Khoshnood et al. (2011), used mating type markers to determine the genetic structure of *A. rabiei* isolates collected from seven provinces. Both mating types were present in all populations, with the majority of the isolates belonging to Mat1-1 (64%) but within populations the proportions of each mating type were not significantly different from 50%.

Ali et al. (2012), used 32 *A. rabiei* isolates representing six geographical regions of Pakistan to compare them with a United States *A. rabiei* population for mating type frequency and genetic variation. Mating type results showed that the Pakistani population had an apparent skewed (3 Mat1-2: 1 Mat1-1) distribution,

although Chi-square tests showed non-significant deviation from equal distribution due to small sample sizes. The US population showed a 1:1 distribution of the two mating types.

The uneven distribution of mating types indicates that sexual reproduction among the Pakistani is rare due to either unavailability of both mating types or lack of conducive environment.

In Syria, Atik et al. (2013), used multiplex MAT-specific PCR with three primers to study the existence and distribution of two mating types, both mating types were found in all provinces in different ratios which were not significantly different from 1:1 ratio, suggesting that there is random mating of pathogen population under natural conditions.

Likewise, Barve et al. (2003), found an equal distribution of mating types in two different fields from the Pacific Northwest of the United States, and Kaiser and Okhvat (1996), found a close to 1:1 ratio in mating type in 9 isolates from Iran**.**

2.3. Genetics of virulence in *Ascochyta* spp.

The genetics of interactions between plants and fungal pathogens have been studied for many plant diseases and important advances in plant pathology have come for genetic analysis of fungi (Ahmed and Morrall, 1996). Beata et al. (2003), studied the inheritance of virulence in *A. lentis* to the lentil cultivar Northfield by crossing Northfield attacking isolate (AL4) with an avirulent isolate (AL36) the F_1 progeny segregated on 3:1 ratio for high virulence/low virulence suggesting that the virulence of *A. lentis* may be controlled by 2 independently segregating genes.

In another study, Ahmed and Morrall (1998), studied the inheritance of virulence in *A. fabae* f.sp. *lentis* by crosses between isolates of heterothallic fungus *A. fabae* f.sp. *lentis* with different virulence and tow lentil cultivars the F_1 progeny of some crosses included intermediate and trans-aggressive segregants of both cultivars, suggesting that inheritance of virulence is non Mendlian.

Hernandes et al. (2006), made interspecific crosses *A. pisi* with *A. fabae, A. vicia-villosa* with *A. lentis* those crosses produced pseudothecia with visible

ascospores and the hybrid statues of ascospore progeny was verified by (AFLP) markers, interspecific progeny was morphologically normal, but exhibited more phenotypic variation compared with progeny from intraspecific crosses, mating type and the majority of AFLP markers segregated in Mendelian 1:1 ratios in both intraspecific and interspecific crosses.

Kaiser et al. (2007), did crosses between isolates of *A.fabae* and *A.lentis* which succeed to produce hybrid pseudothecia but the inoculation with single ascospore from this progeny did not induce disease on both hosts.

2.4. Molecular microsatellite markers (SSR markers)

Molecular genetic markers represent one of the most powerful tools for the analysis of plant genomes and the association of heritable traits with underlying genetic variation. One form of sequence based marker, Simple Sequence Repeats (SSRs), also known as microsatellites, now predominate applications in modern plant genetic analysis and are one of the most powerful genetic markers.

Microsatellites are tandemly reiterated short DNA sequence motifs (usually 2-5 bp long) that occur at multiple sites (up to 10^5) in eukaryotic genome. (Beckmann and Weber, 1992; Wang et al., 1994; Field and Wills,1996).

A key feature of this class of repetitive DNA is an extra ordinarily high level of variation among taxa , mainly expressed as variation in the copy number of tandem repeats at a particular locus. Several techniques have been developed to exploit this variability for DNA profiling and molecular marker generation. Firstly, synthetic oligonucleotides complementary to microsatellite motifs can be used as hybridization probes for DNA fingerprinting, revealing restriction fragment length polymorphisms (RFLPs) at multiple loci simultaneously (reviewed by Wising et al., 1995a). Secondly, length variation of individual microsatellites can be detected by PCR using at flanking pairs of primers, electrophoretic separation of the amplification products, and visualization by autoradiography or staining (reviewed by Powel et al., 1996).

Molecular markers of this type have been termed sequence-tagged microsatellite (STMS) markers by (Beckmann and Soller,1990). Thirdly, mini- or microsatellite-specific oligonucleotides can be used as single PCR primers to amplify inter repeats regions in genomic DNA (Heath et al., 1993; Meyer et al., 1993; Gupta et al., 1994; Zietkiewicz et al., 1994).

Electrophoretic separation of such microsatellite-primed PCR (MP-PCR) products commonly results in DNA profiles that resemble RAPD patterns (Williams et al., 1990; Weising et al., 1995b).

In fungi the ubiquitous presence of microsatellites was first demonstrated by DNA fingerprinting using microsatellite-specific oligonucleotide as hybridization probes (reviewed by Rosewhich and McDonald, 1994; Weising et al., 1995a). Both yeast like and filamentous fungi have also been investigated by microsatellite-primed PCR (Meyer et al., 1993; Mayer and Mitchell,1995; Buscot et al., 1996; Longato and Bonfante, 1997).

However only few recent studies have been devoted to the cloning of fungal mini and microsatellites and\or the generation of STMS markers (Groppe et al., 1995; Osciewacz et al., 1996; Andersen and Nilsson-Tillgren,1997).

Ascochyta rabiei is a suitable module system for the study of microsatellites in fungi since at least 25 microsatellite motifs have been detected in its genome by extensive oligonucleotide fingerprint analysis (Weising et al., 1991; Morjan et al., 1994; Geistlinger et al.,1997).

Target sequences recognized by the probes $(CA)_8$, $(CAA)_5$, $(GACA)_4$, $(GGAT)_4$, and $(GTTTGG)_3$, were found by particular abundant, and provided highly informative DNA fingerprints.

3. MATERIALS AND METHODS

3.1. Survey and plant sampling:

Survey was done in Uzbekistan, Turkey, Lebanon and Syria during 2011-2012 and 2012-2013 season, samples were collected from different chickpea planting areas, infected chickpea samples was kept in paper bags which labeled by the first letter of the country name and the area name. About 150 samples were collected from all countries and 96 single spore isolates were obtained from these samples. The isolates were 33 isolates from Uzbekistan, the four pathotypes and six races discovered in Syria, 25 isolates from Turkey, 15 isolates from Syria, and 13 isolates from Lebanon.

3.1.1. pathogen isolation from diseased plant samples

The plant samples with the lesions (Figure 3.1) were disinfected with 5% hypochlorite for five minutes and dried on paper towel, the dry samples were placed in 2% water agar and difco potato dextrose agar (PDA) in 9 cm diameter plastic petri dishes and incubated at 21-23° C under florescent light to induce the sporulation from these isolates, single spore isolates was taken and cultured on chickpea dextrose agar (CDA) the single spore isolate was cultured at 21-23°C under florescent light 16 h photoperiod.

3.1.2. Single spore isolation

Single spore was prepared by a series of dilutions from the mother colony a small amount of spores was taken by a sterilized needle and added to a 20 ml glass tube containing 10 ml of sterilized water the tube was shaked to get spore suspension by using sterilized pipet 0.5 ml was added to another tube containing 10 ml sterilized water. From each tube one drop was added to a petri dish containing water agar using sterilized elastic needle, the petri dishes were incubated under the culture room

conditions 21-23^0 C for 24 hours, after 24 h and under stereoscope one germinated separated spore was selected carefully picked up by a sterilized needle and transferred to a petri dish that contains (CDA) (Atik et al., 2011).

Figure 3.1. The shape of *A.rabiei* lesion on chickpea stem in field.

3.2. Fungal isolates

Four fungal pathotypes and six fungal races were obtained from a legume pathology lab at the international center for agricultural research in the dry areas (ICARDA) that were stored in Eppendorf tubes at -20° C, the pathotypes were grown in plastic petri dishes containing chickpea dextrose agar for one week inside culture room at 21-23°C and florescent lights 16 h photoperiod, these pathotypes and races were obtained from single spore colonies.

Moreover the progeny isolates that were used to inoculate chickpea genotypes were obtained from a single ascospore and multiplied in chickpea dextrose agar culture in plastic Petri dishes for one week in culture room at 21-23°C using

florescent lights (16 h photoperiod). additional 86 isolates were prepared from plant samples using the single spore isolation technique, and these isolates were used for mating type and diversity studies.

3.3. Media preparation

In the current study media were used of three media types, water agar, chickpea dextrose agar, and potato dextrose broth. Water agar was prepared by adding 18 g of agar agar (Merck) to 1 liter of distilled water inside glass Erlenmeyer flask and autoclaved at 121° C for 20 min, then the solution was poured in 9 cm diameter plastic Petri dishes.

Chickpea dextrose agar was prepared by adding 40 g chickpea seeds to a glass Erlenmeyer flask containing 1 liter distilled water that was autoclaved at 121°C for 30 min, the liquid was filtered and made to one liter with distilled water to the filtered solution, 18 g of agar agar and 20 of dextrose were added (Atik et al.,2011), autoclaved at 121°C for 20 min, then poured inside 9 cm diameter plastic Petri dishes.

For DNA extraction Potato dextrose broth was prepared by suspending 24 g in 1000 ml distilled water, the mixture was heated to 40° C till complete solubilization, then the one liter solution was divided to 50 ml and poured in 250ml glass Erlenmeyer flasks for each isolate, finally the solution (each flask) was autoclaved at 121°C for 20 min.

3.4. Dry chickpea stem pieces

Suitable amount of yellow dry chickpea stem pieces were obtained from ICARDA station in Terbol to be used in a crossing trial, these stem pieces were cut into 6-8 cm long, placed inside Aluminum foil and autoclaved for 20 min.

3.5. Plant materials

Two chickpea genotypes used to study inheritance (ILC-263 and ICC 12004) were obtained from ICARDA genetic resources unit, the ILC-263 was a kaboli chickpea that is susceptible to ascochyta, whereas ICC 12004 was desi type chickpea that is resistant to ascochyta.

Seeds were sterilized with 2% of NaOCl, were planted in a glass house at 18-20°C, 16h photoperiod inside 12 cm diameter plastic pots five seeds per pot containing sterilized soil mixture the soil sterilization conditions were 115°C for 45 min. To screen sub-set chickpea collection 200 chickpea accessions were obtained from the genetic resources unite of ICARDA to study their resistance against pathotype four which is the highly virulent pathotype. The genotypes were from different sources and countries Iraq, Portugal, Turkey, Syria, Greece, Cyprus, Italy and Azerbaijan (Table 3.1).

Table 3.1. Country of origin and number of FIGS subset screened for Pathotype-IV resistance. for their reactions to Pathotype-IV.

Country	Number of accessions
Azerbaijan	5
Bulgaria	1
Cyprus	4
Algeria	7
Spain	1
Greece	1
Iraq	1
Palestine	3
Italy	4
Lebanon	1
Portugal	26
Syria	66
Turkey	78
Ukraine	2

The seeds were sterilized using 2% NaOCl to be planted in a glass house under 18-20°C, 16h photoperiod in 12 cm diameter plastic pots five seeds per pot containing sterilized soil mixture the soil sterilization conditions were 115°C for 45 min.

3.6. Spore suspension (inoculum preparation) for inheritance and screening subset

A ten days-old culture of each isolate (each progeny and parental isolates also for pathotype IV to inoculate sub set) was divided and put inside a glass flask containing 50 ml distilled sterilized water, The flask was well shacked to get a spore suspension, The spore concentration was adjusted using haemocytometer to approximately $5x10^5$ conidiospores/ml throw the addition of sterilized distilled water, Tween-20 was added to the final concentration of 0.02% to help the spores to be well distributed inside the liquid which insure equal distribution for the spores in the liquid, the spore suspension was transferred to sterilized hand sprayer for each isolate from the progeny, and it was located at sterilized motorized sprayer to inoculate sup set by pathotype IV.

3.7. Plant inoculation

A four to six real leaves stage chickpea plants were inoculated with conidiospore suspension until runoff using 250 ml hand sprayers for progeny isolates to study the inheritance with one sprayer for each isolate, also one motorized sprayer was used to inoculate a sub set with pathotype IV. In the inheritance trial the plants inside pots then were covered by 60x40 cm polyethylene bags one bag to each pot for 72 h to provide humid conditions to help the conidiospores to initiate infection. The pots were covered with one sheet of polyethylene for all of pots together (the sub set trial), after 72h the plants were removed from the bags and kept under glass house conditions at 21-23°C under florescent lights16 h photoperiod and mist irrigation each 1 h 30 sec of mist to keep the humidity between 85-95%.

3.8. Disease rating

The incubation period and latent period at the leaves and stems was registered (Stem IP, LP) (Leaf IP, LP) after removing the bags. IP: means the beginning of infection at the plant organ. LP: means the developed stage of infection (pycnidia formation). A fifteen days from inoculation the disease score was registered using 1-9 rating scale described by (Chen *et al*., 2004) originally developed by Reddy and Singh, (1984).

3.9. Molecular analysis

3.9.1. DNA extraction

Four discs of 5 mm diameter of *A. rabiei* single-spore isolates were inoculated into 250 ml flasks containing 50 ml of potato dextrose broth (PDB) medium. After 4-6 days of incubation on a rotary shaker set at 60 rpm and 22°C, The mycelia were harvested from the flasks by vacuum filtration using two layers of sterilized cheese cloth, lyophilized for three days then stored at -30°C. Approximately 50 mg of the lyophilized mycelium was transferred to sterilized ceramics mortar containing enough amount of liquid nitrogen. Mycelium of each isolate was ground to a fine powder using the mortar in liquid nitrogen. DNA was extracted using a modified mini-preparation protocol of the cetyl trimethyl ammonium bromide (CTAB) method. (Chongo et al., 2004; Vail and Banniza,, 2009; Atik et al., 2011).

About 0.05 g of lyophilized mycelium was well grounded by liquid nitrogene in ceramic mortar to get very fine powder, the total amount of powder was transferred to 2 ml sterilized eppendorff tube and solved in 1.2 ml of CTAB solution. Eppendorf tubes were placed in 65° C water bath for 1 h, then 600 µl of chloroform isoamyl (4:1) was added to the liquid in the tube and the tubes were hand shacked for 15 min. The tubes were centrifuged at 13000 rpm for 20 min and the upper part of liquid was transferred to new eppendorf tube and 700 µl of very cool isopropanol

was added to each tube, the tubes were hand shacked gently for 3-4 min and placed inside freezer -20° C for 10 min. After placing the tubes in the freezer, the tubes were centrifuged again at 13000 rpm for 15 m and the upper part of liquid was discarded. Finally 1 ml of ethanol 70% was added to each tube to clean the obtained DNA pellet from all the sides, and again to centrifuge 13000 rpm for 15 min to get the pure pellet in the bottom of the tube, and again 1 ml of 70% Ethanol (this step was repeated twice to get very pure pellet). The tubes were allowed to by very well air dried and the pellet was dissolved using 60µl of 1_XTE buffer.

3.9.2. Mating type (MAT) and SSR primers

Three MAT primers (mating type primers) were used together in multiplex PCR method to study the mating type primers were: MAT1-1 specific primer Sp21 (ACAGTGAGCCTGCACAGTTC), MAT1-2 specific primer Tail 5 (CGCTATTTT ATCCAAGACACACC) and Flanking region-specific primer Com1 (GCATGCCAT ATCGCCAGT). In addition eight microsatellite (SSR) primers were used to study the diversity (genotyping) of *A. rabiei* isolates, the primers were shown in (Table 3.2).

Table 3.2. The eight microsatellite primers SSR that used to test the diversity of 96 *A.rabiei* isolates collected from different countries.

Primer SN	Primer	Sequence
1	ArA03T-F	TAGGTGGCTAAATCTGTAGG
	ArA03T-R	CAGCAATGGCAACGAGCACG
2	ArA06T-F	CTCGAAACACATTCCTGTGC
	ArA06T-R	GGTAGAAACGACGACGAATAGGG
3	ArR08T-F	GTGAGCTACTTAGCACCTCTGT
	ArR08T-R	GCTGTGTCGGGTTGAGTAAC
4	ArH02T-F	CTGTATAGCGTTACTGTGTG
	ArH02T-R	TCCATCCGTCTTGACATCCG
5	ArH05T-F	CATTGTGGCATCTGACATCAC
	ArH05T-R	TGGATGGGAGGTTTTTGGTA
6	ArH06T-F	CTGTCACAGTAACGACCAACG
	ArH06T-R	ATTCCAGAGAGCCTTGATTG
7	ArR04D-F	GCTTAGTTGGGCTTGTTACTT
	ArR04D-R	CACACCTCTCTACCAATGAGAC
8	ArH08D-F	ACTTTGACTTCGACTTCGACT
	ArH08D-R	GTGGAAGAGAAGTGGATTGAC

3.9.3. Agarose, polyacrylamide gel preparation

Agarose gel was prepared to determine the quality of obtained DNA and to separate PCR products for mating type after multiplex PCR. 1.5% agarose gel prepared by adding 1.5 g of pure agarose to 100 ml of 1x TBE buffer, and was well mixed by magnet for 5 min, then it was moved to microwave oven for 1 min to be completely dissolved, at 45°C temperature 8 µl of Ethidium bromide was added carefully to the liquid, mixed using magnet finally it was poured in suitable electrophorese chamber. Polyacrylamide gel was prepared by using 75 ml of gel mixture 8% with 75µl of TEMED (Tetramethylethylendiamine), and 360 µl of APS.

3.9.4. Detecting DNA quality

4 µl of obtained DNA was stained with 3 µl of blue agarose dye and was placed inside agarose gel each isolate in one well , agarose gel was staind with 8 µl of ethidium bromide and placed inside electrophorese device adjusted to run for 30 min at 80V. 100 bp ladder was used as indicator finally the DNA quality was observed by placing agarose gel at the surface of UV device.

3.10. Inheritance study experimental design

Three experiments were designed using GENESTAT statistical program each experiment was randomized completely design with three replications. Each experiment was to study the progeny obtained from one cross, were two treatment factors affecting the trial, the progeny that was obtained from the cross 20 progenies in with two parental pathotypes as control and chickpea genotype ILC-263 and ICC12004, the total number of pots was 132 for each trial with five seeds, Chi square test was performed using GENESTAT program.

3.11. Screening sub set chickpea collection experimental design

The experiment was designed using GENESTAT statistical program as randomized completely design with two replications were the treatment factor one was chickpea genotype (200 chickpea genotypes obtained from ICARDA in addition to four check lines), and the second treatment factor is Pathotype IV the highly virulent pathotype. The total number of plots (pots) was 412 plots, each pot contained four seeds.

3.12. Crosses trial

Cross was done between the pathotypes according to Kaiser *et al*., (1997). Ascochyta colonies grown at CDA for one week from each pathotype was used to prepare spore suspension, and the number of spores was adjusted using special haemocytometer. The crosses was done using weakly virulent Pathotype-I (MAT1-2) with three pathotypes (Pathotypes II- III and Pathotype IV) which are MAT1-1 with moderate to high virulence reactions. The crosses was done between Pathotype-I and Pathotype-II; Pathotype-I and Pathotype-III and Pathotype-I and Pathotype-IV. The procedure for crossing was similar to that used by Kaiser *et al*., (1997). Sterile chickpea stem pieces 6 to 8 cm long was placed in test tubes (2.2 by 17.5 cm) that contained 20 ml of a conidial suspension of a single isolate, or a mixture of one pathotype with MAT1-1 and other pathotype with MAT1-2. The spores concentration was $7x10^5$ spores / MI for both individual and mixed pathotypes.

After 1 h, the spore suspension was decanted and the stem pieces was allowed to drain for 10 to 15 min in the tubes. The inoculated stem pieces was then placed on 10 layers of sterile filter paper moistened with 15 ml of sterile deionized water in 10-cm-diameter Pyrex Petri plates. After 24 h at 21 to 23^{O}C, the dishes were incubated in the dark at 10°C for 6-9 weeks inside special incubator. Each Petri plate contained three pieces of sterilized chickpea stem, one inoculated with spores of both parents (the cross) and two control pieces, each inoculated with one parent alone to check for self-fertility (Ahmed and Morrall, 1998). The Petri plates were not sealed

with Parafilm. After 7-9 weeks, the stem pieces were air-dried at 21 to 23° C in a laminar flow hood for enough time to be very well dried.

Ascospores were discharged from inoculated tissues by placing pieces of stem (2.5 cm long) on a Water Agar (WA) block that was placed on the inner surface of a Petri plate which was inverted over a bottom dish that contained WA. Ascospores discharged downwards onto the surface of the plate and were incubated at 21 to 23°C for 24 h to observe viability and germination of the ascospores (Figure 3.2).

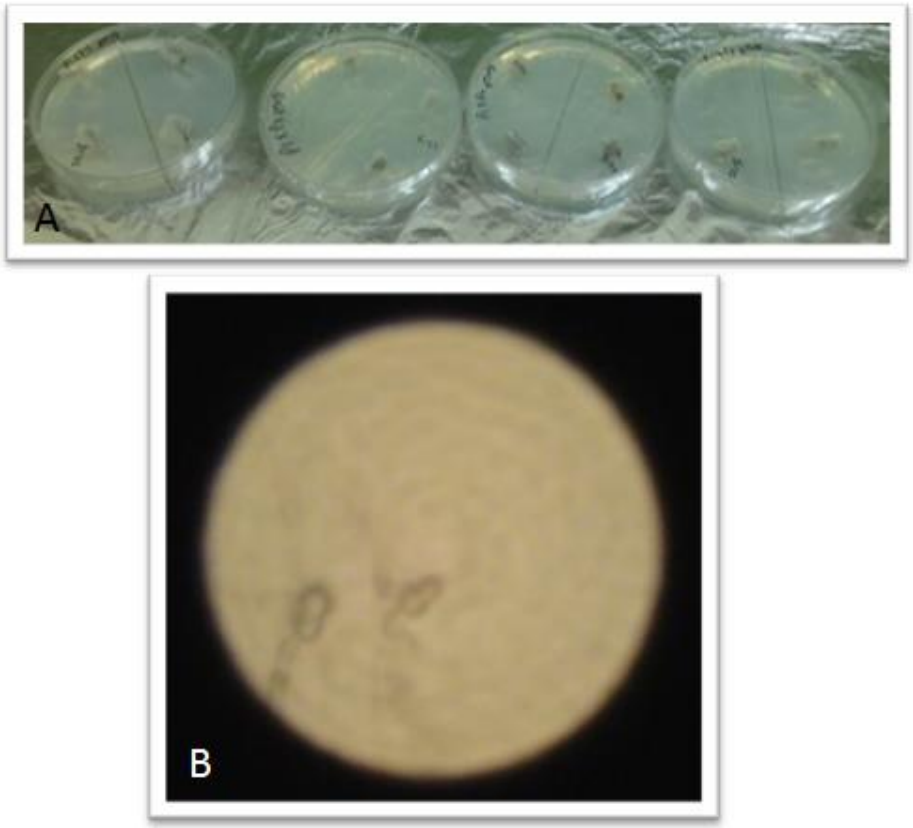

Figure 3.2. The small pieces of stem inside water agar blocks to enhance the discharging of ascospore. (A), Ascospores of *Didymella rabiei* after discharging from pseudothecia that placed in water agar block (B).

After discharging, 30 randomly single ascospores was selected and placed on CDA and after one week under 22-23°C each isolate was kept inside eppendorf tube under -20°C for further use to study the inheritance of virulence, these colonies

were considered to be the F1 progeny for each cross. The selected single ascospores were later referred as A,B,C letters, so A refers to the progeny obtained from cross between PIxPII, B from cross between PIxPIII, and C from cross between PIxPIV, 20 random single ascospores were obtained from each cross.

3.13. Inheritance study of virulence trial

20 randomly selected colonies from the new progeny from each cross was prepared using CDA to prepare spore suspension for each isolate from the progeny to inoculate two chickpea varieties ILC 263 and ICC-12004. ILC-263 is susceptible to Ascochyta, ICC-12004 is resistant. Three separated trials were done to study the inheritance of virulence in the Glass house. Sterile soil mixture was prepared inside sterilized plastic pots, the mixture was 75 % sterilized soil with 25% peat. The Mixture was filled inside the pots and three trials were designed using GENESTAT statistical program. Each trial was randomized completely design three replications as follow: 20 progenies in addition to the two parents PI and PII, PIII, PIV

Each trial was 132 pots and the trials as follow: The trial between pathotype one with Pathotype two and their progeny was as follow: Pathotypes and their progenies were incubated at chickpea dextrose agar for 15 days before inoculation.

Four seeds from each variety ILC 263 susceptible, ICC12004 resistant were planted inside the sterilized soil mixture in the plastic pots according to the experimental design when chickpea plants reached between four to six leaves stage, they have inoculated with spore suspension each pot according to its isolate, inoculation was done using small hand sprayer. Inoculum was as spore suspension from ten-days old for each isolates which was divided and put inside glass flask containing 50 ml of distilled sterilized water, the flask was well shaked to get the spore suspension, the spore concentration was adjusted using haemocytometer to approximately $5X10^5$ conidiospores/ml by adding sterilized distilled water, Tween 20 was added to the final concentration of 0.02 % to help the spores to be well distributed inside the liquid which insure equal distribution for the spores. Each pot was sprayed with spore suspension till runoff and was covered with 40x60 cm

polyethylene bag for 72 hours period. After 72 h the polyethylene bags were removed and the glass house conditions was adjusted to be 19-22°C temperature, mist irrigation to keep the moisture between 80-95% and 16 h artificial lightning to complete the sun period. After removing the bags incubation period and latent period at the leaves and stems was registered (Stem IP,LP) (Leaf IP,Lp)

IP: means the beginning of infection at the plant organ.

LP: means the developed stage of infection (picnidia formation).

After 15 days disease score was registered using 1-9 rating scale described previously (Chen et al., 2004) originally developed by Reddy and Singh, (1984).

3.14. Screening chickpea subset trial against pathotype IV the highly virulent pathotype

200 chickpea accessions were received from genetic resources unite from ICARDA in addition to six check genotypes to study their resistance against pathotype four which is the highly virulent pathotype. The trial was designed using GENESTAT program, completely randomized design with two replications. Soil mixture was prepared using 75% sterilized soil mixed with 25% sterilized peat and was filled inside sterile plastic pots. Each chickpea line (accession) was planted inside the pot according to experimental design four seeds per pot. Pathotype four was multiplied at CDA for 15 days before inoculation.

When chickpea plants reached to four leaves stage the plants were inoculated with spore suspension $5x10^5$ spores/ml. Plants was inoculated using motorized sprayer to cover all the plants till runoff , after inoculation the pots were covered with polyethylene roll to make moist chamber for 72 h, after 72 h the cover was removed and glass house condition was adjusted to: 19-22°C, artificial lightning for 16 h to complete the sun shine, and mist irrigation to keep the humidity between 75-95%. After 15 days from inoculation disease score was registered using 1-9 rating

scale described previously (Chen et al., 2004) originally developed by Reddy and Singh, (1984).

3.15 Mating type trial

MAT specific PCR was conducted using three primers mixed together at equal concentration Sp21 (ACAGTGAGCCTGCACAGTTC) MAT1-1 specific, Tail5 (CGCTATTTTATCCAAGACACACC) MAT1-2 specific, and flanking region specific Com1 (GCATGCCATATCGCCAGT), amplification was performed inside PCR machine, the total reaction volume was 25 µl contained 2 µl of DNA sample, 1µl of 10x PCR buffer, 1 µl of mixed dNTPs with concentration of 2mM, 0.1 µl of Taq polymerase, in addition to 2 µl of each primer in the same reaction the mixture was completed to 25 µl by adding ultra-pure distilled sterilized water. Cycling conditions consisted of primary incubation at 95°C for five minutes followed with 35 thermal cycles 30 sec 95°C, 30 sec 60° C, 5 min 72°C, and final extension 72°C for five minutes. PCR products were separated by electrophoresis on 1.5 % of agarose gel that stained with ethidium promide and photographed under UV illumination. And compared with 100 bp DNA Ladder.

3.16. Diversity trial

Eight microsatellite primer pairs, specific to *A. rabiei* were used to amplify genomic DNA (Table 3.2), these primers selected on the basis of their high PIC as described by different researchers (Geistlinger et al., 2000; Rhaiem et al., 2008; and Nourollahi et al., 2010). The PCR was essentially performed as described by Geistlinger et al. (2000),. PCR reactions were carried out in 25 µl volumes containing 2 µl of template DNA, 1µl 10x PCR buffer, 0.2 mM dNTP's, one units of Taq DNA polymerase, 2 µM of each primer. Cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of 94°C for 20 s, 57°C for 25 s, 67°C for 23 s, and a final extension at 72°C for 5 min. Amplified products were separated in 8% of polyacrylamide gels and compared with 100 bp DNA

ladder. After electrophoresis the DNA was stained with ethidium bromide and photographed under UV illumination.

4. RESULTS AND DISCUSSION

4.1. Inheritance of virulence results and discussion

Pseudothecia were observed 7-9 weeks after incubation from all the crosses. All parent pathotypes and their progenies were virulent on ILC-263 (Figure 4.1). The genotype ICC-12004 was only susceptible by Pathotype-IV but resistant to the other three parents and all progenies In all the three crosses there were progenies that cause less or more disease severity on the susceptible to ILC-263 than their respective parents (Figures 4.2, 4.3, 4.4).

Figure 4.1. Different reaction for two chickpea genotypes against progeny isolates obtained from the crosses between pathotypes in the glass house (LARI 2014).

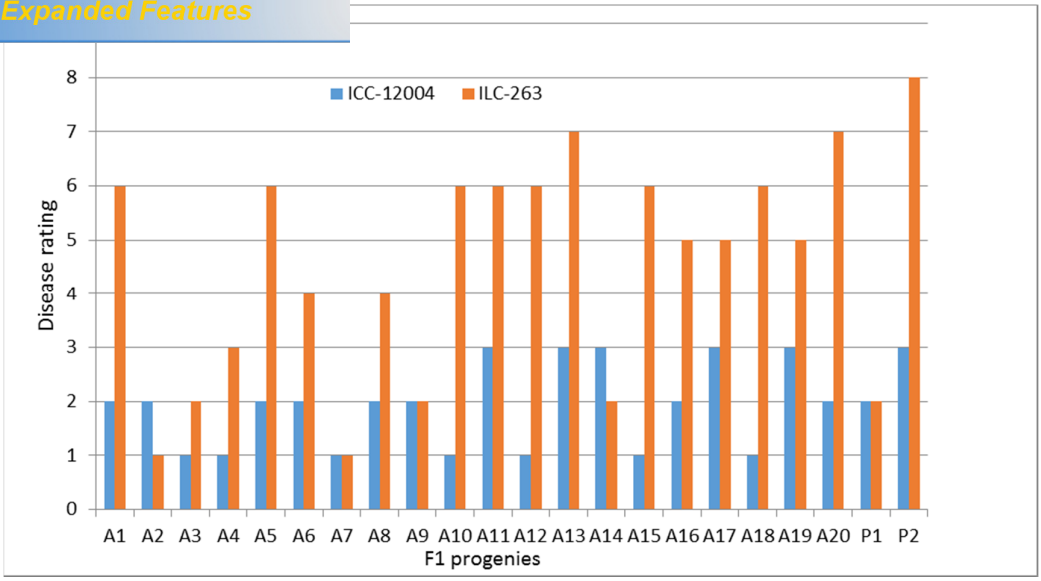

Figure 4.2. Frequency distribution of avirulence-virulence of F_1 progenies (A1-A20) of Pathotype-I X Pathotype-II cross and parents on two chickpea genotypes

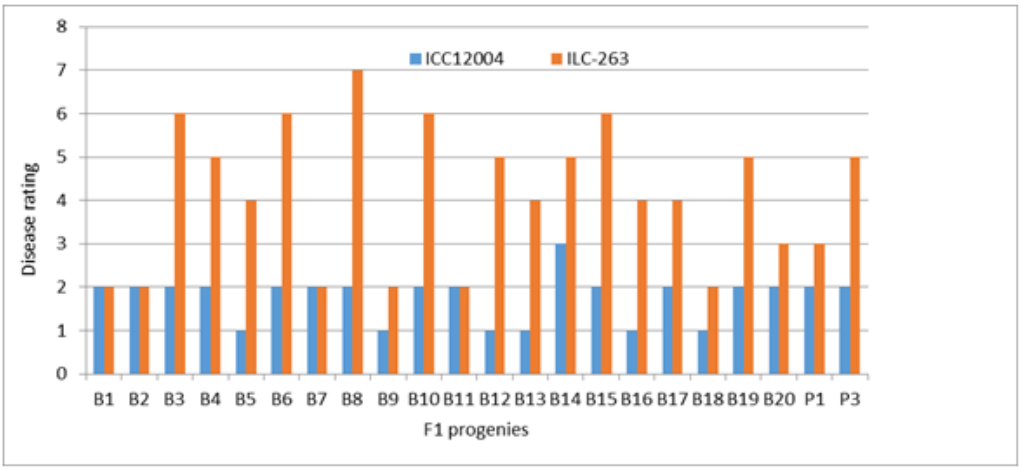

Figure 4.3. Frequency distribution of avirulence- virulence of F1 progenies (B1-B20) of Pathotype-1 X Pathotype-III cross and parents on two chickpea genotypes

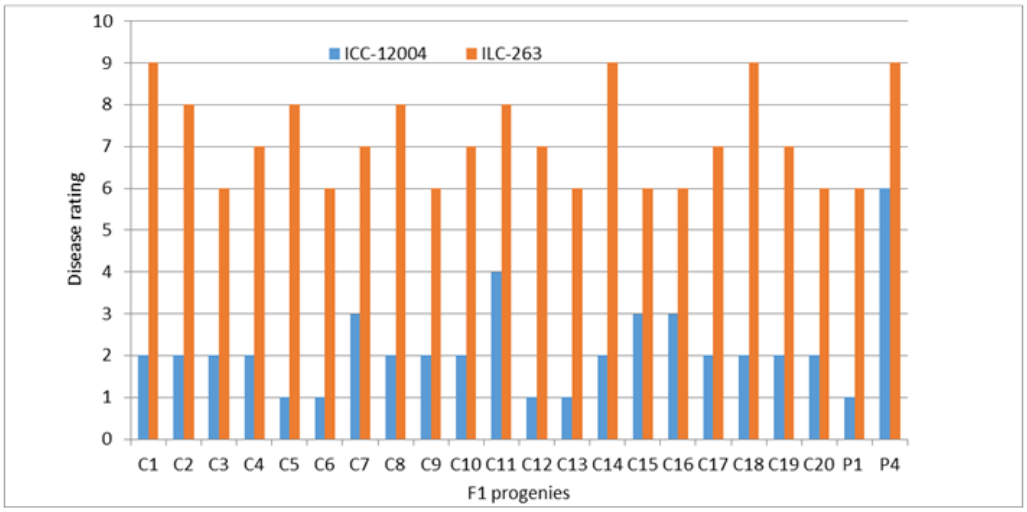

Figure 4.4. Frequency distribution of avirulence -virulence of F1 progenies (C1-C20) of Pathotype-I X Pathotype-IV cross and parents on two chickpea genotypes.

Moreover, some genotypes mainly from Pathotype I by Pathotype-IV showed more disease severity on the resistant ICC-12004. The inheritance results showed a 1:1 segregation in two crosses (Pathotype-I X Pathotype-II and Pathotype-I x Pathotype-III) tested on ILC-263 suggesting a single gene governing virulence in *D. rabiei* (Table 4.1).

Accordingly Ascochyta blight on chickpea will remain as a major biotic yield limiting factor on winter and spring planted chickpea in many countries. The major strategy to combat the disease is mainly through resistance breeding but integrated disease can be used when weather conditions are more favorable for disease development, using fungicides to support popular cultivars. The major threat of resistance breeding is the evolution of virulent *A. rabiei* population in many regions.

Table 4.1. Segregation of avirulence-virulence in *Ascochyta rabiei* using random ascospore progenies on two chickpea genotypes

Crosses	Chickpea genotypes	Avirulent	Virulent	Ratio	Expected ratio	X^2 calculated	X^2 probability	P
Pathotype-1 x Pathotype-2	ILC-263	6	14	6:14	1:1	3.2	0.1-0.05	.07
	ICC-12004	20	0	20: 0	1:0			
Pathotype-1 x Pathotype-3	ILC-263	7	13	7:13	1:1	1.8	0.5-0.1	.17
	ICC-12004	19	1	20:0	1:0	0:0		
Pathotype-1 x Pathotype-4	ILC-263	0	20	0:20	0:1	0:0		
	ICC-12004	20	0	19:1	1:0	0:0		

So far four Pathotypes are reported from Syria and ICARDA Kabuli chickpea program exposed all breeding lines to all pathotypes both under field and controlled conditions (except pathotype four just under controlled conditions) to share different elite lines to national programs.

This study attempt to shade light whether sexual reproduction played a role for the appearances of highly virulent pathotypes since the two mating types are reported in many countries. The result showed that many progenies were more virulent than their parents attacking ILC- 263 and a trend of high infection on ICC-12004. Moreover, highly virulent isolates can be introduce to new places through germplasm exchange through infected seeds and can be a concern for the chickpea industry (Kasier, 1997). The expansion of conservation agriculture where chickpea straw are left in the field will play a key role in generating new aggressive isolates that can attack nearby chickpea fields.

The existence of sexual reproduction can help the pathogen population to develop resistance to commonly applied fungicides to manage Ascochyta blight (Chang, *et al*., 2007; Wise *et al*., 2008). The inheritance of virulence showed in other *Ascochyta*-lentil pathosystem showed that virulence is controlled by single genes (Ahmed and Morrall, 1998) and by two independently segregating genes operating in

mutual epistasis (Beata and Pang, 2003). Unlike the finding of Peever *et al*., (2012), no significant isolate by genotype was found in all crosses in this study (*data not shown*). This study showed that there is high probability that sexual reproduction has placed a key role in rapid evaluation of virulent pathogen population that have affected many cultivars released for winter planting. It is worth to study how new virulent pathogen population respond to popular fungicides used in managing Ascochyta blight. Moreover, it worth to test popular cultivars and parent lines roundly used in crossing program for newly emerging aggressive isolates as part of anticipatory breeding.

4.2. Mating type distribution results and discussion

All isolates showed a single amplicon of the expected size of the two mating types (MAT1-1= 700 bp and MAT1-2 470 bp) (Figure 4.5).

In all farmer fields visited in the four countries, MAT1-1 was found except in Adiyaman, Turkey (Table 4.2). ICARDA Research Station at Tel Hadya, Syria, both Mating types were recorded but in the two research stations in Lebanon, all isolates were MAT1-2. The X^2 analyses showed no significant deviation from 1:1 ratio for Turkey and Uzbekistan populations showing that there could be random mating if weather conditions for sexual reproduction is favorable. Moreover, Syria, Turkey and Uzbekistan isolates were more MAT1-1 than MAT1-2 in contrast the isolates were more MAT1-2 than MAT1-1 in Lebanon.

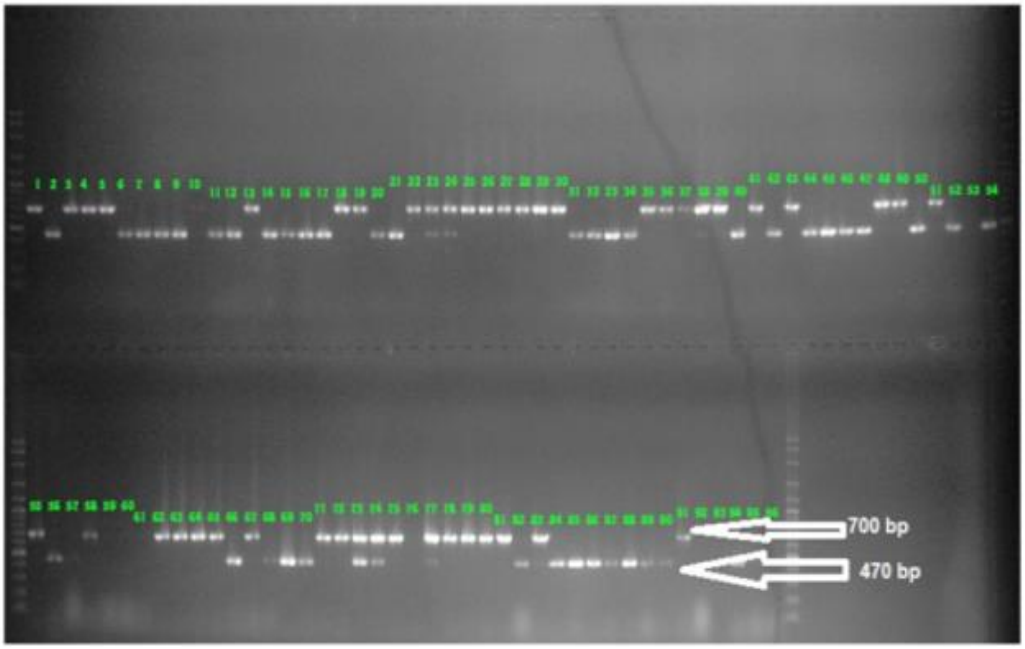

Figure 4.5. Multiplex PCR assay to determine the mating types of Turkish, Syrian, Lebanese and Uzbekistan *Ascochyta rabiei* isolates in chickpea using MAT specific primers.

On the other hand X^2 analyses showed significant deviation from 1:1 ratio for Syrian and Lebanese populations (X^2=4.56 , P= 0.03 and X^2=6.4, p=0.013) respectively. According to mating type distribution results we could expect that both winter and spring planted chickpea in the Mediterranean, West and Central Asia countries will suffer from the devastating effects of Ascochyta blight. The breeding program of ICARDA and other national breeding programs are working to develop high yielding and Ascochyta blight resistant chickpea varieties that can be widely grown by farmers.

Table 4.2. Mating type distribution for 96 isolates of *Ascochyta rabiei* in farmers and research fields in Lebanon, Syria, Turkey and Uzbekistan.

Country	**Collection sites**	**MAT1-1**	**MAT1-2**
Uzbekistan	Parkent	3	1
	Galla Orol	3	2
	Gizzakh Bahmal	2	10
	Samarkand Payarik	9	3
Turkey	Adana Balcalı	4	9
	Adana Sarıçam	6	0
	Adıyaman	0	2
Lebanon	Kfardan Research station	0	3
	Terbol Research station	0	6
	Tanayel	1	0
Syria	Tel Hadya Research Station	6	3
	Jableh	5	0

There are many reports of high genetic variability of *A. rabiei* in many countries that threaten the efforts of breeders to develop durable resistant varieties (Udupa *et al*., 1998; Pande *et al*.,2005; Atik *et al*., 2013). The major possible causes of genetic variability of *A. rabiei* could be sexual reproduction, selection pressure from growing resistant chickpea varieties and gene flow through germplasm exchange (Kaiser, 1997; Bayraktar *et al*., 2007b; Ali *et al*., 2012 Atik *et al*., 2013). There is a tendency of high MAT1-1 than MAT1-2 in the *A. rabiei* population in this study. Other researchers reported similar trend in Turkey, USA, Syria and Iran was observed in Syria, Turkey and Uzbekistan populations which is similar to most reports on mating types of the pathogen (Kaiser and Kusmenoglu, 1997; Nourollahi *et al.,* 2011; Atik *et al*., 2011; Ali *et al*., 2012). However, MAT1-2 was found dominant in Pakistan *A. rabiei* population (Ali *et al*., 2012). The presence of sexual reproduction can affect fungicide seed treatment; crop rotation since ascospores can travel long distance and initiate primary infections. Introduction of chickpea in cereal rotation under conservation agriculture will play a key role for sexual reproduction on left over chickpea straw and proper Ascochyta blight management is required to protect the crop from Ascochyta blight. Sexual reproduction can lead to the development isolates resistant to fungicides which are major complement of

integrated Ascochyta blight management. There are studies some commonly used fungicides of *A. rabiei* population developed isolates resistant to commonly used fungicide to manage the disease (Chang *et al.*, 2007; Wise *et al.*, 2008; Wise *et al.*, 2011; Delgado *et al.*, 2013).

Besides reducing quantity and quality of chickpea yield, the development of highly aggressive/virulent Ascochyta pathogen population in the West Asia can threaten the genetic diversity of chickpea including the wild relatives (Ozkilinc *et al.*, 2010; Atik *et al.*, 2013).

This study showed that both mating type exist in pathogen populations affecting both winter and spring planted chickpeas. Furthermore, the study contributed to knowledge on the mating type situation under farmers' fields in Uzbekistan which was not reported before. It is important to study why MAT1-1 is more dominant than MAT1-2 in many countries.

4.3. Diversity study

The aim of this study was to assess the genetic diversity among 96 *A.rabiei* isolates obtained from four different countries by using eight microsatellite primers (Table 3.2). A total of 29 bands were scorable out of 26 were polymorphic, the results showed that five SSR markers out of eight succeeded to give polymorphic bands (primers ArA03T, ArR08T, ArH06T, ArA06T, and ArR04D) whereas the other primers gave monomorphic bands (Figure not shown).

The eight primers that we used in our study are a part of 20 SSR *A.rabiei* specific primers developed by (Geistlinger *et al.*, 2000), these eight primers selected on the basis of their high PIC as described by different researchers (Geistlinger *et al.*, 2000; Rhaiem *et al.*, 2008; and Nourollahi *et al.*, 2011). Our using for SSR genetic markers showed highly genetic diversity between the isolates that collected from different countries, and this was similar to results for another studies conducted at the same fungi in Tunisia (Rhaiem *et al.*, 2008; Morgane *et al.*, 1994; Geislinger et al., 2000), Iran (Nourollahi *et al.*, 2010) , Syria (Atik *et al.*,2013), Italy (Fischer *et al.*, 1995) Lebanon and Syria (Udupa *et al.*, 1998), Spain (Navas-cortes *et al.*, 1998), Pakistan (Jamil *et al.*, 2000), and India (Santra *et al.*, 2001).

A total of 29 bands were scorable out of 26 were polymorphic, the results showed that five SSR markers out of eight succeeded to give polymorphic bands (primers ArA03T, ArR08T, ArH06T, ArA06T, and ArR04D) whereas the other primers gave monomorphic bands (Not shown).

The analyze of molecular variance (AMOVA) showed that 33 % of variation is because of the genetic difference among populations, and 67 % of variation is because of the genetic difference within populations (Table 4.3) and this result is probably due to the occurrence of sexual stage in these populations because of the presence of both mating types in the studied countries (Attar et al. unpublished data), the sexual stage will occur and more genetic variation will observe within these population. This is similar to what Atik et al. (2013), found where the variation within population was higher than variation between populations.

Table 4.3. Analysis of molecular variance AMOVA using Gene Alex software to detect the variation between and within populations of *A.rabiei*.

Summary AMOVA Table

Source	df	SS	MS	Est. Var.	%	Stat	Value
Among Pops	4	43.831	10.958	0.54	33%		
Within Pops	91	99.627	1.095	1.095	67%	PhiPT	0.33
Total	95	143.458	12.053	1.635			

This is similar to what Atik et al. (2013), found where the variation within population was higher than variation between populations.

Gene diversity index and polymorphism information content (PIC) were calculated and variation was observed between SSR markers regarding to number of alleles, genetic diversity and polymorphism information content (PIC) (Table 4.4).

Table 4.4. Genetic diversity, polymorphism information content (PIC), number of alleles and allele frequency in SSR primers.

Marker	**Major**	**Sample**	**No. of**	**Allele**	**Availability**	**Gene**	**PIC**

	Allele Frqquency	Size	obs.	No		Diversity	
ArA03T	0.3229	96.0000	96.0000	12.0000	1.0000	0.8331	0.8170
ArA06T	0.8125	96.0000	96.0000	2.0000	1.0000	0.3047	0.2583
ArR08T	0.5000	96.0000	96.0000	3.0000	1.0000	0.6215	0.5499
ArH06T	0.6563	96.0000	96.0000	4.0000	1.0000	0.5046	0.4479
ArR04D	0.3333	96.0000	96.0000	5.0000	1.0000	0.7248	0.6741
Mean	0.5250	96.0000	96.0000	5.2000	1.0000	0.5977	0.5494

The maximum number of alleles (12 alleles) was registered when using primer (Ar A03T) with minimum value of major allele frequency (0.32), maximum genetic diversity (83%), and maximum polymorphism information content (PIC=0.81) (Table 11), whereas minimum number of alleles (two alleles) was registered when using primer (Ar A06T) with maximum value of major allele frequency (0.81), minimum genetic diversity (30%), and minimum polymorphic information content (PIC=0.25) (Table 4.4).

The genetic distance was calculated between populations and maximum genetic distance registered between Turkish and Uzbekistani populations (0.64) whereas the minimum genetic distance registered between Turkish and Syrian populations (0.33) (Table 4.5).

Table 4.5. Genetic distance among *A.rabiei* populations using single sequence repeat Primers

Country	LB	SY	TK	Uzb
LB	0.0000	0.3631	0.3858	0.5594
SY	0.3631	0.0000	0.3360	0.4836
TK	0.3858	0.3360	0.0000	0.6451
Uzb	0.5594	0.4836	0.6451	0.0000

*LB : Lebanese population, SY: Syrian population, TK: Turkish population, Uzb: Uzbekistani population.

The unweight pair-group method arithmetic average (UPGMA) was used to cluster 96 isolates , the cluster analysis was performed using PAST software version 1.62 (Hammer *et al*., 2001), the dendrogram obtained from binary matrix (present1 or absent 0) clustered 96 isolates into ten groups at an arbitrary level of 38% similarity (Figure 4.6).

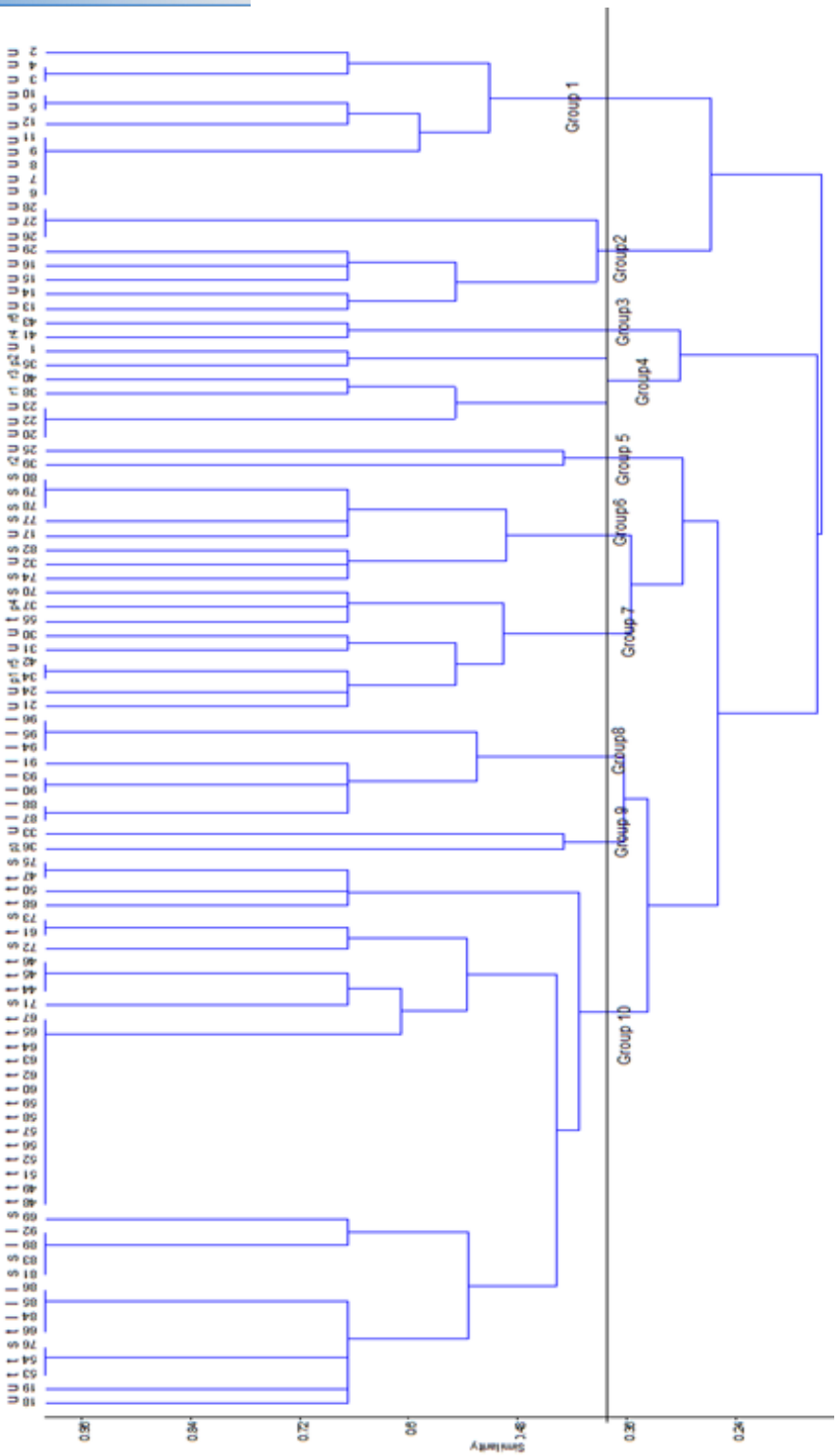

Figure 4.6. Dendrogram depicting similarities among 96 isolates of *A.rabiei* based on SSR markers. u=Uzbekistan, t=Turkey s=Syria, l= Lebanon, p= pathotype, r= Race

The similarity index value between 96 isolates ranged from 15-100%.

- Group 1: consisted of 11 Uzbekistani isolates (seven from Jizzah bahmal, one from Galla orol, and three from Parkent).
- Group 2: consisted of eight Uzbekistani isolates (four from Samarkent payarik, and four from Jizzah bahmal).
- Group 3: consisted of race 4 and race 6.
- Group 4: consisted of pathotype 2 , race 1, race 3 and four Uzbekistani isolates (one from Parkent, one from Galla oroll and two from Samarkent payarik).
- Group 5: consisted of race 2 and one Uzbekistani isolate from Samarkent payarik.
- Group 6: consisted of eight isolates where six Syrian isolates (three from ICARDA research station ,and three from Jableh coastal area), and two Uzbekistani isolates (one from Jizzah bahmal and one from Samarkent payarik).
- Group 7: consisted of pathotype 1, pathotype 4 , race 5 in addition to six isolates where one Syrian isolate from ICARDA station, one Turkish isolate from Adana Balcali, and four Uzbekistani isolates (three from Samarkent payarik , and one from Galla oroll).
- Group 8: consisted of eight Lebanese isolates (seven from ICARDA Terbol station and one from farmer,s field Tanaayel).
- Group 9: consisted of pathotype 3 and one Uzbekistani isolate from Samarkent payarik.
- Group 10: consisted of 39 isolates from all studied countries and could be divided into three sub groups:
- Sub group1: contains four isolates where three Turkish isolates (Adana - Balcali) and one Syrian isolate (ICARDA station).
- Sub group2: contains 21 isolates where three Syrian isolates (ICARDA station) and 18 Turkish isolates (seven from AdanaBalcali, two from Adiyaman Sanliurfa, and nine from Sofulu).

- Sub group 3: contains 14 isolates where four Syrian isolates (two from ICARDA station and two from Jableh) , five Lebanese isolates from (three from Kfardan station and two from Terbol), three Turkish isolates from Adana Balcali, and two Uzbekistani isolates from Galla oroll.

Our study showed genetic similarity between pathotype 1 and race 5 and this was also reported by Atik et al.(2013),

In addition to the presence of 16 cases of 100% similarity between isolates (out of 96 isolates) obtained from Same country (like similarity between 14 Turkish isolates) and similarity between isolates from different countries (like the similarity between some Syrian and Turkish isolates, between Lebanese and Turkish isolates, and between Lebanese and Syrian isolates).

The similar isolates were as follow:

- ✓ Turkish isolates Tk-10, Tk11 from Adana-Balcali were similar to Syrian isolate Sy-8 obtained from ICARDA research station in Aleppo.
- ✓ Lebanese isolates Lb-1, Lb-2, Lb-3 from ICARDA research station in Kfardan north Bekaa were similar to Turkish isolate Tk-23 obtained from Adana balcalI.
- ✓ Lebanese isolates Lb-6, Lb-9 obtained from Terbol station were similar to Syrian isolates Sy-13, Sy-15 obtained from Jableh coastal area.
- ✓ 14 Turkish isolates out of 25 isolates were completely similar and these isolates were (Tk-5, Tk-6, Tk-8, Tk-9) from Adana-Balcali from two farmers' fields, isolates (Tk-13, Tk-14) collected from Adiyaman Sanli Urfa, and isolates (Tk-15, Tk-16, Tk-17, Tk-19, Tk-20, Tk-21, Tk-22, Tk-24) originated from Sofulu.
- ✓ Turkish isolates (Tk-1, Tk-2, Tk-3) originated from Adana-Balcali from the same farmer's field.
- ✓ Syrian isolate Sy-7 obtained from ICARAD station was similar to Turkish isolate Sy-7 from Adana-Balcali.
- ✓ Lebanese isolates (Lb-4, Lb-5) obtained from ICARDA Terbol station.
- ✓ Lebanese isolates (Lb-7, Lb-10) obtained from ICARDA Terbol station.

- ✓ Lebanese isolates (Lb-11, Lb-12, Lb-13) obtained from ICARDA Terbol station.
- ✓ Syrian isolates (Sy-10, Sy-11, Sy-12) from Jableh coastal area.
- ✓ Uzbekistani isolate (Uz6-3) from Galla oroll, was similar to isolates (Uz7-1, Uz7-2) obtained from Samarkent.
- ✓ Uzbekistani isolates (Uz8-1, Uz8-2, Uz8-3) obtained from Samarkent Payarik.
- ✓ Uzbekistani isolates (Uz3-1, Uz3-2, Uz3-3, Uz3-4, Uz4-2) obtained from Jizzah Bahmal from two farmers' fields.
- ✓ Uzbekistani isolate (Uz2) obtained from Galla oroll was similar to (Uz4-1) from Jizzah Bahmal.
- ✓ Uzbekistani isolates (Uz1-3, Uz1-4)obtained from Parkent.

The genetic distance was calculated between populations and maximum genetic distance registered between Turkish and Uzbekistani populations (0.64), whereas the minimum genetic distance registered between Turkish and Syrian populations (0.33) (Table 4.5). In case of Uzbekistani isolates the similarity was just within the Uzbekistani population and no similarity was observed between Uzbekistani isolates and the remaining countries isolates (genetic distance was relatively higher between Uzbekistani population and the other populations) (Table 4.5).

But even in Uzbekistani population itself there was also diversity regarding to isolates collected from the same field in Jizzah Bahmall (Uz4-1, Uz4-2, Uz4-3, Uz4-4) these four isolates were completely different from each other , in addition the maximum genetic distance in this study was observed between two Uzbekistani isolates (Uz1-2 from Parkent and isolate Uz6-1 from Galla Oroll) about 250 Km distance between these two locations .

We could suggest that similarity between Syrian and Turkish populations because both of Syria and Turkey are neighbors and there is shared borders in addition to the import and export of chickpea seeds between these countries which may be contributed to the movement of isolates from one place to another, similarly between Lebanon and Syria there is wide borders area, but regarding to Uzbekistan it

is completely separated country from the remaining studied ones, similarly the range of genetic distances between these adjacent countries (Syria, Lebanon and Turkey) was concerned between (0.33-0.38) which is smaller than the range of genetic distances between Uzbekistani population and these countries (0.48-.064).

Gene diversity index and polymorphism information content (PIC) were calculated from the relation between number of alleles at each locus obtained in the studied isolates (Vuylsteke *et al*., 2000) using Power Marker software V3.25 (Liu and Muse, 2005).

4.4. Screening sub set chickpea results and discussion

None of the FIGS sub-set were resistant to Pathtoype-IV (rating 1-3) and only Genesis 090 showed moderately resistant reaction rating of 4 (Figure 4.7).

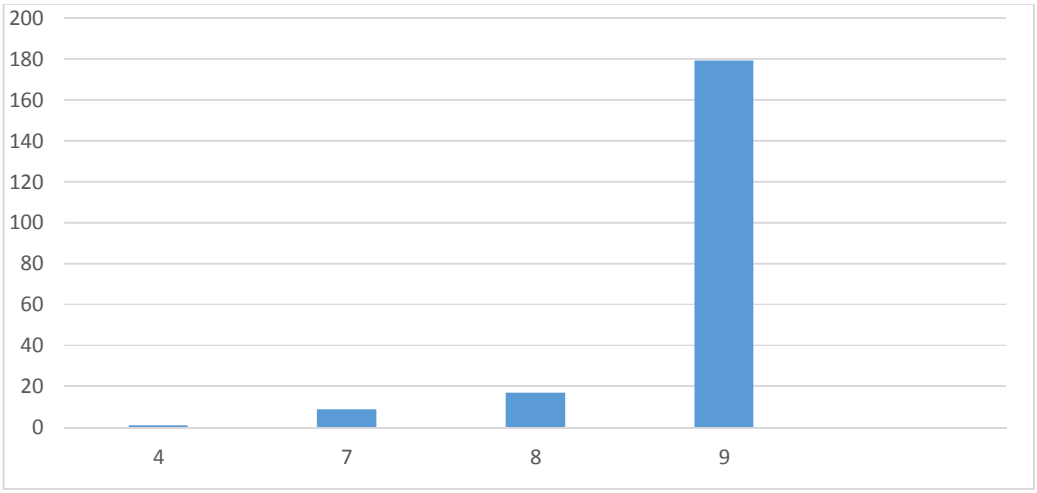

Figure 4.7. Reactions of FIGs set obtained from different countries against *A.rabiei* pathotype IV the highly virulent pathotype.

Seven subset accessions and two control genotypes showed susceptible reaction (rating of 7) and the they originated three from Turkey, two from Syria and two sub set from Italy. The remaining genotypes showed highly susceptible reaction (17 genotypes rating 8 and 179 genotypes rating 9) (Table 4.6).

Table.4.6. Reaction and rang of infection for FIGs sub set genotypes against pathotype IV (regardless to control genotypes reaction).

Number of Accessions	The Mode Disease Score	Range of Infection	Reaction
7	7	6-7	Susceptible
16	8	8-9	Highly susceptible
177	9	8-9	Highly susceptible

In this study the highly virulent pathotype was used to screen chickpea accessions and only one genotype showed moderately resistant reaction. Several sources of resistance to ascochyta blight have been identified in studies conducted in different chickpea growing areas of the world Some of the resistance sources were also released as cultivars (Reddy and Singh,1984; Singh *et al*., 1981; Guar and Singh, 1996).

This includes chickpea lines screened for ascochyta blight resistance at International Center for Agricultural research in the dry Areas (ICARDA) Syria, at ICARDA over 25000 chickpea lines have been screened for ascochyta blight resistance and 14 stable source of resistance have been identified ILC200, ICC 12004, ICC4475, ICC6328, and ILC 6482 were found to be resistant to six races of ascochyta blight in repeated fields and green house evaluation (Singh and Readdy, 1992). Regarding to ICC 12004 which reported as source of resistance it is resistant to the three pathotypes found in Syria but it showed susceptible reaction to pathotype IV (Imtiaz *et al*., 2011).

Thus we tried in this study to find source of resistance to pathotype IV which reported as new virulent pathotype, and this study belongs to host plant resistant strategy which is the essential part of integrated disease management for ascochyta blight around the world. Although FIGS sub sets were effective in capturing resistant genotypes in other diseases and insect pests, the chickpea Ascochyta blight Subsets were susceptible to pathogen population which developed virulence in the courses of selection by growing resistant chickpea genotypes. This preliminary finding showed that there is a need to refine the methodology of identifying subsets for highly virulent pathogen populations like *A. rabaiei* and also to conduct more targeted collections.

5. CONCLUSION

Chickpea (*Cicer arietinum* L.) is a major cool-season food legume in many parts of the world including Turkey and Syria. Chickpea productivity is low due to abiotic and biotic factors on both in spring and winter season production. The major biotic constraints of spring and winter planted chickpea is Ascochyta blight caused by *Ascochyta rabiei* (Teleomorph: *Didymella rabiei*). Some Ascochyta blight resistant chickpea cultivars were susceptible due to appearances of highly virulent/aggressive *A. rabiei* populations. Resistance breakdown of popular chickpea cultivars is the greatest challenge to chickpea breeding for Ascochyta blight resistance in many countries. Limited studies have been made in determining the driving forces that lead to the development of new virulent/or aggressive Ascochyta pathogen populations affecting cool-season food legumes. One of the evolutionary forces leading to new variants is the presence of sexual reproduction in the pathogen populations.

Our study was conducted to:

1- To determine the mating type distribution and genetics of virulence of *A. rabiei*.
2- To determine the pathogenic diversity of *A. rabiei* isolates from different countries.
3- And to screen mini-core chickpea accessions against the highly virulent pathotype 4.

In mating type distribution study: Mating type specific molecular markers were used to detect mating type distribution for *A.rabiei* isolates collected from Turkey, Syria, Uzbekistan and Lebanon and the study showed that:

1. No significant deviations from 1:1 ratio and most of the isolates from farmers' field and Tel Hadya Research Center were MAT1-1.

2. Both mating types exist in pathogen population affecting both winter and spring planted chickpea.
3. It is first report about the existence of both mating types in Uzbekistan.

In genetics of virulence study: Inheritance of virulence *A. rabiei* was studied using four known pathotypes (Pathotypes I-IV).

Three crosses were made and 20 progenies/cross with their respective parents were tested on susceptible (ILC-263) and resistant (ICC-12004) chickpea genotypes.

The results after crossing and testing showed that:

1. Pseudothecia were developed 7-9 weeks after incubation at 10^{O}C.
2. The Chi square test showed that inheritance of *A. rabiei* was controlled by single gene.
3. More virulent isolates were observed in all crosses.

   It is recommended to investigate the high Frequency of MAT1-1 comparing with MAT1-2.

Moreover it is worth to study how new virulent pathogen population respond to popular fungicides used in managing Ascochyta blight, and it worth to test popular cultivars and parent lines roundly used in crossing program for newly emerging aggressive isolates as part of anticipatory breeding.

In diversity study of *A.rabiei:* Eight microsatellite (SSR) specific primers were used to study the diversity of 96 *A.rabiei* isolates collected from Syria, Turkey, Uzbekistan, and Lebanon.

The study showed that:

1. Five SSR markers out of eight succeeded to give polymorphic bands.
2. Highly genetic diversity between the isolates that collected from different countries.

3. The analyze of molecular variance (AMOVA) showed that 33% of variation is due to genetic difference among populations, and 67 % of variation is due to genetic difference within populations.
4. Maximum genetic distance was registered between Turkish and Uzbekistani populations.
5. Minimum genetic distance was registered between Turkish and Syrian populations.
6. The dendrogram clustered 96 isolates into ten groups at an arbitrary level of 38% similarity.
7. Similarity index value between 96 isolates ranged from 15-100%.
8. Presence of 16 cases of 100% similarity between isolates collected from same country and different countries.

It is recommended to use more isolates from each country separately to cover more wide geographical area in each country, in addition the high range of similarity between Turkish isolates should be investigated.

Finally and concerning to screening minicore chickpea accessions against the highly virulent pathotype: 200 chickpea sub set accessions were received from ICARDA genetic resources section in addition to six check genotypes were evaluated for their reactions to *A.rabiei* Pathotype-IV in a glasshouse.
The results showed:

1. None of the (FIGS) genotypes were resistant to Pathotype-4.
2. Only one released cultivar (Genesis 90) in Australia showed moderately resistant reaction (rating of 4).

The focused identification of germplasm strategy (FIGS) is an important approach to in gene mining from large collections of plant genetic resources conserved in Gene banks. ICARDA Genetic Recourses Section has developed many chickpea FIGS sub sets for many important traits like drought, insect pests and disease resistances.

Although FIGS sub sets were effective in capturing resistant genotypes in other diseases and insect pests, the chickpea Ascochyta blight Subsets were susceptible to pathogen population which developed virulence in the courses of selection by growing resistant chickpea genotypes. This preliminary finding showed that there is a need to refine the methodology of identifying subsets for highly virulent pathogen populations like *A. rabaiei* and also to conduct more targeted collections.

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CURRICULUM VITAE

I was born on 10th of August 1981 in Aleppo, Syria. I attended university of Aleppo Faculty of Agriculture in 2000 and completed B.Sc. in Agricultural Engineering Department of plant protection in 2006. In 2010, I joined INTERNATIONAL CENTER FOR AGRICULTURAL RESEARCH IN THE DRY AREAS (ICARDA) as research assistant legume pathology. And according to pre agreement between ICARDA and CUKUROVA university I was accepted as master student in Çukurova University, Institute of Natural and Applied Sciences since February 2012. I moved from ICARDA Syria to ICARDA Lebanon due to the current situation in Syria and in Lebanon. I started establish my pathology lab inside LEBANESE AGRIGULTURAL REASEACRH INSTITUTE (LARI) since December 2012. I have published two papers belong to plant pathology during my work with ICARDA.

SUPPELMENTARY MATERIALS

Supplementary Table 1A. The country and origin of collecting chickpea FIGs sub set.

Origin	province	site	Taxonomic name
IRQ	Ninawa	Tall Kayf	Cicer arietinum
PRT	Evora		Cicer arietinum
TUR	Izmir		Cicer arietinum
TUR	Manisa		Cicer arietinum
TUR	Canakkale		Cicer arietinum
TUR	Bursa		Cicer arietinum
TUR	Sakarya	Adapazari	Cicer arietinum
TUR	Aydin		Cicer arietinum
TUR	Siirt		Cicer arietinum
LBN	Nabatiye	Nabatiyet et Tahta	Cicer arietinum
DZA	Constantine	From Coop. Agricole Generale, Chael-Rasses, Constantine	Cicer arietinum
SYR	Idlib	Jisir Al Shoughur market	Cicer arietinum
SYR	Tartous		Cicer arietinum
GRC	Crete	Monokhonon region	Cicer arietinum
TUR	Antakya	Hassa; shops	Cicer arietinum
TUR	Gaziantep	Hassa shops; 16 km from Fevzipasa on Maras road	Cicer arietinum
TUR	Aydin	Cine	Cicer arietinum
TUR	Gaziantep	Islahiye	Cicer arietinum
TUR	Sakarya	Geyve	Cicer arietinum
TUR	Mugla	Datca	Cicer arietinum
TUR	Mugla	Bodrum	Cicer arietinum
TUR	Edirne	Enez	Cicer arietinum
TUR	Edirne	Meric	Cicer arietinum
TUR	Bursa	Mudanya	Cicer arietinum
TUR	Balikesir	Bandirma	Cicer arietinum
Origin	province	site	Taxonomic name

TUR	Izmir	Menemen	Cicer arietinum
TUR	Kastamonu	Inebolu	Cicer arietinum
TUR	Izmir	Urla	Cicer arietinum
TUR	Izmir	Karaburun	Cicer arietinum
TUR	Antalya		Cicer arietinum
SYR	Aleppo	Kerzehel-Afrin	Cicer arietinum
SYR	Aleppo	Midanki-Afrin	Cicer arietinum
SYR	Aleppo	Mabatly-Afrin	Cicer arietinum
SYR	Aleppo	Aljeh-Azaz	Cicer arietinum
SYR	Aleppo	Boustans-Afrin	Cicer arietinum
SYR	Aleppo	Jenderis-Afrin	Cicer arietinum
SYR	Aleppo	Bablet-Afrin	Cicer arietinum
SYR	Aleppo	Tellef-Afrin	Cicer arietinum
SYR	Hama	Eyn Sulaymu, Jisr ash Shughur	Cicer arietinum
SYR	Idlib	Chaumick-Al Jisir	Cicer arietinum
SYR	Aleppo	Treandeh-Afrin	Cicer arietinum
SYR	Idlib	Frikeh-Ariha	Cicer arietinum
TUR	Icel	Mersin	Cicer arietinum
TUR	Bursa	Mustafa Kemalpasa	Cicer arietinum
TUR	Bursa	Karaagac	Cicer arietinum
TUR	Istanbul	Kandilli	Cicer arietinum
ESP	Andalucia	Carmona	Cicer arietinum
CYP	Paphos	2.5 km W Neokhorio	Cicer arietinum
CYP	Nicosia	0.5 km S Peristerona	Cicer arietinum
CYP	Paphos	Lysos	Cicer arietinum
CYP	Paphos	1.5 km NW Lysos	Cicer arietinum
PRT	Beja	Beja; Nucleo de Beja	Cicer arietinum
PRT	Beja	Salvada	Cicer arietinum
PRT	Beja	Salvada	Cicer arietinum
PRT	Santarem	Pacos das Oliveiras	Cicer arietinum

PRT	Beja	Beringel	Cicer arietinum
PRT	Beja	Monte Coural; 4 km de Beja	Cicer arietinum
Origin	province	site	Taxonomic name
PRT	Evora	Cooperativa de Sao Mancos	Cicer arietinum
PRT	Evora	Monte do Bussalfao	Cicer arietinum
PRT	Evora	Aldeias de Montoito	Cicer arietinum
PRT	Evora	Vendinha	Cicer arietinum
PRT	Evora	Santa Suzana	Cicer arietinum
PRT	Evora	Ald de Pias; Alandroal	Cicer arietinum
PRT	Evora	Monte dos Artistas; Sta Maria	Cicer arietinum
PRT	Portalegre	Sousel	Cicer arietinum
PRT	Portalegre		Cicer arietinum
PRT	Santarem	Cortico; 3 km de T. Novas	Cicer arietinum
PRT	Castelo Branco	Courela da Estacao; Freg; Sao Pedro	Cicer arietinum
PRT	Evora	Estremoz	Cicer arietinum
PRT	Aveiro	4 km after de Veiros	Cicer arietinum
PRT	Portalegre	Fronteira	Cicer arietinum
PRT	Beja	Castro Verde	Cicer arietinum
PRT	Portalegre	Vale des Telhas; region Alentejo-Estremoz-Ervedal	Cicer arietinum
PRT	Santarem	Cem Soldos	Cicer arietinum
PRT	Santarem	Olaia	Cicer arietinum
PRT	Santarem	Alvorao	Cicer arietinum
TUR	Denizli	10 km after road separation from Denizli to Usak	Cicer arietinum
TUR	Denizli	4 km after Sivasnak from Denizli to Usak	Cicer arietinum
TUR	Bursa	16 km after Karacabey to Bandirma	Cicer arietinum
TUR	Balikesir	Bizirci village	Cicer arietinum
TUR	Canakkale	5 km after Gonen to Biga	Cicer arietinum
TUR	Canakkale	Guyemalan village	Cicer arietinum

TUR	Canakkale	Goktepe village; 12 km after Biga to Canakkale	Cicer arietinum
TUR	Canakkale	25 km to Lapseki from Biga	Cicer arietinum
TUR	Canakkale	Adatepe village; 12 km to Lapseki from Biga	Cicer arietinum
TUR	Canakkale	Yapildak village; 21 km after Lapseki to Canakkale	Cicer arietinum
TUR	Canakkale	Edge of Canakkale coming from Lapseki	Cicer arietinum
UKR	Crimea	village Gaspra	Cicer arietinum
Origin	province	site	Taxonomic name
SYR	Hama	Sad El Asharneh; farm store	Cicer arietinum
SYR	Hama	Sekelbeiyeh; farm store	Cicer arietinum
SYR	Aleppo	Atareb; Kafar Nasej; farm store	Cicer arietinum
SYR	Idlib	Batabo; farm store	Cicer arietinum
SYR	Idlib	Kafr Yahmoul; farm store	Cicer arietinum
SYR	Idlib	Sahl Al Rouj; farm store	Cicer arietinum
SYR	Idlib	Mhambel; farm store	Cicer arietinum
SYR	Idlib	Sheikh Yousef; farm store	Cicer arietinum
SYR	Al Hasakah	Ayn Diwar; farm store	Cicer arietinum
SYR	Al Hasakah	Daireek; farm store	Cicer arietinum
SYR	Al Hasakah	Ayn al Khadra; farm store	Cicer arietinum
SYR	Al Hasakah	Ayn al Khadra; Ghamer; farm store	Cicer arietinum
SYR	Aleppo	Afrin; merchant store	Cicer arietinum
SYR	Aleppo	Afrin; Tall el Taweel; merchant store	Cicer arietinum
SYR	Aleppo	Afrin; Jendaris; merchant store	Cicer arietinum
SYR	Aleppo	Afrin; Bableet; farm store	Cicer arietinum
SYR	Aleppo	Afrin; Kafer Batra; farm store	Cicer arietinum
SYR	Aleppo	Afrin; Tellef; farm store	Cicer arietinum
SYR	Aleppo	Afrin; Tall Hamo; farm store	Cicer arietinum
SYR	Aleppo	Afrin; Frereyeh; farm store	Cicer arietinum
SYR	Aleppo	Afrin; Sheikh Abdul Rahman;	Cicer arietinum

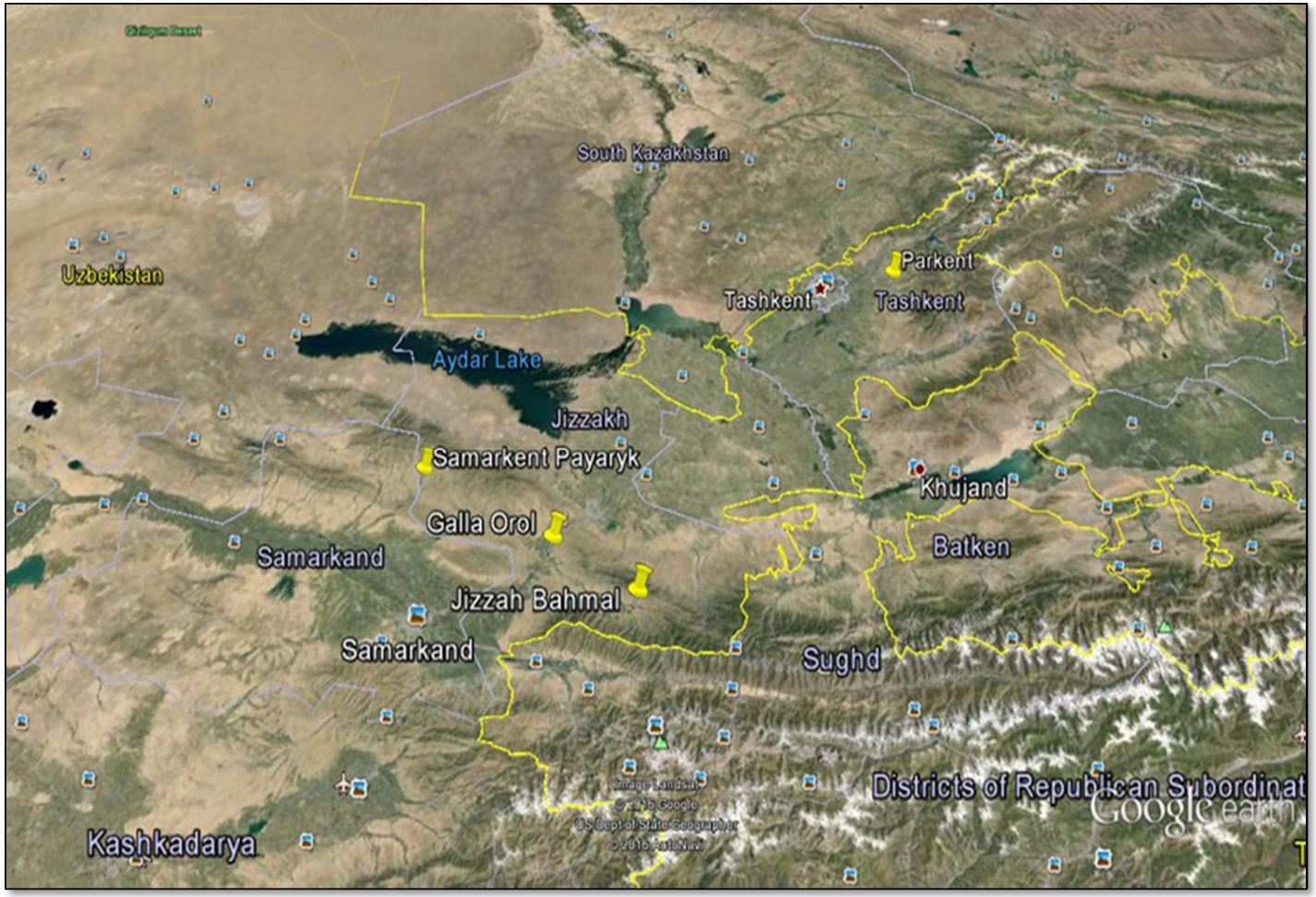
		farm store	
SYR	Aleppo	Jendaris; Ramadeiyeh; farm store	Cicer arietinum
SYR	Aleppo	Jendaris; merchant store	Cicer arietinum
SYR	Aleppo	Eskan; farm store	Cicer arietinum
SYR	Aleppo	Ghazzaweyeh; farm store	Cicer arietinum
SYR	Aleppo	Deir Samaan; farm store	Cicer arietinum
TUR	Icel	North, 19 km of Aydincik, Aydincik to Gulnar road	Cicer arietinum
TUR	Icel	Roadside field, rocky soil, 27 km SE of Gulnar	Cicer arietinum
TUR	Antakya	Roadside field, stony red soil, 2 km N of Senkoy turnoff on road to Antakya	Cicer arietinum
TUR	Gaziantep	Threshing area, Kazikle village, road to Gaziantep	Cicer arietinum
TUR	Gaziantep	Threshing area, 8 km N of Kazikle village, road to Gaziantep	Cicer arietinum
Origin	province	site	Taxonomic name
TUR	Bursa	16 km from Karacabey on way to Bandirma	Cicer arietinum
TUR	Balikesir	Threshing area, Bezirci village, 26 km to Gonen on way to Canakkale	Cicer arietinum
TUR	Balikesir	Harvested piles in field, 5 km after Gonen on road to Biga	Cicer arietinum
TUR	Balikesir	Threshing area for Vicia faba, 14 km after Gonen on road to Biga	Cicer arietinum
TUR	Canakkale	Storage, Guvemalan village, 34 km after Gonen on road to Biga	Cicer arietinum
TUR	Canakkale	Storage, 12 km after Biga on road to Canakkale	Cicer arietinum
TUR	Canakkale	Small garden, 25 km to Lapseki on road from Biga	Cicer arietinum
TUR	Canakkale	Thin stand, near Adatepe village, 10 km to Lapseki on road from Biga near Dardanelle S	Cicer arietinum
TUR	Canakkale	Field, Yapilak village, 21 km after Lapseki on road to Canakkale	Cicer arietinum

TUR	Canakkale	Plants drying in field, edge of Canakkale, on road to Lapseki	Cicer arietinum
SYR	Idlib	Beshmaroon village	Cicer arietinum
SYR	Idlib	Menlis village	Cicer arietinum
SYR	Idlib	Near Harem	Cicer arietinum
SYR	Idlib	Maaret Al Shalif	Cicer arietinum
SYR	Idlib	Hazzano	Cicer arietinum
SYR	Aleppo	Kazzaweia; 112 km	Cicer arietinum
SYR	Aleppo	before Rajo (170 km)	Cicer arietinum
SYR	Aleppo	Rajo	Cicer arietinum
SYR	Aleppo	Rajo; 191 km from Aleppo	Cicer arietinum
SYR	Aleppo	Afrin; 55 km from Aleppo	Cicer arietinum
SYR	Aleppo	13 km after Afrin	Cicer arietinum
SYR	Aleppo	Haj Iskandar; after Jendaris	Cicer arietinum
SYR	Aleppo	Marwa Tahtani; after Jendaris and before Sheikh El Hadid	Cicer arietinum
SYR	Aleppo	Sheikh Chackalli	Cicer arietinum
SYR	Aleppo	between Rajo and Midan Ikbis	Cicer arietinum
SYR	Aleppo	Adamanli; after Rajo and before Midan Ikbis	Cicer arietinum
SYR	Idlib	Salla	Cicer arietinum
SYR	Idlib	3 km from Salla to Aleppo	Cicer arietinum
SYR	Idlib	Kafer Meed	Cicer arietinum
SYR	Idlib	Kenisse	Cicer arietinum
Origin	province	site	Taxonomic name
SYR	Idlib	10 km after Kenisse	Cicer arietinum
SYR	Idlib	Sarari	Cicer arietinum
SYR	Tartous	Al Hanafiye	Cicer arietinum
SYR	Tartous	Raybi	Cicer arietinum
ITA	Basilicata	Potenza	Cicer arietinum
TUR	Antakya	Kuzuculu; 7 km from Dortyol to Yesilkent	Cicer arietinum
TUR	Antalya	1 km from Tasagil on road to	Cicer arietinum

		Beskonak	
TUR	Izmir	Menemen; 11 km from Menemen on road to Izmir	Cicer arietinum
TUR	Gaziantep	32 km W of Kilis to Islahiye road	Cicer arietinum
DZA	Bouira	19 km SW of Tikjda	Cicer arietinum
DZA	Tizi Ouzou	10 km N of Tizi Ouzou	Cicer arietinum
DZA	Boumerdes	34 km N of Tizi Ouzou	Cicer arietinum
DZA	Boumerdes	25 km S of Tigzirt; Tikobain	Cicer arietinum
DZA	Boumerdes	30 km E of Tizi Ouzou	Cicer arietinum
SYR	Al Hasakah	Ayn Diwar; on the border of Turkey-Iraq	Cicer arietinum
TUR	Canakkale	4.2 km after Biga towards Lapseki	Cicer arietinum
TUR	Canakkale	15.3 km after Biga towards Lapseki	Cicer arietinum
TUR	Canakkale	18.3 km after Alcitepe to Istanbul junction	Cicer arietinum
TUR	Canakkale	9.2 km to Gelibolu on Eceabat-Gelibolu road	Cicer arietinum
TUR	Tekirdag	14.3 km to Malkara	Cicer arietinum
TUR	Tekirdag	2.8 km after Kumbag towards Gazikoy	Cicer arietinum
TUR	Canakkale		Cicer arietinum
TUR	Balikesir		Cicer arietinum
TUR	Balikesir	Tepeoren	Cicer arietinum
TUR	Balikesir	Gundogdu	Cicer arietinum
TUR	Balikesir		Cicer arietinum
TUR	Balikesir	Susurluk	Cicer arietinum
TUR	Bursa	Gulluk	Cicer arietinum
TUR	Bursa		Cicer arietinum
AZE	Lankaran	town Lenkoran' (Lankaran)	Cicer arietinum
Pal		Bet Dagan	Cicer arietinum
TUR	Adana	village Dikilitas	Cicer arietinum
Origin	province	site	Taxonomic name
TUR	Adana	village Buruk	Cicer arietinum

TUR	Icel	town Tarsus	Cicer arietinum
TUR	Izmir	village Menemen	Cicer arietinum
Pal	Northern part	town Safad (Zefat)	Cicer arietinum
Pal	Northern part	town Nazareth, market	Cicer arietinum
TUR	Bursa	region town Bursa	Cicer arietinum
UKR	Respublika Krym	village Miskhor	Cicer arietinum
AZE	Lankaran	village Girdany	Cicer arietinum
AZE	Lenkoran'	region town Lenkoran	Cicer arietinum
AZE	Lankaran		Cicer arietinum
BGR	Burgas	Yambol	Cicer arietinum
TUR	Samsun	Samsun bazar	Cicer arietinum
SYR	Lattakia		Cicer arietinum
TUR	Izmir	Izmir	Cicer arietinum
DZA		region Kabylie	Cicer arietinum
AZE	Celibad	Lathyrus and Cicer crop in village of Aghchay	Cicer arietinum
ITA	Sardegna	village Sedini	Cicer arietinum
ITA	Sardegna	village Macomer	Cicer arietinum
ITA	Sardinia	village Sarule	Cicer arietinum

*IRQ: Iraq, PRT :Portugal , TUR: Turkey, LBN: Lebanon, DZA: Algeria, SYR: Syria, GRC : Greece, ESP: Spain, ITA: Italy, CYP: Cyprus, UKR: Ukraine, AZE: Azerbaijan , BGR: Bulgaria.



Supplementary Figure 1A. Map showing collection sites *of Ascochyta rabiei* isolates, Uzbekistan, 2012 cropping season depending on GPS data.