UNIVERSITY OF CÓRDOBA

DEPARTMENT OF GENETICS

PhD THESIS

Resistencia a *Fusarium oxysporum* f. sp. *lentis* en lenteja (*Lens culinaris*): mecanismos de resistencia y variabilidad patogénica
(Resistance to *Fusarium oxysporum* f.sp. *lentis* in lentil **(***Lens culinaris):* mechanisms of resistance and pathogenic variability)

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TITULO: *Resistencia a Fusarium oxysporum f. sp. lentis en lenteja (Lens culinaris): mecanismos de resistencia y variabilidad patogénica*

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TÍTULO DE LA TESIS: Resistencia a *Fusarium oxysporum* f.sp. *lentis* en lenteja (*Lens culinaris*): mecanismos de resistencia y variabilidad patogénica

DOCTORANDO: Hamid Reza Pouralibaba

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma)

El trabajo titulado "Resistencia a *Fusarium oxysporum* f.sp. *lentis* en lenteja (*Lens culinaris*): mecanismos de resistencia y variabilidad patogénica" se considera finalizado y reúne los requisitos para su exposición y defensa como tesis Doctoral.

Esta tesis ha obtenido resultados relevantes para la mejora de las lentejas para resistencia a la fusariosis vascular, realizando estudios tanto en el patógeno como en el huésped. Así, se han identificando nuevas fuentes de resistencia a esta enfermedad y se han caracterizado los mecanismos de resistencia implicados en dicha resistencia; se han identificado por primera vez patotipos en *Fusarium oxysporum* f.sp. *lentis* y desarrollado un set de líneas diferenciales capaz de distinguirlos; se han desarrollados nuevos marcadores SSR para este patógeno y se han utilizado para caracterizar su variabilidad genética. Estos trabajos han requerido el aprendizaje de distintas técnicas tales como la evaluación de los síntomas de la fusariosis vascular en condiciones de cámara y campo, el desarrollo y análisis de marcadores moleculares y el manejo de distintos programas y análisis estadísticos. Los resultados de la tesis han dado lugar a tres artículos publicados en la revista SCI European Journal of Plant Pathology y a otro que está en proceso de revisión en esta misma revista. Además, los resultados se han presentado en dos congresos internacionales. Dichas contribuciones se reseñan a continuación:

Artículos:

Pouralibaba HR, D Rubiales y S Fondevilla (2015) Identification of resistance to *Fusarium oxysporum* f.sp. *lentis* in Spanish lentil germplasm. European Journal of Plant Pathology, 143:399-405.

Pouralibaba HR, D Rubiales y S Fondevilla (2016) Identification of pathotypes in *Fusarium oxysporum* f.sp. *lentis*. European Journal of Plant Pathology,144: 539-549.

Pouralibaba HR, A Pérez-de-Luque y D Rubiales (2017) Histopathology of the infection on resistant and susceptible lentil accessions by two contrasting pathotypes of *Fusarium oxysporum* f.sp. l*entis*. European Journal of Plant Pathology, 148:53–63.

Pouralibaba HR, Z Satovic, MJ Cobos, D Rubiales y S Fondevilla. Genetic diversity and structure of *Fusarium oxysporum* f.sp. *lentis* isolates from Iran, Syria and Algeria. European Journal of Plant Pathology, enviado

Contribuciones a congresos:

Pouralibaba HR, D Rubiales y S Fondevilla (2013) New sources of resistance to *Fusarium oxysporum* f. sp *lentis* in lentil. First Legume Society Conference. Novi Sad, Serbia

Pouralibaba HR, Z Satovic, MJ Cobos, D Rubiales y S Fondevilla (2017) Identification of pathotypes and analysis of the genetic structure of *Fusarium oxysporum* f.sp.*lentis* populations. "15th Congress of the Mediterranean Phytopathological Union". Córdoba, España, p. 61. Presentación oral.

Pouralibaba HR, D Rubiales, A Perez-de-Luque y S Fondevilla (2017) Sources of resistance to *Fusarium oxysporum* f.sp. *lentis* in Spanish lentil germplasm. "15th Congress of the Mediterranean Phytopathological Union". Córdoba, España, p. 154.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 09 de Octubre de 2017

Firma del/de los director/es

Fdo.: Diego Rubiales Fdo.: Sara Fondevilla Aparicio

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I would like to give my special thanks to Professor Antonio Trapero-Casas (UCO, Spain), Dr. Asghar Mehrban (AREEO-DARI, Iran), for their motivation, helps and technical supports; and Dr. Shiv Kumar, Dr. Seid Kemal (ICARDA, Syria), Professor Bassam Bayaa (University of Aleppo, Syria), Dr. Naser Mohammadi (Tarbyat Modarres University, Iran) and Dr. Lakhdar Belabid (Mascara University, Algeria) for their efforts in supplying fungal materials and lentil germplasm to be used in this thesis.

To my wife who supported me emotionally and truthfully, in all stages of doing the thesis

The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.

AVICENNA (PERSIAN: ابن سینا), 980 IN BALKH – 1037 IN HAMEDAN

Abstract

Lentil (*Lens culinaris* Medikus *subsp. culinaris*) is one of the most important cool season food legumes. World production of lentil is estimated at 4.95 million tons from an estimated 4.34 million ha with an average yield of 1140 Kg/ha. Lentil production is threatened by biotic and abiotic stresses. One of the most destructive diseases, especially in the warm and drought conditions, is wilt disease, caused *Fusarium oxysporum* f.sp. *lentis* (*Fol*). Under favorable conditions this disease can cause the complete loss of the crop. The most effective, economical and environmentally friendly method to control the disease is the use of resistant cultivars. In this thesis we studied different aspects of this disease.

In chapter one, 196 Spanish lentil landrace accessions were evaluated for resistance to this disease under controlled conditions using a detailed disease scoring system. Resistant accessions were further screened in the adult plant stage under naturally infested field conditions in Bileh-Savar, Iran. This study identified twelve accessions showing good levels of resistance to the disease under controlled and field conditions which could be exploited in breeding programs.

In chapter two the mechanisms of resistance acting against two different pathotypes of FOL were studied in six lentil accessions showing different levels of resistance. After inoculation with patothype 1, a lower number of colonies were recovered from roots of the resistant accessions compared to the susceptible check. In the inoculation with patothype two no differences between accessions were observed for this trait. At the hystological level, qualitative resistance was detected in accession BGE019696 inoculated with pathotype 1. Thus, cells with condensed areas in the cytoplasm were observed. Furthermore, the presence of phenolic compounds was observed for this accession × pathotype interaction. This phenomenon was not observed in any other interaction.

In chapter 3 the pathogenic variability of FOL was studied. Although differences in aggressiveness have been observed between FOL isolates, the presence of pathotypes has not been reported in this pathogen. The objective of chapter 3 was to check for the presence of pathotypes in FOL. As a first step to check for the presence of isolate × genotype interaction in *Fol*-lentil pathosysthem, 28 resistant lentil accessions were inoculated by six *Fol* isolates with different geographic origins. A significant isolate×genotype interaction was detected. According to this differential reaction, four accessions were selected to analyse the virulence patterns in a

collection of 52 *Fol* isolates from Iran, Syria and Algeria. Seven different patterns of virulence were identified within this population.

In chapter four the genetic structure of the *Fol* collection described above was analyzed using twelve SSR markers. Eight of these SSRs were developed in this thesis. AMOVA showed that there is a high molecular variation both within regions and among regions, differing Iranian populations from non-Iranian populations. Additionally, STRUCTURE and Fitch-Margoliash analysis identified two ancestral lineages, one present in all regions and the other present only in Iran. No significant relationship was found between phylogenic groups and virulence patterns.

Resumen

Las lentejas (*Lens culinaris*) son una de las leguminosas más importantes a nivel mundial. La producción mundial de lentejas se estima en 4,5 millones de toneladas, con una superficie cultivada 4,25 ha. El rendimiento de este cultivo se ve severamente afectado por diversas enfermedades, entre las que destaca la fusariosis vascular, causada por *Fusarium oxysporum* f. sp. *lentis* (FOL). Este patógeno puede causar la pérdida total de la cosecha en condiciones favorables para el desarrollo de la enfermedad. La resistencia genética se muestra como el método más efectivo, económico y respetuoso con el medio ambiente para controlar la enfermedad.

En el primer capítulo de esta tesis se evaluó, en condiciones controladas, la respuesta a FOL de 196 variedades locales españolas utilizando un método detallado de evaluación desarrollado en esta tesis. Aquellas entradas que resultaron resistentes fueron también evaluadas en condiciones de campo en Bileh-Savar, Iran. Como resultado se han identificado 12 entradas de lenteja que muestran buenos niveles de resistencia tanto en condiciones controladas como en campo y que pueden ser de gran utilidad como fuentes de resistencia en programas de mejora.

En el capítulo 2 se estudiaron los mecanismos de resistencia que actúan frente a dos patotipos de FOL en seis entradas de lenteja con distintos niveles de resistencia. Tras inocular con el patotipo 1, de las raíces de líneas resistentes se rescataron un menor número de colonias que en el testigo susceptible. En la inoculación con el patotipo 2 no se observaron diferencias entre líneas para este parámetro. A nivel celular se detectaron mecanismos cualitativos de resistencia en la entrada BGE019696 inoculada con el patotipo 1. Así, se observaron en esta línea células que mostraron áreas condensadas en su citoplasma. Además, en esta combinación entrada-patotipo se observó la presencia de compuestos fenólicos. Este fenómeno no se detectó en el resto de las interacciones.

En el capítulo 3 se analizó la variabilidad patogénica FOL. Aunque se han descrito diferencias en agresividad entre aislados de FOL (Belabid y Fortas, 2002), hasta ahora no se habían descrito patotipos. El objetivo de este capítulo fue el estudiar si existen patotipos en FOL. Como un primer paso, para testar la existencia de interacciónes aislado x genotipo en el patosistema lenteja-FOL, se inocularon 28 genotipos de lenteja descritos como resistentes con 6 aislados del FOL de distintos orígenes geográficos. Los resultados mostraron una significativa interacción aislado x genotipo. En función de los resultados obtenidos se seleccionaron cuatro entradas de lenteja que mostraron una reacción diferencial frente a los distintos aislados para caracterizar el patrón de virulencia de una colección de 52 aislados de FOL provenientes de Iran, Siria y Argelia. Como resultado se identificaron 7 patotipos con distinto patrón de virulencia.

En el capítulo 4 se estudio, utilizando doce marcadores SSR, la estructura y variabilidad genética de la colección de aislados de FOL descrita en el capítulo 3. Los análisis AMOVA mostraron que había una gran variación genética tanto dentro de regiones como entre regiones, difiriendo las poblaciones de Irán del resto de poblaciones. Por otro lado los análisis realizados con el programa STRUCTURE y el árbol de Fitch-Margoliash mostraron la existencia de dos líneas ancestrales en FOL, una que sólo estaba presente en Irán y otra que estaba distribuida por todas las regiones estudiadas. No se encontró relación entre los grupos filogenéticos y los patotipos.

Objectives

The objectives of this thesis are:

1) To identify new sources of resistance to *Fusarium oxysporum* f.sp. *lentis* in a collection of Spanish lentil landrace

2) To characterize the mechanism of resistance acting against *Fusarium oxysporum* f.sp. *lentis* at the cellular level

3) To study *Fusarium oxysporum* f.sp. *lentis* pathogenic variability

4) To study the genetic diversity in *Fusarium oxysporum* f.sp. *lentis*

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General Introduction

1. The Host

Legumes: botany and importance

Legumes (= Leguminosae or *Fabaceae*) is a group of plans containing 727 genera and 19,327 species, being the third largest group of flowering plants. Legumes have been divided into three subfamilies including Caesalpinioideae, Mimosideae and Papilionoideae which are subdivided into groups of genera called tribes. Ecologically they coupe different ranges of habitats, from rain forests to deserts and from lowland to alpine habitats; including giant forest trees, shrubs, lianas, tiny annual herbs and even aquatic species (Doyle and Lukow 2003, Lewis et al. 2005).

Legumes in combination with other products (mainly cereals) are the staple food for a large part of the world population, especially for the low income fragments of the societies. The seeds of legumes are sources of protein, complex carbohydrates (dietary fiber), lipids, minerals, various active phytochemicals and vitamins, as well as several anti-nutritional compounds (Grusak 2002, Wang et al. 2003, Prakash et al. 2007, McPhee and Muehlbauer 2014, Sánchez-Chino et al. 2015). Furthermore grain legumes are good sources of protein for animals feeding (Muñoz et al. 2017).

Legumes are also able to fix atmospheric nitrogen through symbiosis with bacteria. Therefore, they reduce inputs of industrial N fertilizers, improve the sustainability of agricultural ecosystems and increase the biomass yield of the next crop (Vance 2001, Courty et al. 2015). The global amount of nitrogen fixed by legumes and other symbiotic plants was estimated to 40 Tons yr^{-1} (Galloway et al. 1995).

The legumes are the second most important crop after cereals based on area harvested and total production. Grain and forage legumes are grown on 180 million ha, corresponding to 12 – 15% of the earth's arable surface and provide about one-third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance 2003). Despite these advantages, the global trend of legume cultivation has not met the expectations and remains below that of other crops such as cereals, mainly due to low and unstable yields, and susceptibility to biotic and abiotic stresses (Rubiales and Mikic 2015).

Lentil Crop

History

Recent archaeological evidences suggest that lentil was domesticated for the first time in the Pre-Pottery Neolithic villages in ancient near east and Anatolia around 12,000 years ago, beside other crops such as wheat, barley, legumes like pea, chickpea, bitter vetch, and flax (Blockley and Pinashi 2011, Goring-Morris and Belfer-Cohen 2011). From this proposed center of origin, lentil spread rapidly to the Nile Valley, Europe, India and Central Asia. After 1,500 AD the Spanish introduced lentil to South America. More recently, it has been cultivated in Mexico, Canada, the United States, New Zealand, and Australia (Erskine et al. 2016).

Consumption

Lentil as a food is served in various forms across the world such as boiled, fried, snack, bread, cake and soup; alone or in the mix with other foods or flours like rice or wheat. Lentil processing includes cleaning, sizing, de-hulling, splitting, and polishing. Small-seeded red cotyledon lentil is de-hulled and large-seeded yellow cotyledon lentil is used as a whole (Erskine et al. 2016). Lentil seeds, like other grain legumes are a good sources of protein, carbohydrate with starches, total dietary fibers (including both insoluble and soluble dietary fibers), relatively low fat and low energy content (1.4g/100g) including 16.7% saturated fatty acids, 23.7% monounsaturated fatty acids and 58.8% polyunsaturated fatty acids; relatively high ash content (3-5g/100g), high content of minerals such as Mg, P, Ca, and S but low content of Na and K, 7.5 mg/100g Iron, 3.2-6.3 mg/100 g Zn; and Cu, Mn, Mo and B in relatively low amounts. Lentils are a significant source of various types of vitamins including folate (B_9), thiamin (B_1) and riboflavin (B_2), other water soluble vitamins such as niacin, anthithetic acid, pyridoxine, vitamin E, vitamin K; bioactive phytochemicals such as polyphenols (flavonols, tannins, phenolic acids), phytosterols, phytic acid, saponins and lectins, protease inhibitors; dietary fiber (mostly insoluble), resistant starches (25g/100g) and finally high total of antioxidant capacity measured by ferric reducing antioxidant power and total radical-trapping antioxidant parameter (Shahwar et al. 2017).

Several evidences demonstrate that a diet rich in lentil and lentil sprouts is very useful for prevention and management of diabetes, lowering the risk of cancers (especially colon, prostrate,

gastric, breast, colorectal and pancreatic cancers) and Alzheimer's disease. Body weight regulation, lowering serum cholesterol and increasing the saturation level of cholesterol are other effects of lentil consumption on human body health (Świeca and Baraniak 2014, Shawar et al. 2017).

Botany

Lentil is an annual herbaceous plant, generally 20-30cm tall, erect to semi-erect, showing compact growth to much branched low bushy forms. Lentils possess slender tap-root system and are indeterminate- acropetal plants. Their stems are thin, square and ribbed, while the pods are oblong, laterally compressed, bulging over the seeds. Lentil leaves are alternate, compound and pinnate with one to eight pairs of sub-sessile or sessile, ovate, elliptical or lanceolate leaflets. The rachis is 4–5 cm in length and it may terminate in a bristle or simple. Lentil cultivars were grouped into two intergrading clusters based on seed sizes, small-seeded (microsperma) and large-seeded (macrosperma) (Duke 1981, Saxena and Hawtinn 1981, Muehlbauer et al. 1985, Muehlbauer et al. 2002, Gahoonia et al. 2005, Sarker et al. 2005, Saxena 2009). The number of seeds per plant, being an important yield attribute, is closely correlated with the number of pods per plant. The 100-seed weight may range from 1.1 - 4g (small-seeded types) to 4 - 8.2g (large-seeded types).

The four *Lens* species, as proposed by Ladizinsky (1993), are diploid (2n = 14), have a similar karyotype of three pairs of metacentric or submetacentric chromosomes, three pairs of acrocentric chromosomes, and one satellited pair of chromosomes.

Analyzing previous findings based on origin and spread; morphological, cytological, and cytogenetic observations; and more recently on the basis of isozyme and molecular studies, *Lens* taxonomy was reassessed. The genus now consists of seven taxa split into four species (Erskine et al. 2016):

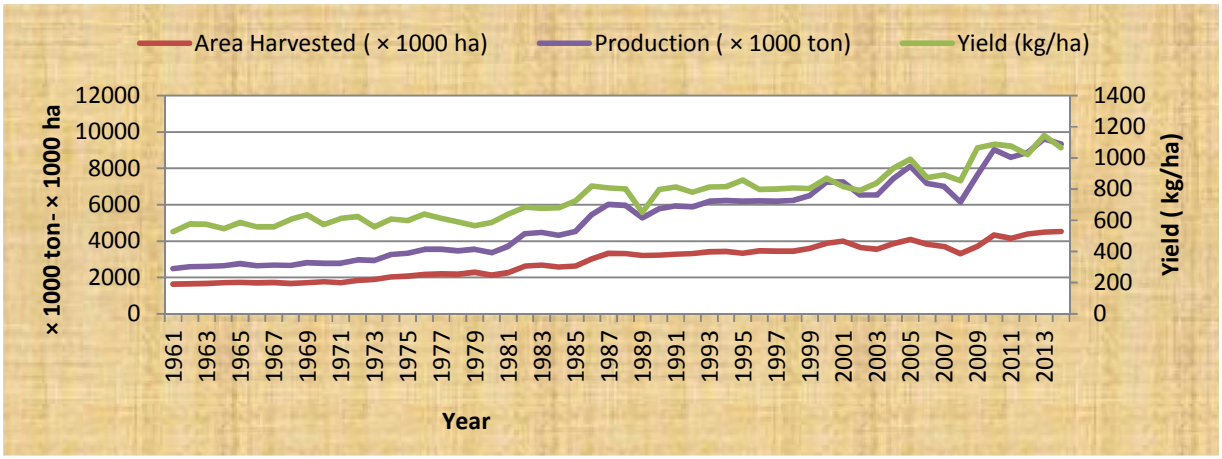
1. *L. culinaris* Medikus
   ssp. *culinaris*
   ssp. *orientalis* (Boiss.) Ponert
   ssp. *tomentosus* (Ladiz.) M.E. Ferguson et al.
   ssp. *odemensis* (Ladiz.) M. E. Ferguson et al.
2. *L. ervoides* (Brign.) Grande

3. *L. nigricans* (M. Bieb.) Godr.
4. *L. lamottei* Czefr.

The International Center for Agricultural Research in Dry Areas (ICARDA) has the global mandate for research on lentil improvement, and thus houses the world collection of *Lens*, which includes around 10,800 accessions (Furman et al. 2009). Other major lentil collections worldwide include those at the Australian Temperate Field Crops Collection (ATFCC) in the Department of Primary Industries, Victoria, Australia (http://149.144.200.50:8080/QMWebRoot/SiteMain.jsp) with 5,250 accessions, Pullman United States Department of Agriculture (USDA) Agricultural Research Service (ARS) with 2,797 accessions (http://www.ars.grin.gov/), the N.I. Vavilov All-Russian Research Institute of Plant Industry (VIR) (http://vir.nw.ru/) with 2,396 accessions, and the National Bureau of Plant Genetic Resources, India with 2,212 accessions (Dwivedi et al. 2006).

Crop Production

The total lentil cultivated area in the world in 2014 is estimated around 4.5 million ha with annual production and productivity of 4.8 MT and 1,080.1 kg/ha respectively, (FAOSTAT, accessed on 29 Oct 2016, http://faostat3.fao.org/). The production of the crop is increasing significantly from year to year through expansion of net cropped area along with productivity. Thus, yield productivity is showing an enormous upward trend, increasing almost more than two-fold during the last 50 years (Fig. 1). This is caused by the introduction of new cultivars and improved management systems.


Area Harvested (× 1000 ha)
Production (× 1000 ton)
Yield (kg/ha)
× 1000 ton- × 1000 ha
Yield (kg/ha)
12000
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1400
1200
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800
600
400
200
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1961
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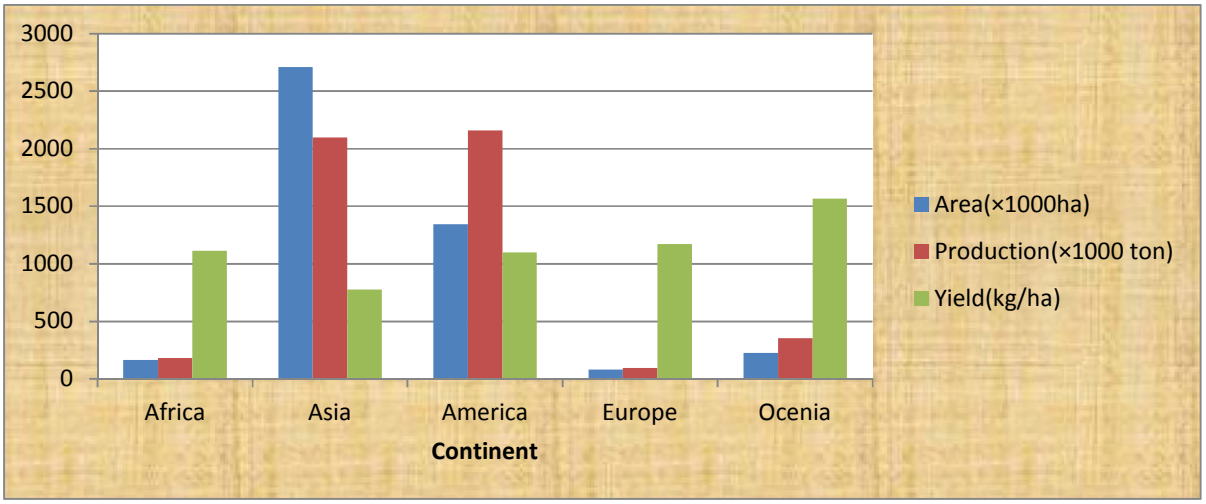
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2000
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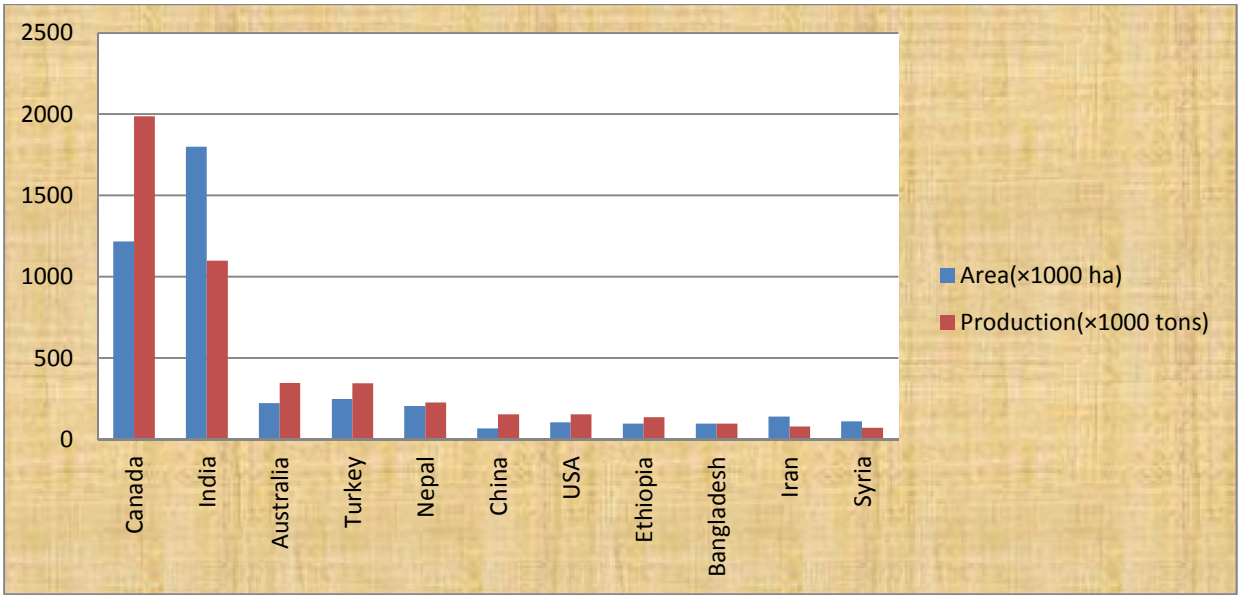
500
0
Africa
Asia
America
Europe
Ocenia
Continent
Area(×1000ha)
Production(×1000 ton)
Yield(kg/ha)


2500
2000
1500
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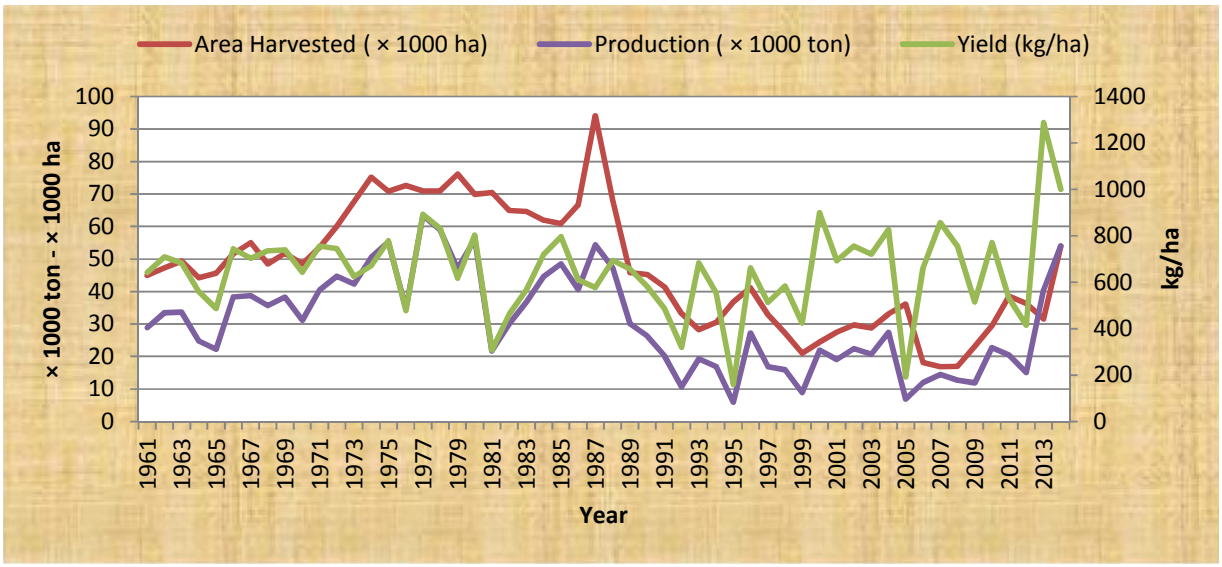
500
0
Canada
India
Australia
Turkey
Nepal
China
USA
Ethiopia
Bangladesh
Iran
Syria
Area(×1000 ha)
Production(×1000 tons)


Area Harvested (× 1000 ha)
Production (× 1000 ton)
Yield (kg/ha)
× 1000 ton - × 1000 ha
kg/ha
Year
0
10
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70
80
90
100
0
200
400
600
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1200
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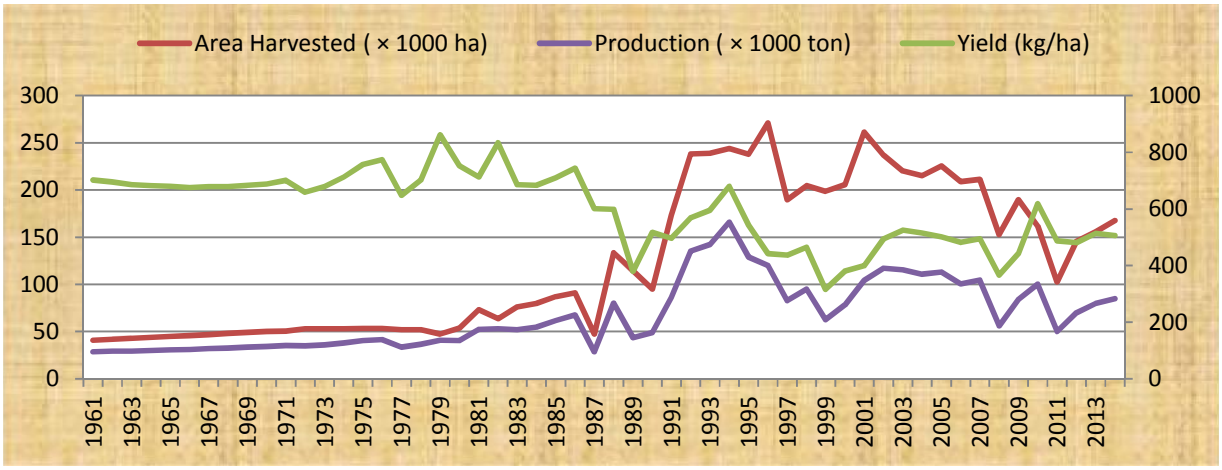
Area Harvested (× 1000 ha)
Production (× 1000 ton)
Yield (kg/ha)
300
250
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individually or in combination, cause the low levels of crop yield and productivity observed across the country (Sabaghpour, 2014)

Average lentil consumption in Iran is estimated in 2.5 kg Person/Year. Iran's production is not able to meet this demand, and therefore the country needs to import an average of 256,000 tons annually.

Factors limiting lentil yield

Abiotic stresses

Abiotic stresses affecting lentil production are cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity. Of these stresses, drought and heat are considered the most important worldwide (Turner et al. 2001). Cold stresses are important in West Asia and North Africa (WANA). Salinity is an important stress factor in Indian sub-continent and to some extent in WANA. Nutrient deficiency and nutrient toxicity is of lesser importance worldwide but important in some specific regions (Buddenhagen and Richards 1998).

Biotic stresses

Ascochyta blight caused by *Ascochyta lentis*, rust caused by *Uromyces viciae-fabae*, botrytis grey mould caused by *Botrytis cinerea* and *B. fabae*, anthracnose caused by *Colletotrichum truncatum*, stemphilium blight caused by *Stemphylium botryosum*, powdery mildew caused by *Erysiphe pisi* and *Erysiphe trifolii*, and sclerotinia stem rot (= white mould) caused by *Sclerotinia sclerotiorum*, are most important foliar diseases of the crop. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lentis* and collar rot caused by *Sclerotium rolfsii* are the most important soil born fungal diseases of the crop, although some other diseases with minor effect on the crop have been reported worldwide (Chen et al. 2009).

Ascochyta blight has worldwide occurrence and causes severe damage to lentil yield and grain quality (Muehlbauer and Chen 2007) and is important in West Asia, Canada and high rainfall areas of India (Chen et al. 2009). Rust is the key yield reducer of lentil in Bangladesh, India, Nepal, Morocco and Ethiopia, with reported losses of up to 70% of yield (Negussie et al. 2005). Stemphylium blight is a major problem in Bangladesh and Nepal, and appeared in fields in North Dakota and Saskatchewan in the recent years (Holzgang and Pearse 2001). Anthracnose, botrytis grey mold and sclerotinia white mold are major problems in North America (Chongo et al. 2002). Recently, powdery mildew has also been reported on lentil in

Canada and USA (Attanayake et al. 2009). Fusarium wilt is globally widespread (except Australia) and collar rot is also important universally, the former is more important in dry areas while the later is more important under humid conditions.

Disease resistance, beside other control approaches such as using disease-free seeds, biological agents, chemicals, cultural and agronomic practices is considered the most effective control strategy. Resistance can be conferred by single, race-specific resistance genes (R genes), usually conferring complete resistance, or by a number of minor genes resulting in a broad-spectrum incomplete resistance. The identification of genes underlying resistance is challenging and a detailed understanding of the interaction between plants and their pathogens at the genetic, histological and molecular level could be helpful to reach this objective (Rubiales et al. 2015). Resistant varieties or sources of resistance were identified for ascochyta blight (Ford et al. 1999, Nguyen et al. 2001, Taylor and Ford 2007), rust (Basandrai et al. 2000, Tikoo et al. 2005, Rubiales et al. 2013), botrytis grey mould (Bayaa and Erskine 1998, Davidson et al. 2004); stemphylium blight (Chen et al. 2009); powdery mildew (Tikoo et al. 2005); fusarium wilt (Bayaa and Erskine 1998, Stoilova and Chavdarov 2006) and *Orobanche crenata* (Fernández-Aparicio et al. 2008).

2. The Pathogen

Fusarium is a genus of filamentous ascomycete fungi (Sordariomycetes: Hypocreales: Necteriaceae) that includes many toxin-producing plant pathogens of agricultural importance, being also human pathogens. *Fusarium* spp. have large scale distribution habitat, from permafrost in the arctic to the sands of the Sahara (Di Pietro et al. 2003, Leslie and Summerell 2006, Ma et al. 2013).

Traditional classification of the genus was based on morphological characters which mainly referred to producing conidia which are clearly visible under fluorescent microscope as light colored raised bodies on the surface of culture using fine magnification. They are long, slender, rather pointed at both ends, dorso-ventrally curved, sickle-shaped, septated, and poses a basal foot cell. The “macroconidia” are phialospores which are initially attached to the conidiophores and are produced one by one, appearing apex end first, from a small opening body at the tip of the conidiophores, named “phialids” (Smith 2007). Recently many researchers have applied molecular approaches to the taxonomy of *Fusarium* species. Consequently, the 300

genealogically phylogenetically exclusive phytogenetic species with most important plant pathogens resided in the four groups, including *F. fujikuroi* species complex, *F. graminearum* species complex, *F. oxysporum* species complex and *F. solani* species complex (Watanabe et al. 2011, Aoki et al. 2014).

Fusarium spp. are soil borne pathogens that can grow in and on living or dead plants and plant products, as well as on living and dead animals (Smith 2007). The fungus can survive in the soil in two different growth phases: an active growth phase (when the soil environment and the remained substrates are suitable for its growth with enough nutrients) and a survival phase (when the soil conditions and environment are harsh with fewer nutrients) (Sungalang et al. 1995). During the survival phase the fungus will form dormant structures naming "chlamydospores" or multi-cellular resting bodies known as "sclerotia", while the main structure of active phase is "macro" and "micro-conidia" (Garrett 1981, Smith 2007). Fungus development and its activity in the soil are affected by environmental conditions such as temperature, humidity, pH, crop rotation, fertilizers use and straw disposal (Bateman and Murray 2001, Gonçalez et al. 2012).

The species *Fusarium oxysporum*, including many pathogenic forms, is the most damaging species of the genus. This pathogen causes a series of symptoms including vascular wilt, yellows, corm rot, root rot, and damping-off, being wilt symptom the most important one (Agrios 1997, Smith et al. 1988, Smith 2007). Strains that are rather poorly specialized may induce yellows, rot, and damping-off, while the more specialized strains produce severe vascular wilt (Smith et al. 1988).

F. oxysporum produces both "macro" and "micro conidia", "sporodochia" and "chlamydospores"; and no perfect stage is known for this species (Di Pietro et al. 2003). Resting spores, known as the ultimate soil-borne propagule of the species, and their rough heavy walls, protect them from all abiotic and biotic stresses, making the fungus able to survive long periods in the absence of the host. In the presence of fresh nutrients, such as root exudates, they come out of resting state, germinate, produce fresh hyphae and grow toward the roots of plants. After germination, infection hyphae adhere to the host roots and penetrate them directly. Small rifts in the root cuticle or a root injury may facilitate the penetration procedure. The hyphae grow in the cortex, inter and intra-cellularly and get inside the vessels. The pathogen inside the vessel is capable to produce micro conidia as well as hyphal extension, being capable of escaping and

avoiding plant barriers, colonizing the entire vascular system fast. Host, on the other hand, respond to the fungal invasion by producing callose and depositing it around paravascular parenchyma, pits and vessel walls where the fungus may attempt to enter. Moreover, secondary metabolites such as phytoalexins, phenolics, indole acetic acid, ethylene and other compounds have an important role in the host defense. The pathogen to counteract these plant defenses produces polygalacturonases, cellulases, alpha- and beta- galactosidases, and other enzymes capable to break down gels, retarding tyloses formation and degrading stress metabolites of the host plant. As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. After the host plant is killed by the pathogen, the fungus can invade the parenchymatous tissue, sporulate profusely on the plant surface and release spores (Talboys 1972, Bishop and Cooper, 1983b, Beckman 1987, Rodriguez-Gálvez and Mendgen1995, Agrios 1997, Di Pietro et al. 2001a, Di Pietro et al. 2003).

F. oxysporum has adapted to colonize different host species with more than 120 *formae speciales* (ff.spp.) reported so far (Di Pietro et al. 2003, Michielse and Rep 2009). Some of the *formae specials* are further divided into subgroups, named races, on the basis of differential virulence patterns in a set of differential cultivars within the same plant species (Snyder and Hansen 1940, Armstrang and Armstrang 1981).

F. oxysporum f.sp. *lentis* (*Fol*) is one of the most destructive diseases of the lentil crop worldwide (except Australia), causing complete yield loss especially under drought and warm conditions. Symptoms of disease can be observed at both seedling and adult plant stages as seed rot and damping off, stunting of plants, wilt of top leaves, shrinking and curling of leaves which start from the lower parts of plant and move up progressively; leading to complete yellow and die back (Khare 1981). The symptoms are intensified with warm and drought conditions. The pathogen can survive in the soil for several years saprophytically, or as a chlamydospore without access to suitable host.

Although there are some reports on variation in *Fol* in term of genetic diversity, differences in *in vitro* growth patterns and aggressiveness (Khare et al. 1975, Belabid and Fortas 2002, Belabid et al. 2004, Taheri et al. 2010, Datta et al. 2011, Mohammadi et al. 2011), races or pathotypes have not been reported in the *Fol*-lentil pathosysthem.

Use of resistant varieties is the most effective, economic and eco-friendly method to control this disease (Bayaa et al. 1995, Kraft et al. 2000) and a number of sources of resistance as

well as several resistant cultivars are available (Pandya et al. 1980, Bayaa et al. 1995, Bejiga et al. 2001, Sarker et al. 2001, El-Ashkar et al. 2003, ICARDA 2003, El-Ashkar et al. 2004a, El-Ashkar et al. 2004b, ICARDA 2004, Machleb et al. 2007, Mohammdi et al. 2012).

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CHAPTER I

Identification of resistance to *Fusarium oxysporum* f.sp. *lentis* in Spanish lentil germplasm

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CHAPTER II

Histopathology of the infection on resistant and susceptible lentil accessions by two contrasting pathotypes of *Fusarium oxysporum* f.sp. *lentis*

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CHAPTER III

Identification of pathotypes in *Fusarium oxysporum* f.sp. *lentis*

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CHAPTER IV

Genetic diversity and structure of *Fusarium oxysporum* f.sp. *lentis* isolates from Iran, Syria and Algeria

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Summary: In this study the genetic structure of a collection of *Fol* isolates from Iran, Syria and Algeria, was analyzed using twelve SSR markers. Eight of these SSRs were developed in this thesis. AMOVA showed that there is a high molecular variation both within regions and among regions, differing Iranian populations from non-Iranian populations. Additionally, STRUCTURE and Fitch-Margoliash analysis identified two ancestral lineages, one present in all regions and the other present only in Iran. No significant relationship was found between phylogenic groups and virulence patterns.

General conclusions

1. The new detailed scoring system developed in this thesis was very efficient to detect different levels of resistance to *Fol* at seedling stage.
2. Promising sources of resistance to *Fol*, showing resistance under seedling, and adult plant stage under field conditions, were identified within Spanish lentil landraces.
3. The main courses of infection of lentil accessions with *Fol* are: (i) attachment of hyphae to the root surface, penetration through epidermis and colonization of the cortex cells within 0-2 *dai*, (ii) hyphal penetration through endodermis layer and invasion of vascular tissues within 2-4 *dai*, (iii) colonization of all plant root tissues by the pathogen, including horizontal and lateral spreading; and presence of different gum-like substances inside the xylem vessels from 4-15 *dai*; and (iv) development of tylosis and accumulation of mucilage inside the vessels, colonization of the hypocotyls and stem, and finally phenotypic expression (yellowing and/or wilt) at 15-30 *dai*.
4. Uniform xylem occlusion with gum-like substances or continues degree of colonization of all host accessions by the pathogen is contributing to quantitative resistance.
5. Secretion of phenolic compounds in the cortex in an early stage (0-4 *dai*) might be observed in specific interactions (i.e. accession BGE01969 challenged with pathotype 1 only) leading to a lower colonization as well as a lower disease index.
6. There are significant *Fol* isolate×lentil accession interactions, suggesting different patterns of virulence: seven pathotypes were identified within a *Fol* population formed by 52 isolates from Iran, Argelia and Syria. This is the first report of existence of pathotypes in this *Fol*.
7. SSRs constitute a valuable resource for molecular studies in *Fol*. We developed 8 new SSRs that ad to the only 9 previously existing ones.
8. In the collection of *Fol* isolates studied, a high molecular variation within regions was observed, although variation among regions also exists, with Iranian populations differing significantly from non-Iranian ones, having some private alleles.
9. Our results suggest the presence of two ancestral *Fol* lineages, being one present exclusively in Iran and closely related to *F. oxysporum* f. sp. *pisi*, while the other was distributed across all the regions studied
10. No clear relationship was observed between *Fol* ancestors and pathotypes.