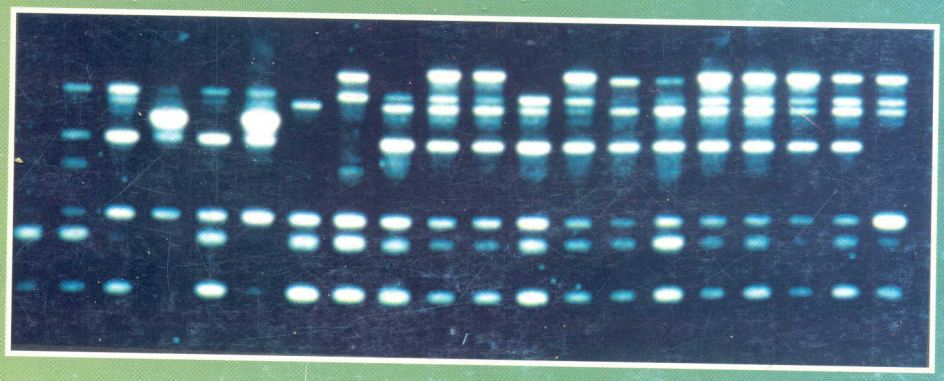
DNA Markers and Breeding for Resistance to Ascochyta Blight in Chickpea



S. M. Udupa and F. Weigand
Editors

International Center for Agricultural Research in the Dry Areas

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Proceedings of the Symposium on
"Application of DNA Fingerprinting for Crop Improvement: Marker-assisted Selection of Chickpea for Sustainable Agriculture in the Dry Areas"

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Edited by

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Contents

Foreword

Today, we are in an era of unprecedented scientific potential in which biotechnology is playing a major role in crop improvement. Biotechnology does not replace traditional disciplines such as plant breeding, genetics and pathology, but rather supports and complements them. The new tools of biotechnology such as DNA markers based primarily on advances in molecular biology, have the potential to be applied in crop breeding programs. DNA markers combined with conventional strategies can help to attain crop breeding objectives more speedily than by conventional techniques alone.

In West Asia and North Africa (WANA), chickpea is an important component in human diets and in the farming systems. Among the biotic stresses which constrain productivity of chickpea, ascochyta blight is the most important. At ICARDA, in collaboration with the National Agricultural Research Systems and the University of Frankfurt, Germany, suitable DNA markers have been developed and are being used for tagging resistance genes and for studying the population biology of the pathogen. This book contains a collection of articles presented in the symposium, "Application of DNA Figerprinting for Crop Improvement: Marker-assisted Selection of Chickpea for Sustainable Agriculture in the Dry Areas," organized at ICARDA to review past progress and to formulate future strategies on application of DNA markers for chickpea improvement. Publication of these papers is intended to provide researchers with an understanding of the variety of DNA marker techniques being used, their relevance and usefulness, and their potential benefits in crop improvement.

Adel El-Beltagy
Director General
ICARDA

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S.M. Udupa
F. Weigand

Preface

In recent years the use of DNA markers has fostered a better understanding of plant pathogen populations and quickened practical plant breeding, including breeding for biotic stresses. However, many markers used in crops such as maize, rice, and *Brassica* spp. are ineffective in detecting polymorphism in chickpea and its pathogens, such as *Ascochyta rabiei* (Pass.) Lab. Our collaborative project with NARS and the University of Frankfurt shows that microsatellite markers solve this problem.

A symposium on "Application of DNA Fingerprinting in Crop Improvement: Marker-assisted Selection of Chickpea for Sustainable Agriculture in the Dry Areas" was held at ICARDA, Aleppo, Syria for the purpose of reviewing recent achievements and formulating new strategies for our collaborative research. Consultants from various disciplines, including chickpea breeding and pathology, also took part.

During the symposium, it became clear that there is tremendous potential for the use of microsatellite markers in chickpea resistance breeding, as well as in the population genetics of pathogens. Future research should focus on pyramiding resistance genes to improve resistance in chickpea, in which DNA markers could play a major role.

We hope that this proceedings will help researchers develop suitable strategies for disease-resistance breeding and plant disease management, not only in chickpea but in other crops as well.

S.M. Udupa
F. Weigand

Population Biology of the Host–Pathogen Interaction

The Teleomorph of *Ascochyta rabiei* and its Significance in Breeding Chickpea

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Abstract

The presence of *Didymella rabiei* (Kovachevski) Arx (Syn. *Mycosphaerella rabiei* [Pass.] Kovachevski), the teleomorph (sexual or perfect stage) of *Ascochyta rabiei* (Pass.) Lab., increases the spread of ascochyta blight in chickpea (*Cicer arietinum* L.) by producing airborne ascospores capable of spreading the pathogen over long distances. This reduces the effectiveness of disease-control practices such as planting clean seed and crop rotation. *D. rabiei* is heterothallic, requiring the presence of two compatible mating types, referred to as MAT1–1 and MAT1–2, for the production of mature ascospores. The teleomorph contributes to heterozygosity within the pathogen population, which could lead to increased general adaptation and new combinations of virulence genes, favoring the appearance of new pathotypes. The two mating types has been found in 15 out of 21 countries sampled, and the teleomorph developed on infested residue from 12 countries on three continents—proof that it may play a significant if unknown role in the spread of the disease. Throughout the world both mating types occur about equally, but for some reason MAT1–2 has not been found in India and is rare in Pakistan. This paper discusses the occurrence of the teleomorph on plants other than chickpea, laboratory methods for mating the fungi and the production of mature sporulating pseudothecia, discharge of ascospores, conditions favoring ascospore germination, and a comparison of the relative effectiveness of ascospores and conidia in infecting chickpea.

Citation: Kaiser, W.J. 1997. Teleomorph of *Ascochyta rabiei* and its significance in breeding chickpea. Pages 3–21 *in* DNA Markers and Breeding for Resistance to Ascochyta Blight in Chickpea. Proceedings of the Symposium on "Application of DNA Fingerprinting for Crop Improvement: Marker-assisted Selection of Chickpea for Sustainable Agriculture in the Dry Areas," (S.M. Udupa and F. Weigand, eds.). 11–12 April 1994, Aleppo, Syria. ICARDA, Aleppo, Syria.

Introduction

Ascochyta blight in chickpea, associated with *A. rabiei*, is one of the most important diseases of chickpea in South Asia, the Middle East, the Mediterranean, and North America (Kaiser and Muehlbauer 1988; Nene 1982; Nene and Reddy 1987; Punithalingam and Holliday 1972). Since first described in chickpea by Butler in 1911 in the Northwest Frontier Province of present day Pakistan (Butler 1918), considerable research has been devoted to various aspects of the disease and its control. However, large gaps still exist in our knowledge of the disease, particularly the role played by the teleomorph (sexual or perfect stage) *D. rabiei* in disease epidemiology and in the variability of growth, sporulation, colony appearance, morphology, and pathogenicity exhibited by isolates of the pathogen. This paper focuses on the teleomorph and new information that has resulted from recent research in Spain (Navas Cortés 1992; Nuñez Cañete et al. 1990, 1991; Trapero-Casas et al. 1988, 1992; Trapero-Casas, unpublished data) and the United States (Kaiser 1992; Kaiser et al. 1994; Trapero-Casas and Kaiser 1987, 1992a).

Discovery

In 1936, Kovachevski discovered the teleomorph in chickpea debris (especially the pods) that had overwintered on the soil surface in southern Bulgaria. He named it *Mycosphaerella rabiei*. Kovachevski found that *M. rabiei* pseudothecia form only on overwintered debris and not on living plants or agar media. Subsequently, the teleomorph was found on overwintered chickpea residue in the former USSR (Gorlenko and Bushkova 1958), Greece (Zachos et al. 1963), Hungary (Kovics et al. 1986), Spain (Jiménez Díaz et al. 1987), Syria (Haware 1987), and the United States (Kaiser and Hannan 1987).

Geographical Distribution

The teleomorph has been reported in Bulgaria, Greece, Hungary, Syria, the United States, and the former USSR (Kaiser 1992). In studies to determine the distribution of *D. rabiei*, Kaiser and Trapero-Casas (unpublished data) incubated naturally infested chickpea debris from different countries under environmental conditions that promoted the development of the teleomorph. Mature *D. rabiei* pseudothecia formed and ascospores were discharged from chickpea debris from Algeria, Bulgaria, Canada, Greece, Morocco, Pakistan, Portugal, Spain, Syria, Tunisia, Turkey, and the United States (Table 1). The teleomorph is thus present in more countries than previously reported. It is likely that *D. rabiei* will be found in even more countries as further samples of infested debris are examined.

Table 1. Countries where *Didymella rabiei* developed on naturally infested chickpea debris.

Country	Teleomorph incidence
Algeria	1/3[†]
Bulgaria	7/15
Canada	1/2
Cyprus	0/3
Egypt	0/1
France	0/3
Greece	2/2
Hungary	0/1
India	0/9
Iran	0/3
Italy	0/3
Libya	0/1
Morocco	2/15
Pakistan	1/23
Portugal	3/7
Spain	3/11
Syria	2/6
Tunisia	4/7
Turkey	11/27
United States	32/52
Total	69/195

[†] Numerator = number of samples from which the teleomorph developed; denominator = total number of samples tested.

Description

At maturity, pseudothecia of *D. rabiei* are usually erumpent, dark brown to black, subglobose, and 120–270 mm in diameter with an inconspicuous ostiole. Asci are cylindrical to subclavate, bitunicate, eight-spored, and measure 50–80 × 10–12 mm. Ascospores are hyaline, two-celled (with the upper cell broader than the lower), constricted at the septum (septum below the middle), and measure 9.5–16 × 4.5–7 mm (Kovachevski 1936; Trapero-Casas and Kaiser 1992a).

Pycnidia and pseudothecia of *D. rabiei* usually occur together on overwintered chickpea debris. Pycnidia frequently outnumber pseudothecia. Under a stereoscopic microscope (X50), it is often difficult to distinguish between the two. However, pseudothecia show several distinguishing characteristics: they have darker walls, larger size, a subglobose and more erumpent shape, lack a conspicuous ostiole, and form a white exudate when moist (Trapero-Casas and Kaiser 1992a).

Genetics of Mating Incompatibility

D. rabiei is a heterothallic fungus, which implies that it has a mating incompatibility system requiring two thalli with different mating alleles for successful fertile crosses to occur (Trapero-Casas and Kaiser 1992a; Wilson and Kaiser 1994). The mating system is unifactorial (bipolar), meaning that sexual incompatibility is controlled by a single factor or locus on a single chromosome, with two different mating types required for a successful cross (Wilson and Kaiser 1994). The two mating types are referred to as MAT1-1 and MAT1-2 (Trapero-Casas and Kaiser 1992a), following the system proposed by Yoder et al. (1986).

Ascospores and conidia are commonly multinucleate and appear to undergo repeated mitotic divisions prior to spore germination. However, somatic hyphae derived from ascospores and conidia are predominately uninucleate (Wilson and Kaiser 1994).

Table 2. Identification of mating types of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, from different countries.

Country	No. of isolates tested	MAT1-1	MAT1-2
Algeria	8	1	7
Bulgaria	6	2	4
Canada	15	3	12
Cyprus	3	0	3
Egypt	1	0	1
France	9	2	7
Greece	2	1	1
Hungary	1	1	0
India	30	30	0
Iran	5	2	3
Israel	2	0	2
Italy	14	1	13
Libya	2	0	2
Morocco	11	2	9
Pakistan	21	20	1
Portugal	14	6	8
Spain	9	5	4
Syria	16	4	12
Tunisia	11	3	8
Turkey	32	23	9
United States	23	13	10
Total	235	119	116

Geographical Distribution of Mating Types

In studies by Kaiser and Trapero-Casas (unpublished data) to determine the mating types of single-spore isolates of *D. rabiei* in various countries, refer-

ence isolates of MAT1-1 and MAT1-2, represented by ATCC 76501 and ATCC 76502, respectively, were used in all pairings. Isolates of *A. rabiei* from 21 countries were tested (Table 2). Both mating types were found in 15 countries. India was the only country where many isolates were tested that failed to show both mating types. More isolates need to be tested from different countries.

Nuñez-Cañete et al. (1990, 1991, 1992) and Trapero-Casas (unpublished data) tested 168 single-spore isolates of *A. rabiei* from various chickpea-growing regions of Spain and from several other countries for sexual compatibility (mating type). All isolates were self-sterile and only formed mature pseudothecia with asci and ascospores when paired with a compatible MAT1-1 or MAT1-2 isolate, confirming that the fungus is heterothallic.

Identification of Mating Types from Different Plant Species

Kaiser (1990, 1992, unpublished data) isolated *A. rabiei* from surface disinfested tissues of various crop and weed species collected from ascochyta blight-infected commercial chickpea plantings in the US Pacific northwest and from a blight screening trial at Pullman, Washington. Single-spore cultures of the blight pathogen isolated from the various species were paired with reference isolates of MAT1-1 and MAT1-2 to determine the mating type. Mating types for *A. rabiei* isolated from 14 plant species were determined (Table 3). Both types were found in isolates of the fungus from *Lamium amplexicaule*, *Solanum nigrum* L., *Thlaspi arvense*, and *Triticum aestivum* L. subsp. *aestivum*. The role of these alternative hosts in the epidemiology of the disease needs to be investigated.

Development of the Teleomorph on Infested Chickpea Debris

Research in different countries has demonstrated the importance of infested chickpea debris to the survival of the blight pathogen from one growing season to the next, and as a source of inoculum for infection of subsequent chickpea plantings (Kaiser 1992). This also establishes the importance of crop management practices, including crop rotation and clean seed, in controlling ascochyta blight.

Table 3. Mating types of *Ascochyta rabiei* isolated from alternative hosts from the Palouse region of eastern Washington and northern Idaho.

Host	No. of isolates tested	No. of isolates	
		MAT1-1	MAT1-2
Amaranthus albus (Tumble pigweed)	1	1	0
Anthemis cotula (Dog fennel)	1	0	1
Asperugo procumbens (Catchweed)	1	1	0
Brassica nigra (Black mustard)	1	1	0
Capsella bursa-pastoris (Shepherds' purse)	1	0	1
Descurainia sophia (Flixweed)	1	1	0
Lamium amplexicaule (Henbit)	2	1	1
Lens culinaris (Lentil)	10	10	0
Medicago sativa (Alfalfa)	3	0	3
Pisum sativum (Pea)	2	0	2
Solanum sp. (Nightshade)	1	0	1
Solanum nigrum (Black nightshade)	2	1	1
Thlaspi arvense (Fanweed)	2	1	1
Triticum aestivum (Wheat)	2	1	1
	30	18	12

Lukashevich (1958), working in the Ukraine, observed that *A. rabiei* developed after harvest as a saprophyte on infested chickpea residue, covering the tissue by the following spring. Colonization of infested chickpea debris by *A. rabiei* after harvest was also observed by Luthra et al. (1935) in India, Navas Cortés (1992) in Spain, Trapero-Casas et al. (1988) in Spain and the United States, and Zachos et al. (1963) in Greece. Zachos observed that stems from a blight-infected chickpea crop left in the field after harvest were covered with *A. rabiei* pycnidia and pseudothecia, and that mature pseudothecia were found by February of the following year.

In the field, the teleomorph of *A. rabiei* only develops on dead, infested chickpea debris after harvest. It does not develop on living plant tissue during the growing season. Only the anamorph (asexual stage) develops on the living host during the crop season (Kaiser 1992).

In the US Pacific Northwest, *A. rabiei* grows rapidly from lesions on chickpea stems and pods, forming pycnidia and pseudothecia (ascocarps) within 4–6 weeks of placement of the tissue on the soil surface in the fall of the year (Trapero-Casas and Kaiser 1992a). Similar events occur on infested chickpea stems and pods in Spain (Navas Cortés 1992). Depending on temperature, pseudothecia in the US are often mature and ready to discharge ascospores by early March (Kaiser and Hannan 1987; Trapero-Casas and Kaiser 1992a). In Spain, mature pseudothecia are found in late January to late March, de-

pending upon the year and location (Navas Cortés 1992; Trapero-Casas et al. 1988). Maturity is delayed at cooler locations.

Maturity of *D. rabiei* pseudothecia in the Pacific northwest, as determined by ascospore discharge, usually begins in February or March, reaching a maximum in April (Kaiser 1992; Trapero-Casas and Kaiser 1992a). Maximum discharge of ascospores varies from year to year, ranging from 400 to 1,600 ascospores/mm^2 of infested chickpea tissue. The discharge of ascospores in the spring often coincides with the early vegetative stage of chickpea. Repeated wetting and drying of naturally infested debris at weekly intervals shows that ascospores are discharged for up to 6 weeks from the same ascocarps. Maximum discharge of ascospores usually occurs within the first 24 hours (Kaiser 1992). Ascospore discharge usually decreases markedly from June to October (Trapero-Casas and Kaiser 1987). Once all ascospores are discharged from the pseudothecia, no more are produced and pseudothecial walls degenerate. New pseudothecia do not develop on the infested chickpea debris during the next crop season (Trapero-Casas and Kaiser 1992a).

In southern Spain, Navas Cortés (1992) found that ascospore maturation usually occurs from late January to late March, depending on the year and location. Maximum ascospore discharge occurs within 2–4 weeks of maturation, but then decreases sharply and is negligible by June. New ascospores do not develop in empty pseudothecia and new pseudothecia do not develop on the debris during the following fall and winter.

Moisture is essential for initiation and development of pseudothecia and for discharge of ascospores. Development of pseudothecia ceases if infested debris is air-dried (Kaiser, 1992; Trapero-Casas and Kaiser 1987), but resumes when the debris is moistened. When naturally infested chickpea stems and pods were incubated at different temperatures and relative humidity (RH), mature pseudothecia were observed at 10° C and 100% RH, and at 5° C and 10° C at a fluctuating RH (100/34%). At 10° C, pseudothecia matured in 10 and 14 weeks when incubated at 100% RH and at a fluctuating RH, respectively. At 5° C, pseudothecia matured within 14 weeks at a fluctuating RH (Navas Cortés 1992). Pseudothecia containing mature asci and ascospores did not develop at 15° C, while at 20° and 25° C, pseudothecia aborted (Trapero-Casas and Kaiser 1992a).

Survival in Infested Debris

Survival of the teleomorph in soil using naturally infested chickpea debris was studied in Pullman, Washington (Kaiser 1992; Kaiser et al. 1987) and in southern Spain (Navas Cortés 1992). In Pullman, discharge of ascospores

from pseudothecia ceased after 27 weeks on stem pieces that had been incubated on the soil surface, and after eight weeks in tissue buried 16 cm deep. Viable ascospores were still being discharged from pseudothecia on stem pieces after 50 weeks at an above-ground weather station at 4–6° C and 30-40% RH (Kaiser et al. 1987). In southern Spain, *D. rabiei* survived in infested debris on the soil surface for at least three months, but lost viability rapidly when buried (Navas Cortés 1992).

Ascospores as Inoculum

Kovachevski (1936) and others have demonstrated that ascospores of *D. rabiei* are pathogenic to chickpea. Only the anamorphic stage (conidia and pycnidia) of *A. rabiei* develops in necrotic lesions on chickpea plants, resulting from infection by ascospores. Similarly, only conidia and pycnidia develop on culture media seeded with ascospores.

When the mature pseudothecia are moistened, the asci protrude through the opening of the pseudothecium and forcibly discharge the ascospores into the air (Kaiser 1992). On infested chickpea debris at 15–25° C, over 70% of the ascospores are discharged from mature pseudothecia within two hours of wetting (Kaiser 1992; Trapero-Casas and Kaiser 1987). Ascospores are carried on wind currents and can be blown long distances. Rain undoubtedly contributes to their spread, as it does with conidia. Ascospores spread the blight fungus faster and over greater distances than conidia. Although ascospores are disseminated by air currents, they require free moisture for discharge from pseudothecia and for infection of chickpea tissues. In the Palouse region of eastern Washington and northern Idaho, pseudothecia usually mature in spring, during the early vegetative stage of the chickpea crop. Chickpea fields may receive showers of ascospores that blight the whole field within days. In 1985–1987, this phenomenon was observed in northern Idaho, where chickpea fields are often separated from one another by several kilometers (Kaiser 1992). Despite planting clean seed and practicing crop rotation, infection of isolated chickpea fields may result from the presence of ascospores from infested debris that has overwintered on the soil surface of a neighboring field affected by ascochyta blight the preceding season. The significance of the anamorphic and teleomorphic stages in the disease cycle of ascochyta blight in chickpea in the Pacific Northwest is illustrated in Figure 1.

Trapero-Casas and Luque-Marquez (1992) studied the germination of conidia and ascospores of *D. rabiei* on glass slides coated with water agar. Germination of conidia and ascospores began after two hours, with maximum germination (>95%) occurring in 15, 9, 7, 6, 7 and 32 hours at 5°,

10°, 15°, 20°, 25°, and 30° C, respectively. Germination times were a function of the fungal isolate. At 0° and 35° C, no germination occurred. At most temperatures, ascospores germinated faster than conidia. Germination of conidia and ascospores declined rapidly as the relative humidity (RH) was reduced from 100 to 97%. No germination occurred at 94% RH or lower. Optimum germination at different RH occurred at 20–25° C. Ascospores germinated over a wider range of RH than conidia.

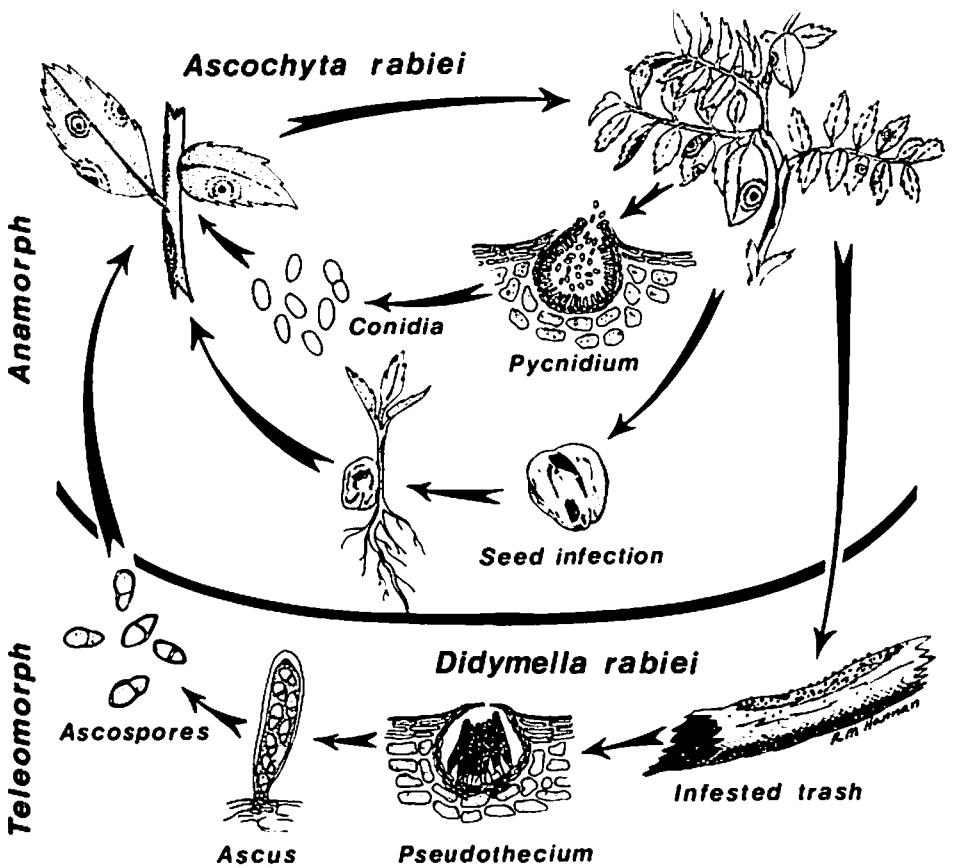

Figure 1. Disease cycle of ascochyta blight of chickpea caused by *A. rabiei* in the Pacific northwest. Both pycnidia and pseudothecia may develop on overwintered infested chickpea debris. (Drawing by R.M. Hannan)

Trapero-Casas and Kaiser (1992b) compared the effects of conidia and ascospores of *D. rabiei* on infection and development of disease in a blight-susceptible chickpea cultivar. Two-week-old plants were inoculated with suspensions (2×10^4 spores/ml) of ascospores or conidia. After inoculation, plants were placed inside moist chambers in growth cabinets at temperatures ranging from 5° to 30° C for 6–96 hours. At an optimum temperature of 20° C, disease severity increased significantly as wetness periods increased. Very little disease occurred at 5° or 30° C. Ascospores were more effective as an inoculum source than conidia and resulted in greater disease severity over the range of temperatures and wetness periods tested.

Induction of Pseudothecia *in vitro*

A reliable and repeatable method has been found to induce pseudothecial formation on sterile chickpea stems *in vitro*. This method is a modification of that used by Trapero-Casas and Kaiser (1992a).

1. Prepare spore suspensions (1×10^6 spores/ml) of single-spore cultures of *A. rabiei* isolates being tested for mating type.
2. Prepare spore suspensions (1×10^6 spores/ml) of reference isolates of MAT1-1 (ATCC 76501) and MAT1-2 (ATCC 76502).
3. Soak dry, sterile chickpea stem pieces (7–9 cm long) in spore suspensions of each isolate, and in mixtures of each isolate with those of MAT1-1 and MAT1-2, for 30 minutes.
4. Pour off liquid and allow stem pieces to drain for 30 minutes.
5. Place inoculated stem pieces on 10 layers of sterile moist filter paper (Whatman no. 1) in glass petri dishes. Add 15 ml sterile water to each dish.
6. Incubate dishes in the dark at 10° C. Do not seal petri dishes or place in a plastic bag.
7. After 5–6 weeks, dry stem pieces at room temperature.
8. To test tissue for discharge of ascospores, affix pieces of dry tissue to a 2% water agar (WA) block (~2 cm^2) on the underside of a petri dish lid and invert the lid over a dish bottom containing WA. Ascospores of *D. rabiei* will be discharged downward onto the surface of the WA in the dish bottom. Discharge of ascospores often begins within 1–2 hours. Ascospores begin germinating in 6–8 hours.

Preservation of Cultures

For long-term storage, single-spore isolates of *A. rabiei* are cultured on sterile chickpea stems. Dry, sterile chickpea stem pieces (4–6 cm) are soaked in a single-spore suspension of each isolate for 20–30 minutes, drained, and

placed on 2% WA in petri dishes. Dishes are incubated at 20–24° C under fluorescent lights (12-hour photoperiod). After two weeks, the colonized stem pieces are dried and stored at -18° C or colder. Using this technique, isolates have remained viable for over eight years. This method of culture preservation minimizes the danger of mutation or other genetic change in the culture.

Formation of the Teleomorph on Different Plant Species

Since 1988, different annual and perennial *Cicer* species in the germplasm collection of the Regional Plant Introduction Station at Pullman, Washington have been screened for resistance to ascochyta blight (W.J. Kaiser, unpublished data). After harvest, tissue from plants included in the screening trials is placed in nylon mesh bags which are incubated outdoors on the soil surface. The bags are collected the following March or April and the tissue tested for discharge of ascospores of *D. rabiei*. Several of the perennial *Cicer* species, including *C. anatolicum*, *C. microphyllum*, and *C. oxyodon* have proved to be highly resistant to ascochyta blight. The teleomorph has developed on 11 *Cicer* species (Table 4), including perennials resistant to blight.

Table 4. Development of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on debris in *Cicer* spp. collected from a screening trial at Pullman, Washington for ascochyta blight of chickpea.

Cicer spp.	No. of accessions developing teleomorph
C. anatolicum	2
C. arietinum	6
C. bijugum	2
C. cuneatum	1
C. echinospermum	3
C. judaicum	3
C. microphyllum	1
C. oxyodon	1
C. pinnatifidum	4
C. reticulatum	5
C. yamashitae	1

Tissue of different plant species was collected in July and August from a screening trial at Pullman, Washington for ascochyta blight in chickpea. In October, dried tissue of each plant species was placed outdoors in nylon mesh bags on the soil surface. The bags were collected the following March and April and the tissue tested for discharge of ascospores of *D. rabiei*. Mature pseudothecia of *D. rabiei* developed and ascospores were discharged

from *Amaranthus albus*, *C. arietinum*, *Lens culinaris* Medik., *Medicago sativa* L. and *Pisum sativum* L. (Table 5).

Table 5. Development of the teleomorph of *Didymella rabiei* on debris of different crop and weed species from the chickpea screening trial at Pullman, Washington for ascochyta blight, and from glasshouse inoculations.

Field	
Amaranthus albus	Tumble pigweed
Cicer arietinum	Chickpea
Lens culinaris	Lentil
Medicago sativa	Alfalfa
Pisum sativum	Pea
Glasshouse	
Cicer arietinum	Chickpea
Melilotus alba	White sweetclover
Pisum sativum	Pea
Triticum aestivum	Wheat

In the glasshouse, *C. arietinum*, *Melilotus alba* Medik., *P. sativum*, and *Triticum aestivum* L. were inoculated with a spore suspension of the reference isolates of MAT1-1 and MAT1-2 and incubated under environmental conditions favoring infection and disease development. After 6–8 weeks, the plants were harvested and dried. Tissue of each plant species was placed in nylon net bags, which were incubated outdoors on the soil surface in the fall and collected the following spring. Discharge of ascospores of *D. rabiei* occurred from the tissue of all four species (Table 5). Kovachevski (1936) found that pseudothecia of *D. rabiei* did not form on sterile stems of *M. alba*. It is possible that he inoculated the stem pieces with a single-spore isolate of *D. rabiei* and not compatible isolates (MAT1-1 and MAT1-2), which are required for the teleomorph to form. Additional research is needed to determine the role of the development of the teleomorph of *D. rabiei* on plant species other than chickpea in the spread and survival of the pathogen.

Cultural and Pathogenic Variability

Luthra et al. (1939) were the first to report that isolates of *A. rabiei* from the Indian subcontinent differ in cultural characteristics. Subsequently, researchers in other countries noted that isolates of *A. rabiei* frequently differ in growth rate, sporulation, colony appearance, and morphology (Bedi and Aujla 1969; Grewal 1984; Kaiser 1973, 1992; Nene and Reddy 1987; Porta Puglia 1992; Punithalingam and Holliday 1972). Until recently, most, if not all, observations on cultural variability of isolates of *A. rabiei* were of cul-

tures derived from conidia (anamorph). Large differences in cultural characteristics are apparent in single-ascospore cultures of *D. rabiei* from various countries (W.J. Kaiser, unpublished data).

Pathogenic variability among isolates of *A. rabiei* was first suspected by researchers in India when resistance to ascochyta blight in cultivars such as C-12/34 began to break down in the early 1960s (Nene 1982; Nene and Reddy 1987). In studies with 11 isolates of *A. rabiei*, Bedi and Aujla (1969) found several physiologic races in the state of Punjab in India. Kaiser (1973) observed differences in pathogenicity among isolates of the pathogen from India, Iran, Pakistan, and Turkey. Subsequently, Vir and Grewal (1974) identified two races (1 and 2) and a biotype of race 2 in India. Qureshi and Alam (1984) report finding five pathogenic groups in Pakistan. Using a standard set of differential cultivars, Reddy and Kabbabeh (1985) at ICARDA in Aleppo, identified six races (1 to 6) from isolates collected in Lebanon and Syria, the most virulent of which was race 6.

Gowen et al. (1989) studied the variation in pathogenicity of isolates of *A. rabiei* from 11 countries. Large differences were found in the pathogenicity of the isolates. They attribute the differences in pathogenicity to variations in aggressiveness rather than in virulence. Isolates from the Indian subcontinent and Western Asia were found to be more pathogenic than isolates from the Mediterranean countries.

Jan and Wiese (1991) studied the virulence of 39 isolates of *A. rabiei* collected from blight-infected commercial plantings in the Palouse region of northern Idaho and eastern Washington. They distinguished 11 different virulence forms, three of which accounted for 21 of the 39 isolates. Seven isolates were similar to race 3 identified by Reddy and Kabbabeh (1985). Jan and Wiese (1991) and others (Porta Puglia 1992) found that there does not appear to be a correlation between cultural and morphological characteristics and pathogenicity.

In Spain, Nuñez Cañete et al. (1990, 1992) observed differences in virulence among 168 isolates of *A. rabiei* collected from various regions of Spain and other countries. Most of the Spanish isolates were similar to race 1 described by Reddy and Kabbabeh (1985). Only one isolate was similar to race 4. Among the foreign isolates, some were similar to races 2 and 3, while the majority were similar to race 1.

Little is known about the relationship between the sexual stage in the development of new pathotypes of the blight pathogen and the degree of virulence (greater or lesser than the parent isolates). To study the role of the sexual cycle in pathogenic variability, Trapero-Casas (unpublished data)

crossed a highly virulent isolate of *A. rabiei* from Fuentesauco with an isolate of low virulence from Córdoba. Fifty six single-ascospore cultures were used to inoculate chickpea cultivars which varied in their resistance to ascochyta blight. The results show a constant gradation in virulence among isolates, from high levels similar to the virulent parent from Fuentesauco to levels of virulence lower than that of the Córdoba parent, proving that sexual reproduction provides a mechanism for recombining genes for virulence and avirulence.

Various mechanisms may contribute to the pathogenic variations observed in isolates of *A. rabiei* by researchers in different countries. These are the sexual cycle, mutation, and parasexuality (Agrios 1978). Mutations may account for some of the differences in virulence observed in the pathogen. However, it is unlikely that mutations alone can account for the large differences reported in pathogenic variation in some areas. With sexual reproduction, a recombination of genetic factors, including those governing virulence, occurs during meiosis. The large number of virulent forms (11 among 39 isolates) identified by Jan and Wiese (1991) came from the US Palouse region, where less than 3,500 hectares of chickpea are cultivated. A possible explanation is that the sexual stage, which is widely distributed in this region (Kaiser 1992; Kaiser et al. 1994), is contributing to the high level of pathogenic variation.

The parasexual cycle provides recombination of genetic factors in some fungi in the absence of sexual reproduction. Parasexuality can occur in fungi that have both anamorphic and teleomorphic stages in their life cycle. This may result in the production of recombinants that differ in virulence from the parental strain (Agrios 1978). It is not known if parasexuality occurs in *A. rabiei*. However, it may be operative in some regions, such as India, where pathogenic variation is reported, although the sexual stage has not been found and only one mating type has been identified among the 30 isolates tested (Table 2).

Use of Molecular Techniques to Genetically Characterize *Ascochyta rabiei*

Molecular techniques are used to study the genetic variability of isolates of *A. rabiei* (Weigand et al. 1992; Weising et al. 1991; Simon et al., unpublished data). Random amplified polymorphic DNA (RAPD) is a relatively new technique (Williams et al. 1990) being applied to the area of genetic characterization of isolates and lines of plants and microbes. The method is based on polymerase chain reaction (PCR) using short (10 base) primers of arbitrary sequence. The reaction results in several bands being amplified

from template DNA taken from the test subject. These bands differ for different isolates and can be used to "type" or "fingerprint" the different isolates (Weising et al. 1991; Welsh and McClelland 1990). This technique has been used to characterize isolates of races of *Fusarium oxysporum* f.sp. *pisi* (Linf.) Snyd. & Hans. (Grajal-Martín et al. 1993).

Current research in our laboratory involves the characterization of our collection of isolates of *A. rabiei* using RAPD analysis. DNA has been obtained from numerous isolates and we have begun RAPD characterization with several primers. Clear differences are seen between isolates with some of the primers (C.J. Simon et al., unpublished data). The study will be expanded to include a larger number of isolates (both single-conidium and single-ascospore isolates) and primers to develop a data set that can be analyzed with computer software designed to characterize genetic relatedness and dissimilarity between isolates. From these studies it should be possible to organize the collection of isolates into potential groups based upon degree of genetic similarity.

Additional applications of this technique involve its use to confirm the success of mating experiments using isolates of the pathogen that vary in pathogenicity. An extension of this approach is to investigate the feasibility of identifying a tag for virulence in the pathogen. Little information is available on the genetics of the virulence reaction in *A. rabiei*, but if it is a simply inherited trait, it should be feasible to tag virulence by mating virulent and avirulent isolates to establish a progeny population that segregates for virulence. Using well-established methods for tagging genes in plants, we should then be able to identify the RAPD patterns associated with virulence.

Significance of the Teleomorph in Breeding Blight-resistant Chickpea

In countries where the teleomorph of *A. rabiei* occurs, chickpea breeders should be aware of potential problems that may arise when the sexual stage is a component of the disease cycle.

1. The teleomorphic stage may contribute to the development of new and more virulent pathotypes of the fungus, resulting from genetic recombination. This may have adverse affects on ongoing resistance breeding programs.
2. The sexual stage aids in the survival of the pathogen from one growing season to the next.
3. Sexual reproduction results in long-distance spread of airborne ascospores that may provide the primary inoculum required for development

of disease epidemics, negating or reducing the effectiveness of other crop practices, such as planting clean seed and crop rotation.

4. The accidental introduction of *A. rabiei* infected seed or chickpea germplasm imported for crop improvement may result in the distribution of new virulence genes in an area that could cause serious problems for commercial production and breeding programs.
5. Chickpea breeders should strive to pyramid genes for blight resistance in the development of new chickpea cultivars.
6. In cooperation with plant pathologists, chickpea breeders may be able to utilize new molecular techniques to identify the presence of new pathotypes of the blight pathogen that may adversely affect their resistance breeding programs.

Integrated Control of Ascochyta Blight

Even with an effective breeding program that develops highly resistant chickpea cultivars, integrated control of ascochyta blight is very important. Blight-resistant cultivars are historically overcome by new pathotypes of *A. rabiei* (Nene 1982; Nene and Reddy 1987). Crop rotation, favorable cultural practices, clean seed, and seed-treatment fungicides that reduce the pathogen population will increase the useful life of resistant cultivars.

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Pathogenic Variability of *Ascochyta rabiei* and Ascochyta Blight Resistance in Chickpea

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Abstract

Pathogenic variability of *A. rabiei* has been demonstrated since the 1960s, but there is no consensus as to whether the variability is due to "race" or "aggressiveness" in a single race. The main argument against variability due to race is that there are no significant reversals in the ranking order of cultivars in their reaction to different isolates. This indicates that only one race has the ability to become highly aggressive under favorable environmental conditions.

Little is known about the resistance of the new generation of chickpea cultivars. Such information is a vital guide to resistance breeding and is particularly important when making adjustments to the screening and breeding procedures that ICARDA has adopted. This paper presents evidence that pathogenic variability of *Ascochyta rabiei* (Pass.) Lab. is a factor of both race and aggressiveness and that the two coexist. It also highlights results from resistance screening in the new generation of breeding lines. Resistance shows strong race/pathotype specificity, but there are good indications that race-nonspecific resistance is present. This calls for deliberate efforts to identify the race-nonspecific resistance and/or identify forms that have characteristics associated with durable resistance.

Introduction

Ascochyta blight caused by *Ascochyta rabiei* (Pass) Lab. in chickpea (*Cicer arietinum* L.) has been reported in 31 countries (Nene and Sheila 1992).

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The pathogen attacks all above-ground plant parts and has the potential to cause severe crop damage, particularly where cool temperatures (15–25° C) and frequent rainfall occur during the crop season. Its destructive nature makes it a major limiting factor to chickpea production, and the most important disease of chickpea in the West Asia and North Africa (WANA) region. Repeated epiphytotics have been reported from several important chickpea growing countries. These include Pakistan, which incurred a 42% yield loss in 1981/82, the US, which lost $ 7.42 million in 1982/83 and $ 1 million in 1987, Tunisia, which incurred a 40% loss in 1981, Spain, with 20–100% losses in 1981, and Syria, with losses of 5–30% in 1981, 30% in 1982, and 60–100% in 1992/93 (Nene and Reddy 1987; ICARDA 1993).

The magnitude of yield losses attributed to the disease varies from year to year, but is largely dependent on weather favorable to disease development. In 1993, northeast Syria received an average rainfall of 100–130 mm per month in April and May when the crop was flowering—a highly susceptible stage. A high-yielding blight-resistant cultivar, ILC 482, released in Syria in 1986, was grown widely throughout the country, only to sustained nearly 100% losses in the northeast of the country. A more resistant cultivar, FLIP 82-150C, sustained 60–70% losses. Even though both cultivars were considered moderately resistant to blight when they were released, they were not able to withstand the high disease pressures in 1993. Similar devastation from ascochyta blight has been reported from other countries, with cultivars that were previously resistant to the disease becoming susceptible. New cultivars are therefore being sought.

Winter sowing of chickpea in the WANA region promises nearly 100% increases in yield (Saxena 1992), but also increases the risk of ascochyta blight. The adoption of winter chickpea technology by farmers depends largely on the development of a successful management strategy against ascochyta blight to keep losses below an acceptable economic threshold. Some of the earlier cultivar releases were thought to have adequate levels of ascochyta blight resistance, although requiring improvement in seed size, earliness, and durability of resistance (Saxena 1992). Newer lines for winter sowing, developed through hybridization, have now reached the level of on-farm trials in several countries and will provide a new generation of cultivars. Second generation problems of winter-sown chickpea are being tackled, with durability of ascochyta blight resistance a high priority in the breeding program. Failure of host resistance is often ascribed to the occurrence of new races (Johnson 1982), therefore an understanding of the pathogenic variability of *A. rabiei* is essential to guide resistance breeding. This paper highlights some of the recent studies on ascochyta blight pathogenic variability in relation to resistance breeding.

Pathogenic Variability of *Ascochyta rabiei*

Pathogenic variability of *A. rabiei* has been demonstrated since the 1960s (Aujla 1964; Bedi and Aujla 1969; Kaiser 1973; Grewal 1984; Vir and Grewal 1974; Reddy and Kabbabeh 1985; Nene and Reddy 1987; Porta Puglia et al. 1986; 1987; and Harabbi et al. 1993). While some of these authors characterize the pathogenic groups as races of different cultivars, others note the difference in aggressiveness without significant reversal of the ranking order, suggesting variability in aggressiveness rather than in virulence (Gowen 1987; Haware 1987). The question of whether we have different races or just differences in aggressiveness of a single race, with the ability to become highly aggressive under favorable conditions, was addressed by Malik and Rahman in the 1989 workshop on disease-resistance breeding in chickpea (Singh and Saxena, 1992). Porta Puglia (1992) points out that the limited information available may not allow the use of a precise term such as "race," but, for practical purposes, we may use such a word to distinguish the differences in pathogenicity that we observe among isolates of the fungus.

Jan and Wiese (1991) characterize the variability of *A. rabiei* isolates from Palouse, USA, but abstain from using precise terminology such as "race" for the same reasons pointed out by Porta Puglia. Recommendations to standardize race characterization have been made since 1989 (Sing and Saxena 1992), but to date standard methodology has still not been agreed upon. This situation needs to be resolved to guide resistance breeding. A better understanding of the pathogen is required to guide the development of appropriate strategies for disease management that will enhance durability of ascochyta blight resistance. A better understanding of resistance available in the new generation of cultivars is important to screening and breeding procedures.

Studies on *A. rabiei* using isolates from different locations in North Africa, the Middle East, India, and the USA have all shown that great pathogenic variability exists within *A. rabiei*. Pathogen populations have been shown to differ in their aggressiveness, and the most aggressive pathotypes tend to be associated with areas where selection pressure is highest (Gowen 1986). High adaptability of *A. rabiei* to its host has also been indicated in host passage studies (Gowen 1986), which may partly explain resistance instability. Evidence of pathogenic differences in virulence patterns, with reverse ranking order of the genotype reaction, has been the main reason for rejecting the idea of races in *A. rabiei*. In addition, the high influence of environmental factors on disease expression affects the repeatability of the results. The affect of the environment on foliar diseases is, however, not unusual,

and without the standardization of the methodologies used in variability studies the question of race or no race will not be resolved.

Table 1. The list of genotype that expressed the best resistance of rate 3-4 on a 1-9 rating scale in the greenhouse and field 1992/93.

1	FLIP 90-76	7	S91292‡
2	FLIP 90-77 †	8	S91345‡
3	FLIP 90-109 †	9	S91347‡
4	FLIP 84-93†	10	S914007‡
5	FLIP 84-92‡	11	S91348‡
6	S91241	12	S91377

† Slight change in ranking to one isolate from 4 to 5. All are S in field in 1993/94 (rated 6–9).

‡ Reconfirmed in greenhouse to the six individual and mixed races in 1993/94, but rated susceptible 79 to Hassake field isolates.

Studies conducted in the USA using 18 differential lines provide clear evidence of the reverse ranking of genotype reaction to different isolates of *A. rabiei* (Table 1; Jan and Wiese 1991). Similar evidence from recent studies conducted on the six races of *A. rabiei* previously characterized at ICARDA (Reddy and Kababbeh 1985) clearly shows reverse ranking (Fig. 1; ICARDA 1993). In another recent study, the virulence patterns of 19 single-spore isolates collected from northeast Syria in 1992/93 were compared with the six races on a set of 26 differential lines. Reverse ranking order of genotype reaction was evident (Table 2), and the analysis of variance showed significant genotype × isolate interaction (Table 3). A physiologic race is defined as: "A sub-specific group of parasites characterized by specialization to different cultivars of one host" (Johnson and Booth 1983). According to this definition, *A. rabiei* populations can be characterized as races if the procedures used in the characterization are standardized. The overall aggressiveness of the isolates can be computed from the mean disease score on all the genotypes, which clearly shows differences in aggressiveness (Table 2; Fig. 1). It is therefore clear that differences in aggressiveness of the "races" also exist.

These results provide evidence that *A. rabiei* differ in both aggressiveness and in their specific virulence patterns. The occurrence of a complex pathogenic variability is not surprising, since the pathogen has a sexual stage, is heterothallic (Kaiser 1992), and can generate new genetic recombinants and broad virulence spectrum. The choice of differential lines, however, determines the degree of variability that can be detected in such studies. To be effective, standard international differential lines must separate the pathogenic groups by resistance and susceptibility over a broad geographical area in a clear-cut fashion. While it is important to understand the extent and dynamics of the variability, it is equally important to focus research efforts

on the issues that best meet the objectives of such studies—to guide resistance breeding and identify new virulence in the pathogen that matches the available resistance—before the population becomes economically important.

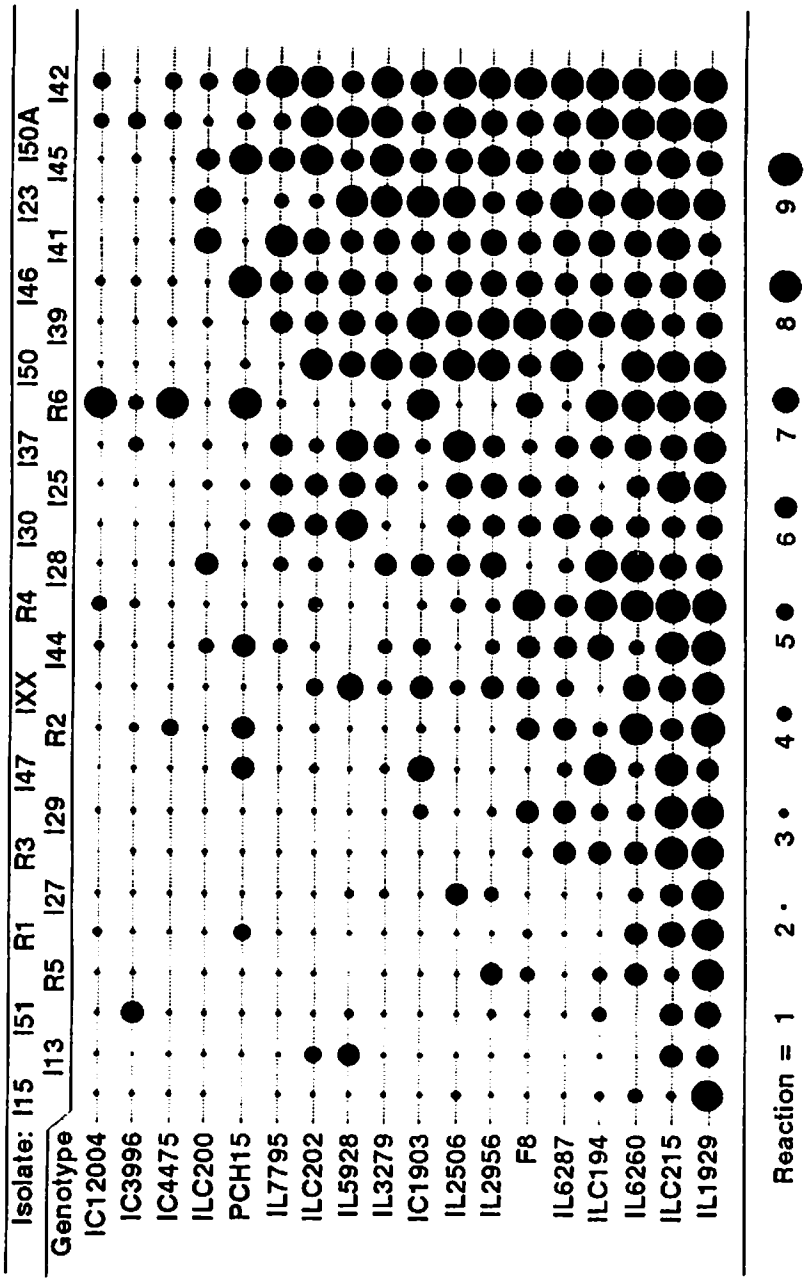

Figure 1.Reaction of 18 differential chickpea lines to different isolates of *Ascochyta rabiei*.

Table 2. Best ascochyta blight resistant lines in the field (1992–1994).

Genotype	1992/93	1993/94	Genotype	1992/93	1993/94
FLIP 82-151°	NT†	4,5‡	FLIP 88-83*	3,3	4,5
FLIP 90-10°	NT	4,4	FLIP 90-27*	4,4	4,5
FLIP 90-79°	NT	4,4	FLIP 92-179	NT	4,4
FLIP 92-194°	NT	4,4	FLIP 91-82	NT	4,5
FLIP 91-131°	NT	4,5	FLIP 91-161	NT	4,5
FLIP 90-112°'	3,4	4,5	FLIP 92-175	"	4,5
FLIP 91-149°	NT	4,5	FLIP 92-178	"	4,5
FLIP 82-32°	NT	4,5			
ICC 2165	NT	3,5	ICC 13629	NT	3,5
ICC 2270	NT	3,4	ICC 2342	"	3,4
ICC 2364	NT	4,3	ICC 3912	"	3,3
ICC 3918	NT	3,3	ICC 3919	"	4,5
ICC 3940	NT	3,3	ICC 3991	"	3,3
ICC 4000	NT	3,4	ICC 4020	"	3,3
ICC 4030	NT	3,3	ICC 4045	"	4,3
ICC 4241	NT	4,5	ICC 4616	"	3,3
ICC 11932	NT	3,3	ICC 11933	"	3,3
ICC 12004	NT	3,2	ICC 13292	"	4,3
ICC 13718	NT	3,2	ICC 13729	"	3,3
ICC 13754	NT	3,3	ICC 14903	"	3,3
ICC 4475	NT	3,5	ICC 6373	"	3,5
ICC 9189	NT	3,5	ICC 4187	"	3,5
ICC 4188	NT	3,5	ICC 76	"	4,3

† NT= not tested, ‡ Scoring in 2 replications on 1-9 scale.

Table 3. Virulence characteristics† of 39 Palouse isolates and races 3 and 6 of *Ascochyta rabiei* on 15 differential chickpea genotypes (Jan and Wiese 1991).

Chickpea	A	B	C	D	E	F	G	H	I	J	K	R3‡	B
ILC 72	W	W	W	H	W	W	W	W	W	W	W	W	W
ILC 194	W	W	W	W	W	W	W	H	W	W	W	W	H
ILC 202	W	W	W	W	W	W	H	W	W	W	W	W	H
ILC 215	W	W	W	W	W	W	W	W	W	W	W	W	H
ILC 249	W	H	H	W	W	W	W	W	W	W	W	W	H
ILC 482	W	W	W	H	H	W	W	W	W	W	W	W	H
ILC 1929	W	H	H	H	H	H	H	H	H	H	H	H	H
ILC 2506	W	H	H	W	W	W	W	W	W	W	W	W	H
ILC 3279	W	W	W	W	W	W	W	W	W	W	W	W	H
ICC 1903	W	H	W	H	W	W	H	W	W	W	H	W	H
ICC 3996	W	W	W	W	W	W	H	W	W	W	W	W	H
ICC 9189	W	W	W	W	W	W	H	W	H	W	W	W	H
F-85-111	W	W	H	W	W	W	W	W	W	W	W	W	H
F-84-84	W	W	W	H	W	H	W	W	W	W	W	W	H
UC-5	H	H	H	H	H	H	H	H	H	H	H	H	H

†Ascochyta blight rated on a modified 1–9 scale according to Reddy and Nene (14). Isolates causing mean disease ratings of 1.0–5.9 and 6.0–9.0 were considered weakly virulent (W) and Highly virulent (H), respectively

‡r3 = International Center for Agricultural Research in the Dry Areas (ICARDA) race 3, r6= ICARDA race 6. Total isolates comprising each virulent form are A, 7; B, &; C, 3; D, 4; E, 1; F, 3; G, 2; H, 7; 1, 2; J, 2; and K, 1.

Whether we call the pathogenic groups "races," "pathotypes," or something else, it is important to be able to compare results from different geographic locations to allow the breeders to keep ahead of the pathogen. This requires at the outset the selection of an appropriate international differential set and the standardization of the controlled conditions (i.e. inoculum level, incubation period, temperature, moisture, and light). Genotypes used as differential lines in Table 2 were selected on the basis of demonstrated broad resistance at multiple locations and use in at least three reported variability studies. It is hoped that these will be further tested at different geographic areas to provide a data base that can be used to select a standard international differential set that will also serve as a source of broad resistance. This system was used in the selection of standard international differential lines for bean rust (*Uromyces appendiculatus*) and the approach has been very practical (Stavely et al. 1983; Mmbaga et al. 1994b).

The questions that remain are whether a weak pathotype can have a severe effect on a genotype that is resistant to an aggressive pathotype, and how to balance the representation of the pathogenic variants in resistance screening. The role of weak pathotypes in generating aggressive pathotypes through accumulation of virulence and genetic recombination is not yet understood and needs to be explored. The use of field isolates in resistance screening representing populations of the pathogen, rather than individual or mixed races, has been suggested (ICARDA 1993; Mmbaga et al. 1994). However, broad resistance that is effective against entire populations is not always available and must be developed through breeding (Singh et al. 1992).

The relatedness of the isolates, on the basis of disease score on all genotypes as well as the specific host parasite interaction, can be determined by a cluster analysis of isolates that show similarity and dissimilarity to the different pathotypes. This analysis has been done using euclidean centrad measuring available in the SPSS computer program (SPSS 1988) and is presented in a dendrogram (Fig. 2). Such results are useful when choosing representative pathotypes that may be used to identify specific resistance groups to be combined through breeding. Similar analysis has been done on the differential lines. Due to the multiplicity of complex and subtle host–pathotype and pathotype–pathotype interactions, the use of other, more detailed, statistical analysis of the data, such as principle component analysis, needs to be explored (Shane 1987).

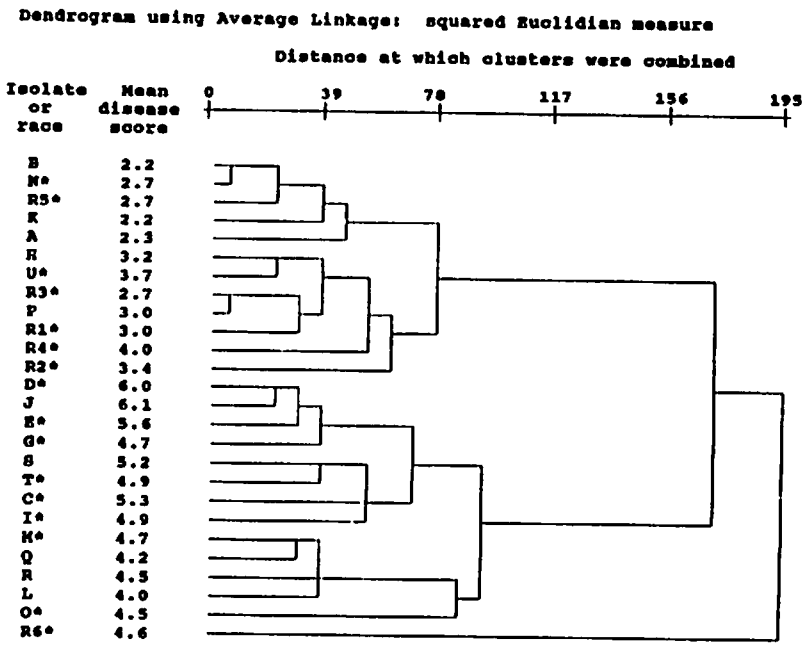

Figure 2. Cluster analysis showing the similarity in virulence of single-spore isolates of *Ascochyta rabiei* on a set of differential cultivars.

Level and Durability of Resistance

Earlier chickpea cultivar releases were thought to have contained adequate levels of ascochyta blight resistance. For example, blight resistance in ILC 482, ILC 3279, and FLIP 82-150C was considered moderate, and it was thought that resistance would be durable over time. ILC 482 has been the most popular and widely grown cultivar because of its high yield potential. However, resistance in ILC 482 is no longer effective in Syria, and resistance in FLIP 82-150C is no longer high enough to withstand high disease pressures, as was evident in the 1993 epidemic in northeast Syria and at ICARDA's disease nursery. Similar devastation from ascochyta blight has been reported in other countries, and varieties that were previously resistant have become susceptible. New varieties are being sought. ILC 3279 has the best resistance of the three cultivars; although resistance is also only moderate, and is not effective against highly aggressive pathotypes, it has always been higher than ILC 482 and FLIP 82-150C.

The durability of ascochyta blight resistance causes concern to breeders and pathologists. The nature of resistance in the new generation of blight-resistant breeding lines needs to be understood. Even though durability can only be tested over time, a focus on the more durable forms of resistance is

required. Partial or moderate resistance cannot be assumed to be more durable than a higher level of resistance unless it is race-nonspecific (Van der Plank 1984; Robinson 1983). On the other hand, it is generally assumed that partial resistance is polygenic and more durable than complete resistance (Parlevliet 1982; Person et al. 1983). Race-specific resistance, or vertical resistance (VR) that is partial can easily be confused with horizontal resistance (HR) that is race-nonspecific, and the two co-exist and are often independent (Ou et al. 1975; Van der Plank 1984). It is therefore important that partial VR is distinguished from HR before the assumption of durability is adopted. Since VR and HR co-exit, care must be taken not to erode components of HR that may be available in the resistant lines. Otherwise, when resistance breaks down, the situation will likely be worse off than before (Robinson 1983; Van der Plank 1984).

If indeed we do not take specific measures to identify and distinguish HR from partial VR in the newer generation of resistant lines, we should focus on obtaining the best resistance under high disease pressures. According to Van der Plank (1984), the abundance, availability, and quality of the resistance influence the success or failure in using VR, and there is little point in using partial VR if genes for complete VR are available. The epidemiological competence of the matching pathotype is not reduced by a weaker resistance, and therefore partial VR does not solve the problem of instability, because such resistance breaks down faster (Robinson 1982; Van der Plank 1984). Our screening procedures have concentrated on looking for a moderate level of resistance in the hope that it will be durable. Evidence in the literature shows that ascochyta blight resistance is simply inherited (Singh et al. 1992), but more recent evidence indicates that the resistance may be mostly polygenic. It is probably time to launch a deliberate effort to understand the type of resistance available and the mechanism of resistance, so that resistance breeding can be adjusted to address the issue of durability.

Johnson (1983), Kranz (1983), and Robinson (1976) address the question of durable resistance. Van der Plank (1984) and Parlevliet (1993) discuss the components of horizontal resistance associated with durability of resistance. There are also examples in the literature of complete VR that are durable (Johnson 1983). Chickpea breeding has adopted a strategy of gene pyramiding to develop resistance that is effective against all pathotypes in the belief that the more resistance (R) genes the pathogen has to match the less likely it is to match them, thus increasing the durability of the resistance (Singh et al. 1384). Evidence that a pathogen can develop multiple virulence that can match many R genes is common. In Australia, using wheat rust, for example, adaptation of *Puccinia graminins tritici* to as many as 15 R genes, the disease has been curbed by wheat breeders' continuous efforts in

R gene management (Van der Plank 1984). While we explore strategies for improving the durability of ascochyta blight resistance, we must also learn from the experience of other pathosystems, which shows that the assumption that many R genes increase resistance stability is not always accurate (Van der Plank 1984).

Moderate resistance (MR) can be of different levels, and disease levels rated 4–6 on a 1–9 scale allow some amount of pathogen population to be maintained in the field. There is no evidence indicating that MR of rate 4 is less stable than rate 6, although the contrary is true for ILC 3279 (rated 4), ILC 482 (rated 5–6), and FLIP 82-150C (rated 5). Even though other factors may have played a role in the better stability of ILC 3279, we must identify the highest level of resistance available alongside the effort to identify moderate resistance. The irony of it all is that complete resistance to ascochyta blight has not been reported, and the best level of resistance available may be just a higher level of moderate resistance.

Resistance Available in the New Generation of Breeding Lines

Evaluation of ascochyta blight resistance to individual and multiple races in 185 elite breeding lines selected for superior resistance in field and greenhouse studies was carried out from 1992 to 1994 at ICARDA. In 1993, it was found that 149 lines (81%) rated 2–4 to the mixture of races in the greenhouse, while only 105 (57%) rated 2–4 in the field. Most of the 1993 results for the six races in the greenhouse were confirmed in 1994. Results of best resistance under greenhouse and field conditions in the 1992/93 season are presented in Table 4. A higher disease pressure existed in the 1993/94 season, and only three of the lines (FLIP 90-112, FLIP 88-83, FLIP 90-27) showed an acceptable level of resistance (rate 3–4.5). More than 35 newer lines that were not tested in 1992/93 were rated 3–5 under the high disease pressure of 1993/94. More than 10 of the lines were kabuli type and at least three (FLIP) were large seeded and high yielding—traits that farmers in WANA like.

The question that must be addressed is why there was such a big difference in genotype reaction in just one year (from 1993 to 1994). Such results show that it is possible for a line to appear resistant in one year and then show susceptibility in the next year. Is it because a new race has evolved in one year, or is it because of the effect of environment on disease development? Study of late blight disease (*Phytophthora infestans*) in potato shows that the resistance breakdown can occur in a short time. In this case, however, the change from resistance to susceptibility was so dramatic that it im-

plies the need for improved screening procedures. The level of disease pressure can be high enough to separate lines with different levels of resistance, but it can also be just high enough to eliminate lines which are highly susceptible and combine all other levels of resistance as one group. What level of disease pressure can be accepted as adequate? Perhaps these results can be used to positively reassess the current screening procedure (Reddy et al. 1984) and guide the adjustment of procedures.

Table 4. Virulence characteristics of 13 isolates of *Ascochyta rabiei* on a set of 16 differential genotypes selected on the basis of broad resistance in multiple locations.

Differential genotypes	Isolate† and mean disease score													
	R1	R2	R3	R4	R5	R6	C	D	E	G	M	N	U	Mean
ILC 72	2	2	2	8	-	2	7	2	5	2	6	2	2	3.7
ILC 200	2	2	1	1	2	2	7	3	6	2	2	2	3	2.7
ILC 215	6	6	7	9	3	7	8	8	7	6	7	3	7	6.4
ILC 482	-	2	2	8	2	8	7	8	7	7	7	3	6	6.0
ILC 1929	8	8	8	8	8	8	8	8	6	7	7	8	8	7.4
ILC 2956	2	2	2	3	6	2	6	8	7	7	6	4	3	4.6
ILC 3856	7	2	2	2	-	2	7	8	7	6	6	3	2	4.1
ILC 5894	3	-	1	1	-	3	7	3	5	6	6	3	5	3.9
ILC 6260	5	7	6	5	6	8	7	8	6	7	5	3	3	5.6
ILC 7374	2	2	1	-	2	2	2	7	3	3	4	2	2	3.0
ICC 3996	2	2	2	2	2	4	2	2	2	2	2	2	2	2.3
ICC 4475	2	5	2	2	1	7	2	3	2	2	2	2	2	2.4
ICC 9189	2	-	1	3	2	3	2	4	1	2	2	2	2	2.0
ICC 12004	2	2	1	3	2	7	2	4	2	2	2	2	2	2.4
F8	2	6	3	8	3	7	7	7	7	7	5	2	5	4.4
PCH 15	5	6	2	2	2	7	2	7	7	8	2	2	4	3.2
Mean	3.0	3.4	2.7	4.0	2.7	4.6	5.3	6.0	5.6	4.7	4.7	2.7	3.7	4.08

† Isolates C-U are representative of 20 isolates from northeast Syria collected in 1992/93 from an epidemic. The selection of isolates C-U was based on the relatedness of the isolates shown in a dendrogram in Fig. 2.

Selection of 16 differential lines is from 26 lines, based on selectedness of the lines in their reaction to 26 isolates of *A. rabiei*.

The high disease pressure in 1994 was created by providing inoculum (infested debris) in January, one month earlier than the usual. The decision to expose the test plants to the pathogen one month early was based on the prevailing weather conditions, which were unseasonably warm (10–15° C), very favorable for infection establishment. Normally, December and January have only a few scattered days when the temperatures warm enough (above 6° C) to allow the establishment of *A. rabiei* (Weltzien and Kaack 1984). Temperatures that are favorable for disease spread (15–25°C) usually start in March. Rainfall declines in March and April. Resistance identified in

March remained fairly stable when inoculum in the form of a spore suspension was provided at the flowering/early pod stage, when the weather is wet and the crop is highly susceptible. The section of the nursery that was inoculated in early February (the normal time) developed less disease, although some of the resistance identified in April did not remain stable at the flowering/early pod stage.

Table 5. Analysis of variance of disease score for 26 genotypes of chickpea, each infected with one of 26 isolates of *Ascochyta rabiei*.

Source of variation	Degrees of freedom	Mean square	Variance ratio	Probability
Main effects:				
Genotypes of chickpea	25	140.1	179.46	<0.001
Isolates of ascochyta	26	108.8	139.40	<0.001
Interaction:				
Genotype × Isolate	635	6.732	8.62	<0.001
Residual	1274	0.781		

Conclusion

- There is enough evidence to show that pathogenic variability of *A. rabiei* can be expressed in the form of differential reactions on a set of differential cultivars, i.e. "race," provided that procedures for race characterization are standardized.
- Pathotypes of *A. rabiei* differ in aggressiveness, and these two forms of variability co-exist.
- Pathogenic variability should be monitored to provide information for resistance breeding.
- Resistance screening should use pathogen samples that represent the entire virulence spectrum found in farmers' fields.
- Resistance to *A. rabiei* in the new generation of genotypes needs to be evaluated, and R mechanisms used to guide resistance breeding.
- Deliberate efforts should be made to identify race-nonspecific resistance and distinguish it from partial VR.
- Identification of the highest forms of resistance should be done concurrently and separately from identification of HR. The disease pressure used in resistance screening should be assessed.
- There is evidence in the literature that R genes may be associated with temperature-sensitive promoters. The relation between resistance and temperature should be studied, especially in terms of winter planting.

- The durability of any host resistance can only be tested over time. Making the right choices improves the probability of achieving the objective, and improving the life span of resistance.

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Pathotyping of *Ascochyta rabiei* Isolates of Syria

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Abstract

Isolates of the ascochyta blight fungus, *Ascochyta rabiei* (Pass) Lab., from different chickpea growing areas of Syria, were tested for pathogenic variability using three differential cultivars with different levels of resistance. A pathogenicity assay was conducted in a growth chamber. The analysis revealed the occurrence of three distinct pathotypes that differ significantly in their aggressiveness. Pathotype III, the most aggressive, has predominated in chickpea growing areas in recent years. This assay has proved to be highly reliable, with reproducible results.

Introduction

Ascochyta blight of the chickpea is caused by the fungi *Ascochyta rabiei* (Pass.) Lab., and is widespread throughout the chickpea growing regions of the world. The disease causes severe crop losses in Mediterranean countries, especially when sown in winter (Porta Puglia 1992). Use of resistant cultivars is a principle method for control of the disease. In former years, varieties were developed that were resistant to pathotypes collected in 1982 (Singh and Reddy 1993). These varieties were released for winter sowing. However, they became susceptible after only a few years. Thus, a detailed analysis of the aggressiveness (pathogenicity) of the prevailing pathogen in the chickpea growing regions is important for the following reasons: (i) to understand the aggressiveness dynamic of the pathogen; (ii) to devise an effective disease-control strategy to reduce the impact of fungal disease; and (iii) to breed cultivars with a high level of durable resistance to the predominant pathotype of the pathogen.

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The methods used by researchers to study the pathogenic variability of *A. rabiei* differ from one another with respect to host line, inoculum concentration, inoculation technique, rating scale, environmental conditions, and age of the plant. The aggressiveness of *A. rabiei* and disease reaction to differential hosts is influenced by host age, inoculum concentration and variability, physiological status of the host and pathogen, temperature, relative humidity, and method of inoculation. Consequently, many inconsistencies have been observed in both green house and field tests. For example, Reddy and Kabbabeh (1985) classified six isolates collected in Syria and Lebanon in order of aggressiveness as 6> 5> 4> 3> 2> 1 under plastic house conditions. The same isolates were grouped by Weising et al. (1991) as 6> 4> 2> 1> 5> 3 in a controlled environment (Conviron growth-chamber). Standardized and more precise methods for screening ascochyta blight resistance are needed. Precise screening is absolutely necessary for tagging resistance genes with DNA markers and for disease-resistance breeding. Hence, attention must be paid to the development of an efficient screening method.

The objective of this study was to re-examine the pathogenic variability of the *Ascochyta rabiei* population in Syria in recent years. The method used by Weising et al. (1991) were refined and used in this study. The isolates from the chickpea growing regions of Syria were sampled in 1982, 1991, 1992, and 1993 and characterized for aggressiveness on three differential hosts under growth chamber conditions and grouped into three pathotypes.

Materials and Methods

Plant material

Seed of differential chickpea cultivars ILC 1929 (susceptible), ILC 482 (moderately resistant), and ILC 3279 (resistant) were obtained from the Germplasm Resources Unit of ICARDA in Aleppo. Single plant progenies of these cultivars were bred for more than three generations to attain genetically homogeneous populations (pure lines). The genetic homogeneity of the population was tested using oligonucleotide DNA fingerprinting.

Fungal material

The 53 isolates used in this study were gathered in 1982, 1991, 1992, and 1993 from different chickpea growing regions in Syria and Lebanon. Table 1 shows the year, place, and number of isolates collected each year. The stem lesions were collected from individual diseased plants and temporarily preserved in silica gel. They were cut into 1 cm lengths, with a small healthy portion on either end, washed in sterile distilled water, and surface steril-

ized in chlorox (10%) for 10 minutes. After being rewashing in sterile distilled water, the lesions were placed in 70% alcohol for 30 seconds, washed three more times in sterile distilled water, and then placed on water agar (20%) plates. After 2–4 days a small section of growing mycelia from a single lesion was transferred onto chickpea seed meal agar plates (CSMA: 4 g of ground chickpea seeds, 3 g dextrose, 1.8 g agar, and 100 ml of sterile distilled water). Single-spore isolates were subsequently obtained from each single lesion as described below.

Stock cultures of the fungi were immersed in sterile distilled water to release spores from the pycnidia. The resulting spore suspension was diluted to a concentration of 3.5×10^5 spores/ml, dispersed on water agar, and incubated at 20° C to induce germination. After 24 hours, a 20 × 20 mm agar block was transferred onto a microscopic slide and cut into smaller squares (1.5 × 1.5 mm) using a multi-blade razor knife. Squares with single germinating spores were microscopically identified and transferred to fresh CSMA medium for culture. The single-spore isolates are referred to hereafter as a numbered version of the original lesion culture.

All the cultures were maintained on CSMA, incubated at 18° C, and illuminated 14 hours per day.

Isolate pathotyping

Pathotypes were determined by a pathogenicity assay using the three cultivars of differential resistance. Good seed of uniform size was hand-picked and germinated in a germination box (watered at regular intervals) for 36 hours at room temperature. The seed with uniform germination was selected, and transferred to Jiffy-7 pots. Seedlings were grown in a growth chamber (Conviron). Temperature and light settings for the experiment are presented in Table 1. After seven days, four seedlings of each cultivar were randomly arranged in a germination box with a transparent cover and a trough at the bottom. Wicks were place in the trough to supply water through capillary movement.

The spore suspension was prepared using a 14-day old culture grown in a liquid medium (CSMA) lacking agar, at the temperature and light described above. The resulting spore suspension was adjusted to 1.6×10^5/ml water and sprayed. Each box (with four plants of each of the three cultivars) was considered a replication and each isolate was sprayed on two boxes. Two hundred ml of spore suspension was used for each treatment. After spraying, the plants were covered with transparent plastic to maintain leaf wetness during incubation. Light intensity was reduced (Table 1), temperature was set at 18° C, and incubated for 40 hours. Plastic covers were then re-

moved and temperature and light were returned to normal. Readings for disease severity for each plant were taken from the third to the 14th day after inoculation using the following scale:

1= no symptoms
2= small round tissue depression or spot
3= elongated spot
4= coalescent spot
5= stem girdling
6= stem breaking
7= lesion growth downward from breaking point
8= whole plant nearly dead
9= plant dead

Table 1. Temperature and light of the growth chamber[†].

Time	Temperature (°C)	Light setting
	Before and after inoculation	
4	16	11
6	16	22
7	18	33
8	20	44
10	22	44
16	22	33
17	22	22
18	21	11
19	20	11
20	20	00
21	18	00
22	16	00
	During inoculation	
4	18	10
20	18	00

[†]The growth chamber used was Conviron, model No. E15, manufactured by Control Environment Ltd., Winnipeg, Manitoba, Canada.

Statistical analysis

Since the experiment was conducted in a controlled environment, the season of planting was not taken into consideration. The mean value of disease severity on the 14th day after inoculation for each replication was used in the statistical analysis. Since the data sets were very small (i.e., between 1 and 9) square-root transformations to determine mean values and analysis of variance were carried out according to Gomez and Gomez (1984), using the isolates and the host genotypes as factors. To establish the precision of the overall experiment, the coefficient of variation (CV%) was calculated for

each experiment and only those experiments with less than 6% CV were considered for statistical analysis. For treatment mean comparison, the standard t-test was performed at $p > 0.01$. Minimum threshold limits of disease severity (transformed value) were fixed for each experiment, and those experiments within the threshold were further analyzed. The mean disease severity values for each isolate on each of the three differential sets were compared for classification of pathotypes.

The area under disease development curve for each isolate on each test cultivar was calculated and compared among the isolates using one-way variance analysis. Since the analysis showed non-significant results, it was not used (data not shown).

Diversity index

Diversity was calculated according to the following equation (Nei 1987):

$$H = [n/(n-1)] \cdot \left[1 - \sum_{i=1} x_i^2\right]$$

where x is the frequency of the i^{th} pathotype in the population, and n is the number of isolates examined.

Extraction of DNA

DNA was extracted from lyophilized fungal mycelia according to Weising et al. (1991), with the difference that the ultracentrifugation step was omitted. DNA was quantified by absorbency at 260 nm.

RAPD analysis

The primer used in the study, OPI-10, was obtained from Operon Technologies (Alameda, California). DNA amplifications were performed in 25 μl containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.001% gelatin), 100 μM each of dATP, dTTP, dGTP, and dCTP, 5 picomoles of a single 10-base primer, 200 ng of genomic DNA, 1 unit of Taq DNA polymerase (Boehringer Mannheim), and topped with a drop of mineral oil. Amplifications were performed in a thermocycler (Perkin Elmer, 9600) programmed for 2 minutes at 94° C; 40 cycles of 1 minute each at 92° C, 1 minute at 36° C, and 2 minutes at 72° C; and a final extension of 5 minutes at 72° C. The reaction mix without template DNA served as a control and was electrophoresed along with the samples. Amplified DNA products were loaded onto 1.2% agarose gels and electrophoresed in 1× TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Gels were stained with ethidium bromide and photographed under UV light.

Results

The disease was caused by all isolates on the cultivars tested. Disease symptoms typically first appeared three days after inoculation on stems as small, pale yellow, water-soaked, restricted lesions, turning into large lesions that often coalesced to girdle the stem. Further progress of the disease resulted in stem breakage and death of the plant. The difference in disease development between resistant and susceptible cultivars was very noticeable 14 days after inoculation. The susceptible cultivars were killed while resistant cultivars started growing new shoots. Thus, the day 14 readings were used in statistical analysis.

Table 2. Disease reaction of *Ascochyta rabiei* on three differential chickpea cultivars and pathotype classification.

Pathotype/Cultivar	Pathotype I	Pathotype II	Pathotype III
ILC 1929	S	S	S
ILC 482	R	S	S
ILC 3279	R	R	S

S = Susceptible reaction
R = Resistance reaction

On the basis of day 14 readings (Table 2), the isolates were classified into three pathotypes, namely, pathotype I (less aggressive), pathotype II (aggressive) and pathotype III (more aggressive). Typical disease development curves for these pathotypes are shown in Figure 1. The average disease severity reading on ILC 1929 for pathotype I isolates exceeded 6 and was significantly higher than the ILC 482 and ILC 3279 readings. An isolate was designated as pathotype II if disease reaction on ILC 1929 and 482 exceeded 6 and was significantly higher than on ILC 3279. An isolates was classified as pathotype III if average disease severity exceeded 6 on all the differentials, and if the differentials were statistically on a par with each other.

The six isolates collected during 1982 were grouped into two pathotypes, I and II. None of them belonged to pathotype III. In 1991, 1992, and 1993, pathotype III was present along with pathotypes I and II (Table 3). In 1991, out of six isolates, four belonged to pathotype III and two belonged to pathotype II. In 1992 only two pathotypes (I and III) were found, and in 1993 all three pathotypes were found. Out of 26 isolates collected in 1992, 20 were pathotype III and six were pathotype I. Out of 15 isolates in 1993, 11 were pathotype III, two were pathotype II and two were pathotype I. Pathotype III was found in all chickpea growing regions, but was most prevalent in northeast Syria.

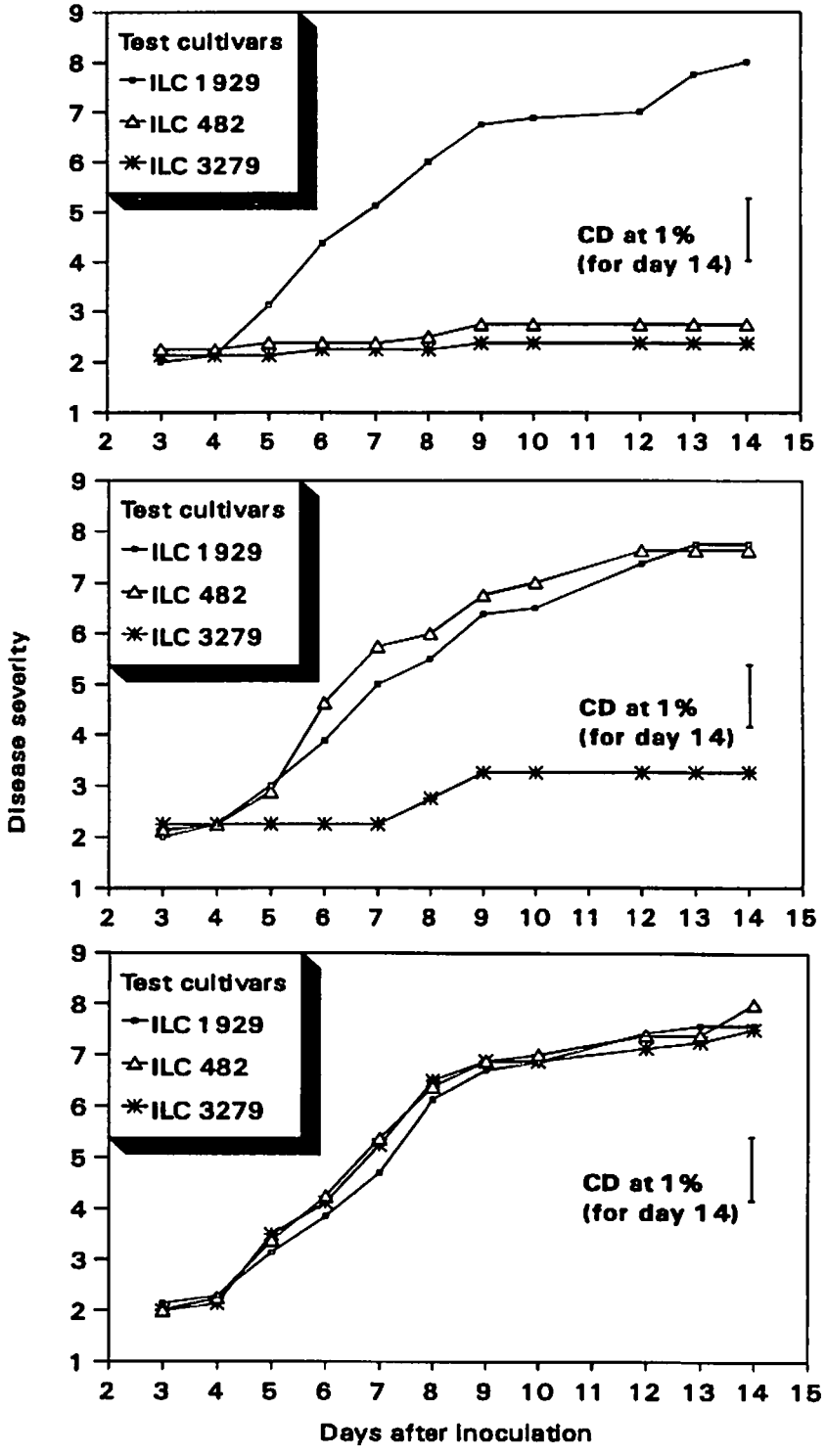

Figure 1. Typical disease development curves for the three pathotypes of *Ascochyta rabiei*.

Table 3. Pathotyping of *Ascochyta rabiei* isolates in Syria.

Year of collection/location	Total No. of isolates collected	No. of isolates			Diversity index
		Pathotype I	Pathotype II	Pathotype III	
1982 Syria/Lebanon	*6*	*4*	*2*	*0*	*0.44*
1991	*6*	*1*	*1*	*4*	*0.50*
Ghab	1	0	0	1	0.00
Izraa	2	0	1	1	0.50
Jinderess	1	0	0	1	0.00
Tel Hadya	2	1	0	1	0.50
1992	*26*	*6*	*0*	*20*	*0.36*
Kamishly	4	2	0	2	0.50
Jinderess	3	0	0	3	0.00
Rouge	3	3	0	0	0.00
Malkieh	15	0	0	15	0.00
Terbol	1	1	0	0	0.00
1993	*15*	*2*	*2*	*11*	*0.43*
Kamishly	4	2	0	2	0.50
Rouge	2	0	0	2	0.00
Malkieh	9	0	2	7	0.35
Over all	53	13	5	35	0.49

The diversity index for pathotypes (Nei 1987; Table 3) shows a decrease in 1992 (0.36) and 1993 (0.43) compared with 1982 (0.44). The index was slightly higher in 1991 (0.50).

To confirm the identity of the pathogen as *A. rabiei*, two approaches were used. The first was proving Kochs' postulates (Agrios 1988). The pathogen was re-isolated from disease symptoms at the first stage of screening. The isolated fungus was re-inoculated on the differential sets. All the re-isolated isolates caused disease at an intensity similar to the first screening. Further repetition of the experiment with both original and re-isolated isolates gave similar results. The second approach was through the use of DNA markers. A PCR-based DNA marker—Random Amplified Polymorphic DNA (RAPD)—analysis was performed using an arbitrary primer (10 bases long) that amplifies a monomorphic DNA fragment pattern among *A. rabiei* isolates. All the isolates were tested and revealed the monomorphic pattern, which is distinct from that of the closely related species *Ascochyta fabae* f.sp. *lentis* (Bond & Vassil.) Grossen et al. (Fig. 2), confirming the identity as well as the purity of the isolates at the DNA level.

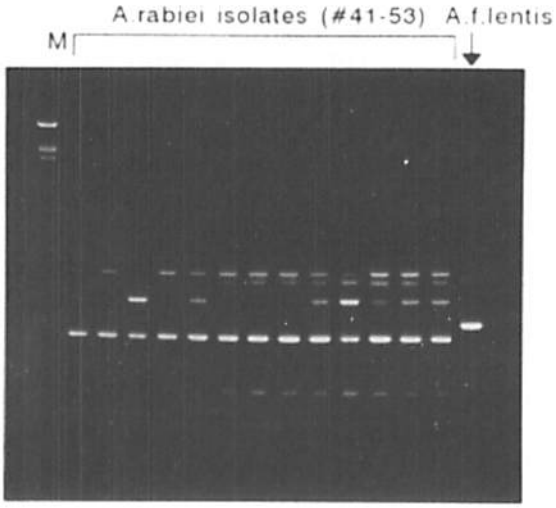

Figure 2. RAPD analysis of *A. rabiei* isolates and *A. fabae* f.sp. *lentis* using the primer OPI-01. Lane M contains molecular weight markers, lambda *Eco*RI-*Hin*dIII digested DNA. This marker reveals the monomorphic pattern among the isolates of *A. rabiei* and distinguishes *A. rabiei* from a closely related species, *Ascochyta fabae* f.sp. *lentis* (Bond & Vassil) Gossen et al.

Discussion

The experimental setup designed for disease severity screening in the present study had a coefficient of variation (CV) of less than 6%. Similar results were obtained after repeating certain sets of treatment. Therefore, the experimental setup designed for disease severity screening is more precise, more reliable, and reproducible.

Based on the results of this study the 53 isolates were classified as pathotype, I, II, or III. This includes the isolates that were classified as six "races" by Reddy and Kabbabeh (1985), which were found to fit into only two pathotypic groups, I and II. The reason for the non-existence of pathotype III in the 1982 collection could be that the pathotype was less prevalent or not collected.

The results show that the population of *A. rabiei* in Syria is highly heterogeneous. This is indicated by the overall diversity index (0.49). Twenty five percent of the isolates are pathotype I, 9.6% are pathotype II, and 65% are pathotype III.

Ascochyta blight epidemics are more common in winter-sown chickpea crops (a relatively recent practice) than in spring-sown (Hawtin and Singh

1984; Porta Puglia 1992). To increase yield in winter-sown crops, and to protect against blight disease, considerable effort has been directed at controlling the disease through the development of resistant cultivars (Nene and Reddy 1987; Singh and Reddy 1991). But this resistance has broken down over the years. At present the most resistant line (ILC 3279) is susceptible to the most predominant pathotype (III) in Syria. From the present study it is evident that pathotype III isolates occur at a very high frequency and can kill the most resistant line, or, given a favorable environment (high rainfall), cause an epidemic. To overcome this problem, breeding of cultivars resistant to pathotype III is an effective strategy.

This study on the population structure of the ascochyta blight pathogen has tempted us to initiate similar projects in other West Asian and North African countries. Future efforts will be directed towards compiling an international reference base of this fungus pathotype, geographical distribution, and genotype. This international atlas of *A. rabiei* will help in selection of resistant germplasm sources, and in the effort to pyramid resistance genes, thus breeding varieties specific to the chickpea growing regions of the world.

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Protein and Isozyme Analysis of *Ascochyta* Species of Food Legumes Using the Isoelectric Focusing Method

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Abstract

Soluble protein and isozyme profiles of single-spore isolates of *Ascochyta* spp. were analyzed using IEF. Species studied included *Ascochyta pisi* Lib., *Ascochyta fabae* Speg., *Ascochyta fabae* f.sp. *lentis* (Bond & Vassil.) Gossen et al., *Ascochyta viciae* Lib., *Ascochyta rabiei* (Pass.) Lab., and *Mycosphaerella pinodes*, which are causal agents of ascochyta blight on grain legumes. Two *Phoma medicaginis* (*P. medicaginis* var. *pinodella* [Jones] Boerema and *P. medicaginis* Malbr. & Roum var. *medicaginis*) were also included. Five enzymes (acid and alkaline phosphatases, malate and shikimic dehydrogenases, and esterases) were studied, and soluble proteins were silver stained. Different *Ascochyta* spp. were distinguished for esterases, with soluble proteins focused between pH 2.5 and 6.5. Intraspecific polymorphism was observed with *A. rabiei*. Patterns for *A. rabiei* were clearly separated from the other *Ascochyta* spp. tested for SKD, with soluble proteins focused between pH 3 and 10. The profiles of all *Ascochyta* spp. tested were similar for MDH, but different from those of *M. pinodes*, *P. medicaginis* var. *pinodella*, and *P. medicaginis* var. *medicaginis*. Isolates of *M. pinodes* and *Phoma* spp. could then be easily differentiated from *Ascochyta* spp. on the basis of soluble proteins and isozyme patterns. Although this study did not involve a wide range of isolates for each *Ascochyta* and *Phoma* sp. tested, the preliminary results suggest the great potential for soluble protein and isozyme analyses to identify *Ascochyta* spp. or to improve taxonomic knowledge of the genus.

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Introduction

Leguminous plants are widely distributed throughout the Mediterranean and include a large number of wild and cultivated species. These crops are important economically because of their nutritional value for human consumption and as animal feed. Their production is limited, however, due to a large number of plant pathogenic fungi responsible for the disease named ascochyta blight. These fungi are seed-transmitted, seed-borne pathogens that infect all the aerial parts of the plant (Richardson 1979).

The most damaging fungal species are: *A. pisi*, *A. pinodes*, and *P. medicaginis* var. *pinodella* on pea; *A. rabiei* on chickpea; *A. fabae* on small and large faba bean; *A. lentis* on lentil; *Phoma medicaginis* var. *medicaginis* on lucerne; and *A. viciae* on vetch. The traditional criteria used to distinguish these species are based on plant host, symptoms, colony aspects, morphological characteristics of conidia, and teleomorph form. *Ascochyta* spp. symptoms are typically circular and grayish brown lesions with a dark brown margin and often concentric picnidia. *Phoma* spp. cause foot rot and occasional spots on aerial parts of the plant. The conidia of *Ascochyta* spp. are hyaline, bicellular, and large (11.5–14.5 mm × 3.5–4.5 mm), while for *Phoma* spp. they are hyaline, mostly unicellular, and small (6–11.5 mm × 2–4 mm). (Sutton 1980).

In practice, species identification is often difficult, due to similar colony morphology. Moreover, spore size is not usually an appropriate character by which to differentiate species. Host specificity is not very clear. For instance, Sprague (1929) and Leach (1962) state that *A. pisi* has been isolated from vetch, while Boerema et al. (1965), Sutton (1980), and Bouznad (1989) note that *P. medicaginis* var. *pinodella* has a broad host range on food legumes. The taxonomy of these fungi has always been confusing: *P. medicaginis* var. *pinodella* (Jones) Boerema and *P. medicaginis* Malbr. & Roum. var. *medicaginis* were in the past named *A. pinodella* Jones and *A. imperfecta* Peck. In the same way, some authors consider *A. lentis* to be a special form of *A. fabae*—*A. fabae* f.sp. *lentis* (Gossen et al. 1986)—the teleomorph stage of which has been described as *Didymella* sp. (Kaiser & Hellier 1993). In addition to the limited number of traditional criteria, some authors have suggested that data on conidiogenesis and formation of crystals *in vitro* would be helpful in *Ascochyta* and *Phoma* spp. taxonomy, but these criteria have already been discussed (Boerema and Bollen 1975; Boerema 1976; Punithalingam 1979; Buchanan 1987; Bouznad 1989; Noordeloos et al. 1993; Kaiser et al. 1994).

Variations in soluble protein patterns and isozyme polymorphisms detected by electrophoresis have been widely used in genetic and taxonomic studies

of fungi. Isoelectric focusing, which provides an excellent resolution of patterns (Adaskaveg et al. 1988; Koch and Köhler 1991; Roux and Labarère 1991; Balesdent et al. 1992; Micales et al. 1992), was performed in this study.

The purpose of this study was to compare several *Ascochyta* and *Phoma* spp. using a biochemical approach (isozyme and protein electrophoresis) in an attempt to examine inter- or intra-specific variations and to correlate the results with classical morphological studies.

Materials and Methods

Fungal isolates

The origins of the isolates and species used in this study are shown in Table 1. Most were collected during surveys in food legume regions in Algeria. Some of them came from the INRA collection of Versailles. All isolates were initially purified by single-spore isolation. Subsequent maintenance was by hyphal subculture on Mathur's medium (1.5 g peptone, 0.5 g yeast extract, 2.8 g glucose, 1.23 g $MgSO_4 \cdot 7H_2O$, 2.72 g KH_2PO_4, 20 g agar, 1 l H_2O) at 4°C.

Mycelium production

Fungal isolates were grown in a 500 ml Erlenmeyer flask containing 150 ml liquid culture of potato dextrose broth or chickpea medium (40 g chickpea seed meal/l H_2O). Each Erlenmeyer was inoculated with two plugs of picnidia (5 mm diam.) taken from a 10-day old solid-plate culture on Mathur's medium. The cultures were incubated on a rotary shaker (100 rpm) at 20° C for 5 days with a 12 hour light period. The mycelium was harvested into a Büchner funnel by vacuum filtration, washed in de-ionized sterile water, dried with filter paper, freeze-dried, and stored at -20° C until use.

Soluble protein extraction

Freeze-dried mycelium of each fungus (50 mg) was ground to a fine powder with liquid nitrogen using a mortar and pestle. An extraction buffer (1.5 ml of 0.01 M Tris-HCl, pH 7, 0.001 M EDTA) was added, and the tubes were placed on ice for 1 hour. The extraction mixture was then centrifuged at 25,000 g for 30 minutes at 4° C. The supernatant was collected and the protein content of the samples was estimated according to the Bradford method using bovine serum albumine (BSA) as a standard (Bradford 1976). The supernatants were freeze-dried in 100 ml aliquots in microcentrifuge tubes, and stored at -20° C until required.

Table 1. Isolates of *Ascochyta* and *Phoma* species used in soluble proteins and isozyme analysis.

Fungus	Study number	Collection number	Host	Origin	Source
A. pisi	38 and Pi†	Pi 10 - Tézier	Pea	Drôme, France	C. Allard(1)
	39	Pi - 91.01	"	Barrahal, Algeria	Z. Bouznad(2)
	40	Pi - L/G	"	Lot-et-Garonne, France	C. Allard
	41	Pi - 91.02	"	Khemisset, Morocco	Z. Bouznad
A. fabae	34	F91.02	Faba bean	A. Temouchent, Algeria	Z. Bouznad
	35	F91.01	"	Collo, Algeria	"
	36	F92.04	"	S.B. Abbes, Algeria	"
	37	F92.03	"	Lamtar, Algeria	"
	Fa†	89.35.5	"	Ille-et-Vilaine, France	B. Tivoli (3)
A. lentis	30	L91.02	Lentil	Zakaria (Tiaret), Algeria	Z. Bouznad
	31	L91.01	"	Saïda, Algeria	"
	32	L91.06	"	Marchouch, Morroco	"
	33	L91.03	"	Tiaret (FSA), Algeria	"
A. viciae	20	V92.01	Vetch (*V. sativa*)	Collo, Algeria	Z. Bouznad
	21	V92.01.1	"	Collo, Algeria	"
	22	V92.01.2	"	Collo, Algeria	"
	23	V92.02	"	Saïda, Algeria	"
	25	V92.03	"	Tiaret (FSA), Algeria	"
	28	V92.01 (Fp1)	"	Collo, Algeria	"
	29	V92.01 (Fp2)	"	Collo, Algeria	"
	26	V92.04	Vetch (*V. monantha*)	Zidane (SBA), Algeria	"
Ascochyta sp.	18† and 27	V92.05	Vetch (*V. monoltha*)	S.B. Abbes, Algeria	Z. Bouznad
A. rabiei	6†	R90.08	Chickpea	Khemis Miliana, Algeria	Z. Bouznad
	7† and 43	R91.01	"	S.B. Abbes, Algeria	"
	7'† and 44	R92.02	"	"	"
	8†	R92.03	"	"	"
	9†	R92.04	"	Tessala (S.B.A.), Algeria	"
	10† and 45	R92.05	"	"	"
	11†	R92.07	"	S.B. Abbes, Algeria	"
	12†	R92.10	"	"	"
	13†	R92.11(1)	"	Sidi Dahou, A. Temouchent	"
	14†	R92.11(2)	"	"	"
	15†	R92.13	"	Khemis Miliana, Algeria	"
	16† and 46	R92.15	"	Guelma, Algeria	"
	17†	R92.16	"	"	"
	42	R90.02	"	Béja, Tunisia	"
	47 and ARC1†	ARC1	"	Clermont-Ferrand, France	D. Tourvieille(4)
	48 and R6†	R6	"	Syria	ICARDA(5)
	49	AR13	"	North Dakota, USA	W.J. Kaiser(6)

Continued next page

Fungus	Study number	Collection number	Host	Origin	Source
M. pinodes	Ps†	Pes 3 - Riou	Pea	Nord, France	C. Allard
P. medicaginis var. *pinodella*	Pa†	Pa 2	Pea	Champagne, France	C. Allard
P. medicaginis var. *medicaginis*	50	PhL	Red clover	O. Smar, Algeria	Z. Bouznad

Isolates were generally cultivated on potato-dextrose broth.

† Isolates cultivated on chickpea meal.

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Electrophoresis

Isoelectric focusing (IEF) was carried out on horizontal slab gels (0.5 mm thick), using a LKB 2217 Multiphor II electrophoresis unit. Ampholines (6% final concentration) were included in 5% T, 3% C polyacrylamide gel to obtain two pH gradients. These pH intervals, 3–10 and 2.5–6.5 (1:1 2.5–5 and 4–6.5 mixture), were prepared with Servalyt (Serva) and Pharmalyte (Pharmacia), respectively. Electrode wicks were saturated with anode fluid 3 (3.3 g L-aspartic acid and 3.7 g L-glutamic acid per liter of distilled water, Serva), and cathode fluid 10 (4 g L-arginine, 4 g L-lysine and 120 ml ethylene diamine per liter of distilled water, Serva) for the 3–10 range, and with 0.1 M H_2SO_4 as anolyte and 0.1 M b-alanine as catholyte for the 2.5–6.5 range. Gels were first prefocused to 250 Vhr; 6 ml of sample was subsequently loaded onto prefocused gels with a plastic applicator strip that was about 3 cm from the anode of the gel, and then focused to 3,000 Vhr. For each gel, permanently cooled to 4° C, current was limited to 25 mA, voltage to 2,000 V and power to 6 and 12 W for prefocusing and focusing, respectively.

Samples were prepared by re-suspending freeze-dried supernatants in deionized water to obtain 2 and 6 mg protein/ml (for soluble protein staining and enzyme activities, respectively) and then centrifuged at 25,000 g for 15 minutes at 4° C to eliminate debris.

Soluble proteins and isozymes staining

After electrophoresis, gels were stained to reveal the total soluble proteins and five enzymes, including acid and alkaline phosphatases, esterases, malate, and shikimic dehydrogenases. Silver staining to reveal total soluble proteins was carried out according to a modified method for the PhastGel silver

kit (instruction manual, Pharmacia). The enzyme abbreviations, enzyme commission (EC) numbers, and stain references are given in Table 2.

Table 2. List of enzymes, abbreviation, enzyme commission number, and stain references used to study *Ascochyta* and *Phoma* species.

Enzyme system	EC number	Abbreviation	Stain reference
Acid phosphatase	3.1.3.2	ACP	Roux & Roux, 1981
Alkaline phosphatase	3.1.3.1	ALP	Brewer, 1970
Esterases	3.1.1.-	EST	Soltis et al., 1983
Malate dehydrogenase	1.1.1.37	MDH	Stenlid, 1985
Shikimic dehydrogenase	1.1.1.25	SKD	Lebrun & Chevallier, 1990

Results

Total soluble protein patterns

Protein samples were focused between two pH gradients: 3–10 and 2.5–6.5. Patterns are presented in Figures 1 and 2. Numerous protein bands were resolved with different strain profiles. For pH 3–10, only minor variations were observed between *A. pisi*, *A. fabae*, *A. lentis*, *A. viciae*, and *P. medicaginis* var. *medicaginis*. Common and different protein bands were detected between these *Ascochyta* spp. and *A. rabiei*, which allows us to clearly differentiate it. The soluble protein patterns of *P. medicaginis* var *pinodella* and *M. pinodes* were different from those of the *Ascochyta* spp., especially the *M. pinodes* pattern, which did not show an important band in the basic region.

On the other hand, for pH 2.5–6.5, all species were observed to have distinct protein bands, in addition to many common bands. Some variation was observed between isolates of the same species. However, protein patterns differed in band intensity. Supplementary tests are necessary to confirm these results.

Isozyme profiles

Four enzymes (alkaline phosphatase, shikimic dehydrogenase, malate dehydrogenase, and esterases) had well-resolved bands (Figs. 3, 4, 5, and 6). With acid phosphatase, bands were difficult to resolve because of streaking or smearing (result not presented).

Position of the sample application mask is indicated by * and negatively stained bands are indicated by arrows.

Alkaline phosphatase (ALP) was analyzed at pH 2.5–6.5; results are shown in Figure 3. Only three *Ascochyta* spp. (*A. pisi*, *A. lentis*, and *A. viciae*) had

clear resolution of bands with respect to the other tested species; *A. fabae* did not show enzyme activity, and the patterns of other species were streaky. These first results suggest that *A. pisi*, *A. lentis*, and *A. viciae* patterns have a similar number of bands that vary in position, but this needs to be confirmed. Isolate patterns for each species differed in band intensity, but this does not necessarily represent true variation.

Shikimic dehydrogenase (SKD) was also analyzed at pH 2.5–6.5 (Fig. 4). The pattern shows a limited number of bands (maximum four). *Ascochyta lentis* and *A. rabiei* have distinctive patterns, while *A. pisi*, *A. fabae*, and *A. viciae* show similarities, although staining bands were more intense for *A. pisi*. The patterns of *M. pinodes* and *P. medicaginis* var. *pinodella* seem identical, but they differ from *P. medicaginis* var. *medicaginis*. Moreover, two negatively stained bands are common to several species; the first one is common to *A. pisi*, *A. fabae*, *A. lentis*, and *A. viciae*; the second, localized in the basic region, is common to *A. rabiei*, *M. pinodes*, *P. medicaginis* var. *pinodella*, and *P. medicaginis* var. *medicaginis*.

Malate dehydrogenase (MDH) banding patterns (Fig. 5) also show two negatively stained bands, involving the same species as SKD. These bands probably correspond to the same oxydase activity, because many tetrazolium systems reveal this enzyme (Pasteur et al. 1987). The localization of the two bands is different for MDH and SKD, because the pH gradients are different for the two enzymes. Similar banding patterns were observed between all the *Ascochyta* spp., while *M. pinodes*, *P. medicaginis* var. *pinodella*, and *P. medicaginis* var. *medicaginis* have very different patterns. Nevertheless, some variation in band intensity was observed among isolates of the *Ascochyta* spp., but this does not necessary represent intra-species variation.

Esterases patterns show a weak reaction to the staining solution, except those of *A. rabiei*, which were cultivated on a chickpea medium (Fig. 6). Nevertheless, this weak staining reaction allows us to observe the differences between each *Ascochyta* sp. tested. Within *A. rabiei* isolates, intra-species variation was observed, e.g. ARC1 and R6 strains show different patterns in the basic region.

Characterization of an *Ascochyta* sp. isolated from *Vicia monoltha*

The patterns of the *Ascochyta* sp. (no. 27) isolated from *V. monoltha* Rehz are very different from those of the *Ascochyta viciae* Lib. isolated from *Vicia sativa* L. On the other hand, this isolate is very similar to the *A. rabiei* patterns in terms of soluble proteins (pH gradient 3–10), ALP, SKD and ester-

ases. Remarkably similar banding patterns are observed with the R6 isolate (no. 48) for soluble proteins (pH gradient 2.5–6.5) and MDH.

Influence of culture media

Most of the isolates were cultivated on a potato-dextrose medium only, but some were also cultivated on a chickpea medium. Clear differences were found in the protein banding patterns (focused between pH 2.5 and 6.5) of some *A. rabiei* isolates grown on the two media (Fig. 2). This was noticeable for isolates R91 01 (nos. 7 and 43), R92 05 (nos. 10 and 45), R92 15 (nos. 16 and 46), and R6 (nos. R6 and 48). Moreover, when SKD was revealed, some variation in banding intensity was noticeable for *A. pisi* (no. Pi and 38) and the same *A. rabiei* isolates as before (Fig. 4). Some bands were more intense when the fungi were cultivated on the potato medium rather than the chickpea, although samples were adjusted at the same protein concentration. On the other hand, protein patterns of *A. rabiei* seemed similar when focused between pH 3–10 (Fig. 1).

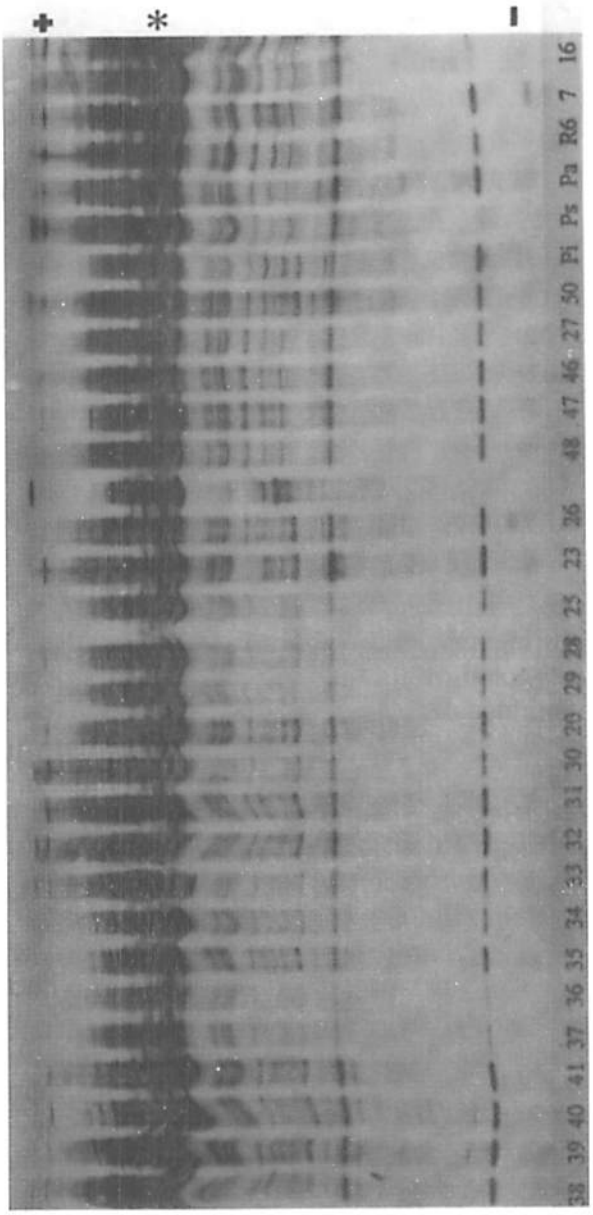

Figure 1. Soluble protein patterns of *Ascochyta* and *Phoma* spp. isolates, after IEF (pH gradient 3-10) and silver staining. Isolates are mentioned in Table 1 (38–41: *A. pisi*; 37–34: *A. fabae*; 33–30: *A. lentis*; 20–26: *A. viciae*; 48–46: *A. rabiei*; 27: *Ascochyta* sp.; 50: *P. medicaginis* var. *medicaginis*; Pi: *A. pisi*; Ps: *M. pinodes*; Pa: *P. medicaginis* var. *pinodella*; R6–16: *A. rabiei*). Position of the sample application mask is indicated by *.

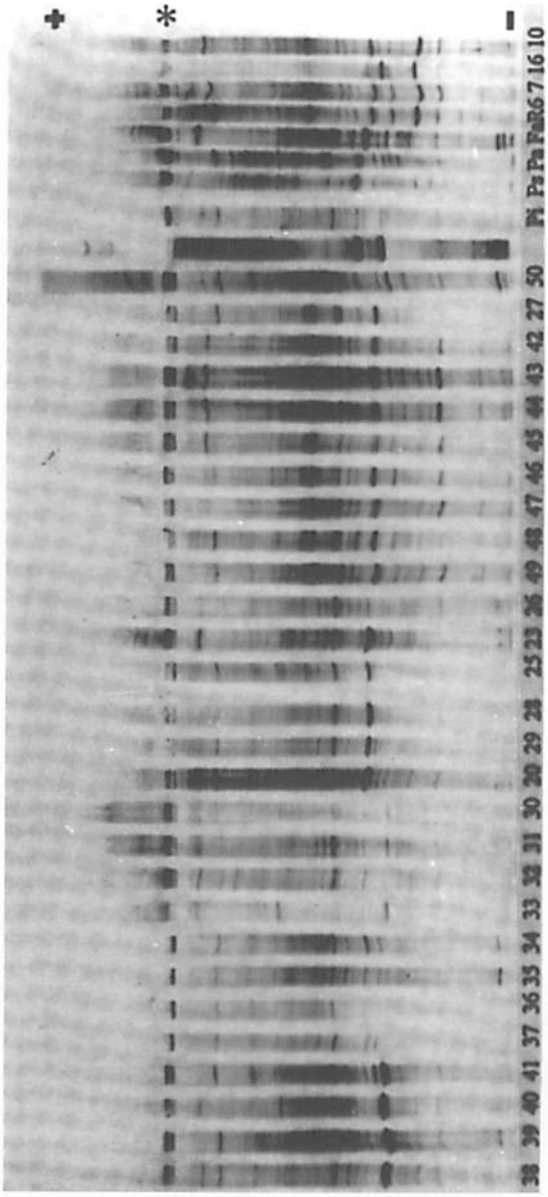

Figure 2. Soluble protein patterns of *Ascochyta* and *Phoma* spp. isolates, after IEF (pH gradient 2.5-6.5) and silver staining. Isolates are mentioned in Table 1 (38-41: *A. pisi*; 37-34: *A. fabae*; 33-30: *A. lentis*; 20-26: *A. viciae*; 49-42: *A. rabiei*; 27: *Ascochyta* sp.; 50: *P. medicaginis* var. *medicaginis*; Pi: *A. pisi*; Ps: *M. pinodes*; Pa: *P. medicaginis* var. *pinodella*; Fa: *A. fabae*; R6-10: *A. rabiei*). Position of the sample application mask is indicated by *.

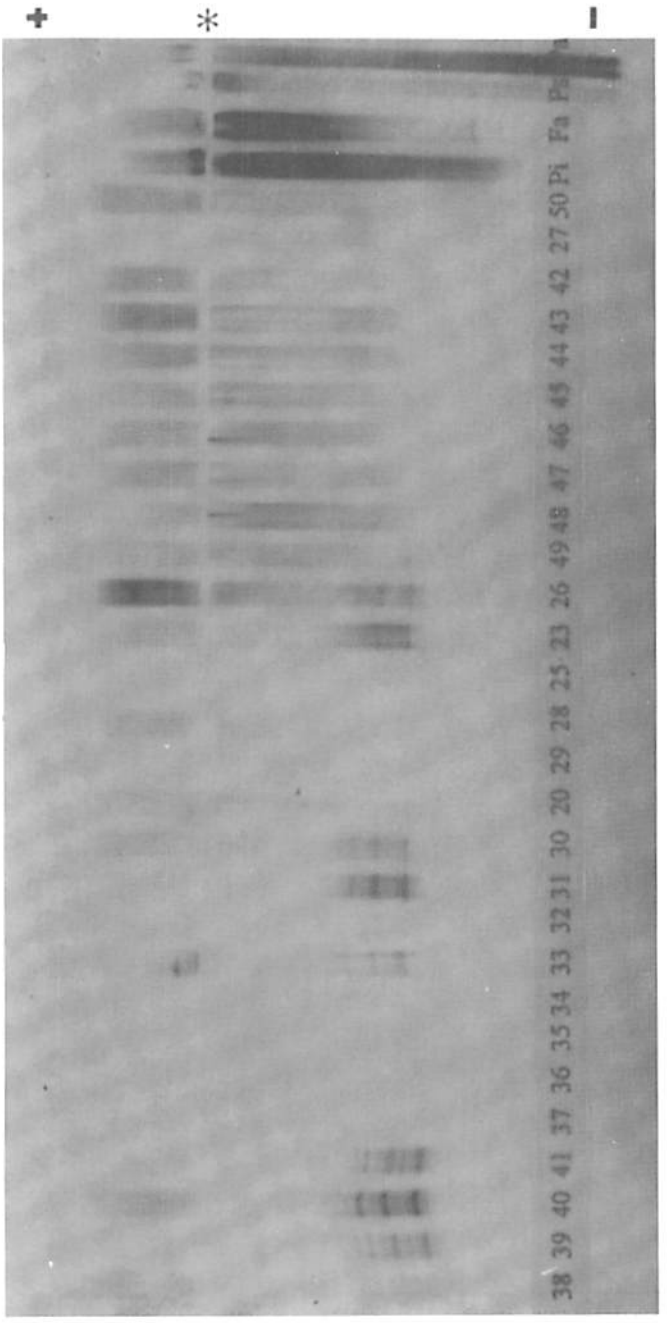

Figure 3. Alkaline phosphatase patterns of *Ascochyta* and *Phoma* spp. isolates, after IEF (pH gradient 2.5-6.5). Isolates are mentioned in Table 1 (38-41: *A. pisi*; 37-34: *A. fabae*; 33-30: *A. lentis*; 20-26: *A. viciae*; 49-42: *A. rabiei*; 27: *Ascochyta* sp.; 50: *P. medicaginis* var. *medicaginis*; Pi: *A. pisi*; Fa: *A. fabae*; Ps: *M. pinodes*; Pa: *P. medicaginis* var. *pinodella*). Position of the sample application mask is indicated by *.

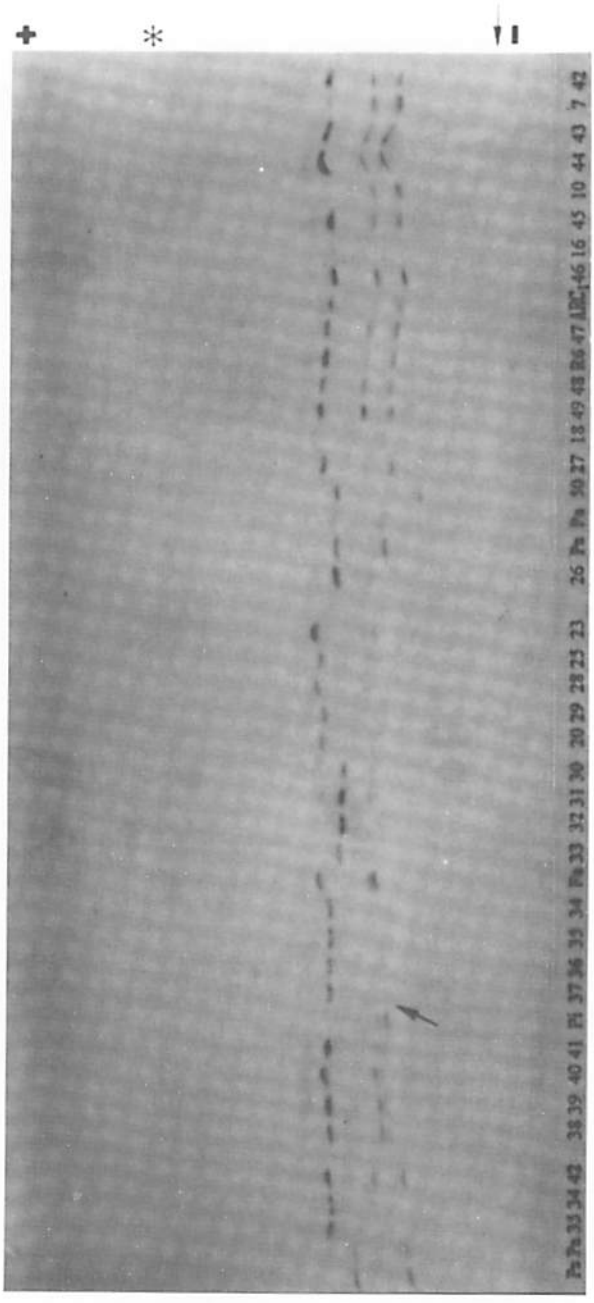

Figure 4. Shikimic dehydrogenase patterns of *Ascochyta* and *Phoma* spp. isolates, after IEF (pH gradient 2.5–6.5). Isolates are mentioned in Table 1 (38-Pi: *A. pisi*; 37-Fa: *A. fabae*; 33–30: *A. lentis*; 20-26: *A. viciae*; Ps: *M. pinodes*; Pa: *P. medicaginis* var. *pinodella*; 50: *P. medicaginis* var. *medicaginis*; 27-18: *Ascochyta* sp.; 49-42: *A. rabiei*).

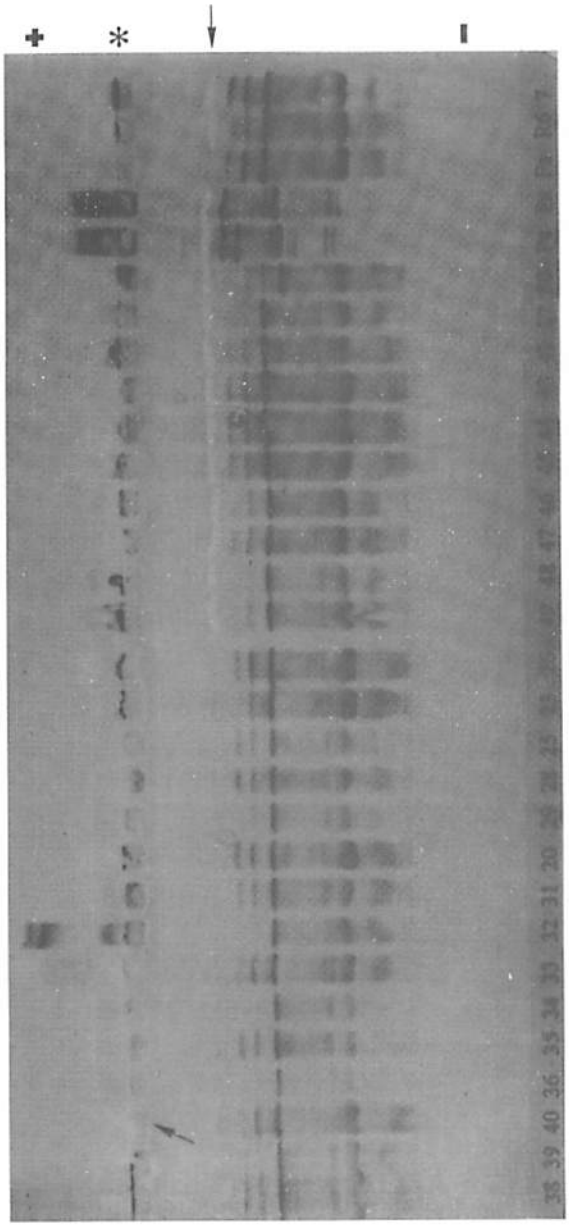

Figure 5. Malate dehydrogenase patterns of *Ascochyta* and *Phoma* spp. isolates, after IEF (pH gradient 3-10). Isolates are mentioned in Table 1 (38-40: *A. pisi*; 36-34: *A. fabae*; 33-31: *A. lentis*; 20-26: *A. viciae*; 49-42: *A. rabiei*; 27: *Ascochyta* sp.; 50: *P. medicaginis* var. *medicaginis*; Ps: *M. pinodes*; Pa: *P. medicaginis* var. *pinodella*; Fa: *A. fabae*; R6-7: *A. rabiei*). Position of the sample application mask is indicated by * and negatively stained bands are indicated by arrows.

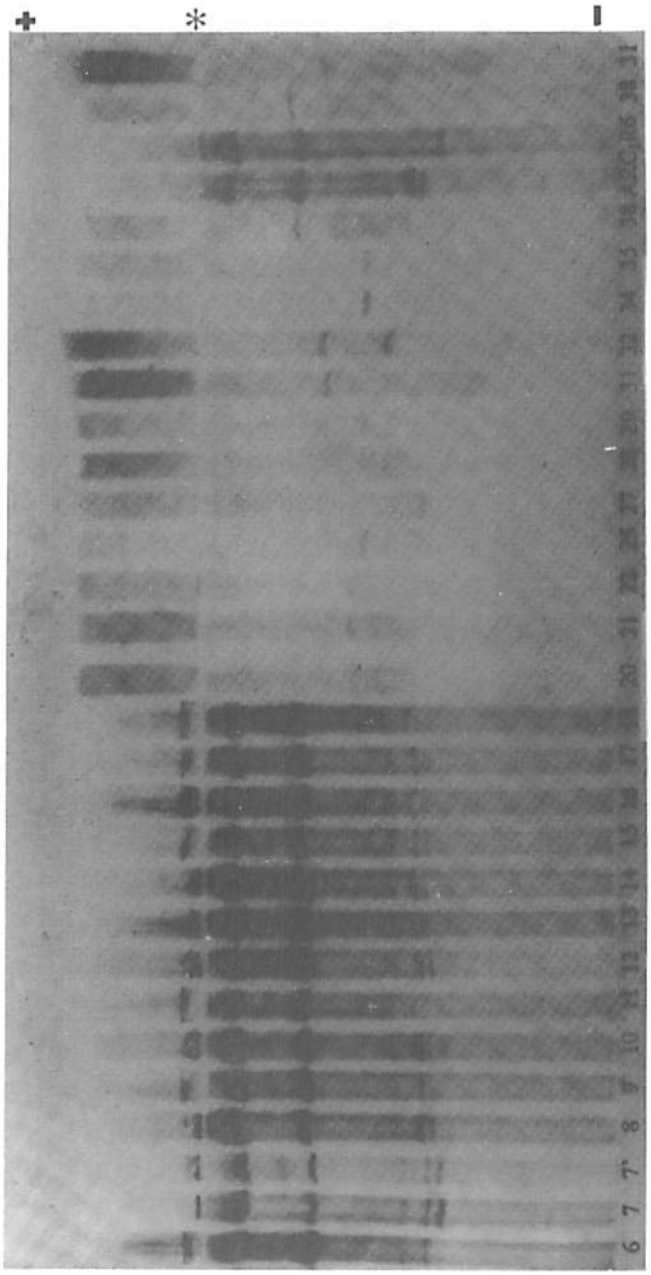

Figure 6. Esterases patterns of *Ascochyta* isolates, after IEF (pH gradient 2.5-6.5). Isolates are mentioned in Table 1 (6-17: *A. rabiei*; 18: *Ascochyta* sp.; 20-25: *A. viciae*; 27: *Ascochyta* sp.; 28-29: *A. viciae*; 31-32: *A. lentis*; 34-35: *A. fabae*; 38: *A. pisi*; ARC1-R6: *A. rabiei*). Position of the sample application mask is indicated by *.

Discussion

Several studies have examined the variation in soluble proteins and isozyme patterns among isolates of plant pathogenic fungi in relation to host specificity, population structure, and geographical location. Researchers have differentiated between different pathogenic species infecting the same host or with a similar host range (Julian and Lucas 1990; Obando et al. 1990; Bonde et al. 1991; Brunner and Petrini 1992; Chen et al. 1992; Priestley et al. 1992). The objective of this investigation was to determine whether protein electrophoresis and isozyme analysis would be helpful in the taxonomy of *Ascochyta* spp. which infect grain legumes.

This preliminary study found differences between patterns of *A. pisi*, *A. fabae*, *A. lentis*, *A. viciae*, and *A. rabiei*, but the variations are not always similar, depending on the protein analysis or enzyme system. Each species can be distinguished from the others by esterases and soluble proteins when focused between pH 2.5–6.5. This staining suggests that an inter-species polymorphism would be revealed by electrophoresis. On the other hand, only minor variations in the patterns of *Ascochyta* spp. are present with MDH. Finally, *A. pisi*, *A. fabae*, *A. lentis*, and *A. viciae* patterns generally show little variation, whereas *A. rabiei* patterns are clearly distinct from them with SKD, soluble proteins focused between pH 3-10, and by the observation of two negatively stained bands present with SKD and MDH. Moreover, one of the negatively stained bands is in the same position for *A. rabiei*, *M. pinodes*, *P. medicaginis* var. *pinodella*, and *P. medicaginis* var. *medicaginis*. Based on these results, *A. rabiei* can be distinguished from the other *Ascochyta* spp. tested. This observation confirms data from conventional taxonomy where this fungus is considered to have similarities with the genera *Ascochyta* (symptoms and cultural characteristics) and *Phoma* (great proportion of unicellular conidia). Further, intra-specific variations are apparent for *A. rabiei*, which is known to be polymorphic on the basis of colony aspect and pathogenicity (Nene 1984; Porta Puglia 1992). However, our comparison does not include a wide range of isolates, and no correlation can be established with geographical origin or pathogenicity of the isolates.

The patterns of *M. pinodes*, *P. medicaginis* var. *pinodella*, and *P. medicaginis* var. *medicaginis* can generally be distinguished from those of *Ascochyta* spp. when soluble proteins and enzyme activities are present. These differences might be used as additional criteria to identify the three fungi, which are pathogens on pea and are sometimes confused when using classical characterization, as noticed by Faris-Mokaiesh et al. (1992). However, *M. pinodes* and *P. medicaginis* var. *pinodella* were analyzed with a single isolate and were not cultivated on the medium generally used for other fungi.

When *A. rabiei* was grown on different media, variations were observed in the number and intensity of protein bands, in silver staining, and in SKD activity. Our results suggest that culture medium may influence electrophoretic patterns. On the other hand, patterns were similar with MDH and soluble proteins focused between pH 3–10. This observation may explain why Adaskaveg et al. (1988) considers species identification from protein banding patterns to be possible when different media are used to culture isolates of *Pythium* spp.

This study has also provided important information about a strain of *Ascochyta* sp. isolated from *V. monoltha*, whose morpho-cultural aspects are similar to those of *A. rabiei*. Electrophoretic studies have confirmed similarities of this strain with *A. rabiei*, since their patterns are mostly identical. However, this fungus has only infected *V. sativa* by artificial inoculation, and has not affected pea, faba bean, lentil, or chickpea (Benelmouffok 1993).

Protein and isozyme analysis appears to be of greater use in species differentiation, although more data are needed, since the number of strains and enzymes tested in this study was small. Further research, that includes a wide range of species and isolates from different geographical locations, is required. Other enzyme activities (GPI, G6PDH, 6PGD, LAP, etc.) that have often been described in the literature (Julian & Lucas 1990; Bonde et al. 1991; Brunner & Petrini 1992; Chen et al. 1992) should also be studied. If banding patterns are consistent, they may be used to develop a rapid electrophoretic technique for routine identification. Finally, analysis of soluble proteins and isozymes, in combination with other methods such as nucleic acid analysis ,would improve the taxonomic and phylogenetic knowledge of the *Ascochyta* genus.

Acknowledgments

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Genotyping with Microsatellite and RAPD Markers Resolves Pathotype Diversity in the Ascochyta Blight Pathogen of Chickpea

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Abstract

The poor definition of pathotype variation in the ascochyta blight fungus has historically hindered strategies to reduce blight disease damage to the chickpea crop in West Asia and North Africa. We employed a probe complementary to the microsatellite sequence $(GATA)_4$ and an RAPD marker to construct a genotype-specific, DNA fragment profile from Syrian field isolates of this fungus. By using conventional pathogenicity tests (see Udupa and Weigand in this proceedings) and genome analysis with a microsatellite sequence and RAPD markers, we demonstrated that DNA markers distinguish the major pathotypes in Syria, and defined the organization of clonal lineages within and among pathotype groups. New opportunities for tracking the population dynamics and evolution of this important crop pathogen were identified.

Introduction

The ascochyta blight fungus *Ascochyta rabiei* (Pass) Lab. is considered the most damaging pathogen of chickpea (*Cicer arietinum* L.). This disease occurs worldwide in chronic epidemic cycles despite the frequent introduction of new resistant chickpea cultivars (Singh and Reddy 1993). The absence of durable blight resistance in the field has been attributed to the appearance of new pathotypes (Singh et al. 1992) and high levels of polymorphism in

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aggressiveness in the pathogen populations (Malik and Rahman 1992; Vir and Grewal 1974; Reddy and Kabbabeh 1985; Porta Puglia 1992). However, the nature of this polymorphism is the subject of much debate (Malik and Rahman 1992; Porta Puglia 1992), marked by strongly contrasting views on the diversity and stability of pathotypes.

The pathotypes of an *A. rabiei* isolate are determined by assaying their infection spectrum, i.e., pathogenicity, on a set of differential chickpea cultivars. The differential set generally includes several cultivars with different levels of resistance to the pathogen. Using such an assay, Vir and Grewal (1974) found more than 10 pathotypes (conventionally called races) among field isolates from India. In similar studies Reddy and Kabbabeh (1985) found six races among 50 isolates from Syria. Nene and Reddy (1987) found five pathogenic groups and several strains among the isolates of Pakistan and Turkey, respectively. The different laboratories used different assay procedures and differential cultivars for pathogenicity tests. Porta Puglia (1992) found that pathogenicity or aggressiveness within an isolate line may vary according to environment. Reddy and Kabbabeh (1985) ranked the aggressiveness of six isolates as 6> 5> 4> 3> 2> 1 using a plastic house. Weising et al. (1991) ranked the same isolates as 6> 4> 2> 1> 5> 3 using a growth chamber (Conviron). Based on these results and similar reports on other pathosystems such as the *Pyricularia grisea* rice pathosystem (Ou and Ayad 1968; Giatgong and Frederikson 1969), it appears that many isolates of *A. rabiei* are pathogenically unstable when subcultured under laboratory conditions. These results indicate that pathotypes may be promiscuously polymorphic and continually changing.

In contrast to the above view, our extensive testing of single-spore-derived cultures did not show changes in pathotype, even after repeated subculturing and prolonged storage. In addition, several researchers have suggested that the lack of standardization in the assay procedures (Reddy et al. 1991; Porta Puglia 1992) and the disease rating scale (Porta Puglia 1992; Harrabi and Halila 1992; Chiha et al. in this proceedings) leads to inflated estimates of pathotypic diversity and variability. The key to resolving these issues is to provide genetic definition to the phenotypic variations observed in pathogenicity assays.

Although a variety of genetic markers can be used to study fungal phytopathogen populations (for review see Michelmore and Hulbert 1987), few have been definitive for the ascochyta blight fungus. For example, Bouznad et al. (in this proceedings) found that the isozyme profiles of *A. rabiei* isolates from Algeria, Tunisia, and Syria are nearly uniform and differ little from other species of genus *Ascochyta*. However, Weising et al. (1991) found

that oligonucleotide sequences complementary to microsatellites can be efficiently used as probes in RFLP analysis to detect genetic diversity in *A. rabiei* isolates.

Here we report the results of experiments designed to resolve the issue of pathotype stability and diversity in Syrian ascochyta blight pathogens by determining the diagnostic value of a microsatellites and RAPDs for pathotype identification. This resolution is based on the following rationale: if pathotypes are stable and represent distinct clonal lineage, they are therefore marked by lineage-associated microsatellites and RAPD markers. This also assumes that the DNA markers used are sufficiently variable to distinguish between clonal lineages but not so hypervariable as to obscure relatedness within lineages. In our tests, microsatellite and RAPD markers were diagnostic for all three pathotypes found in Syria, supporting the view that pathotypes are stable and composed of discernible clonal lineages. We conclude that microsatellite and RAPDs have excellent potential for resolving the population dynamics and the evolution of the ascochyta blight fungus.

Materials and Methods

Fungal material and pathogenicity test

The 53 isolates of *A. rabiei* used in this study were collected in 1982, 1991, 1992, and 1993 from different chickpea growing regions in Syria and Lebanon. These isolates were single-spore purified and pathotyped according to Udupa and Weigand (in this proceedings) using three differential host cultivars (ILC 1929, ILC 482, and ILC 3279) with different levels of resistance to *A. rabiei (*susceptible, moderate, and high), using a growth chamber. The fungus was grown in liquid culture and the mycelia were harvested according to Weising et al. (1991).

Extraction of DNA

DNA was extracted from lyophilized fungal mycelia according to Weising et al. (1991), except that the ultracentrifugation step was omitted. DNA was quantified by absorbency at 260 nm.

RAPD analysis

The primer used in the study, OPJ-01, was obtained from Operon Technologies in Alameda, California. DNA amplifications were performed in 25 μl containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.001% gelatin), 100 μM each of dATP, dTTP, dGTP, and dCTP, 5 picomoles of a single 10-base primer, 200 ng of genomic DNA, 1 unit of Taq

DNA polymerase (Boehringer Mannheim), and topped with a drop of mineral oil. Amplifications were performed in a thermocycler (Perkin Elmer 9600) programmed for 2 minutes at 94° C; for 40 cycles of 1 minute each at 92° C, 1 minutes at 36° C and 2 minutes at 72° C; and a final extension of 5 minute at 72° C. The reaction mix without template DNA served as a control and was electrophoresed along with the samples. Amplified DNA products were loaded onto 1.2% agarose gels and electrophoresed in 1× TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Gels were stained with ethidium bromide and photographed under UV light.

Microsatellite analysis

Genomic DNA was digested either with TaqI or *Bam*H1, and Southern hybridization was performed using digoxigenated $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, and $(GTG)_5$ as probes. Southern hybridization and colorigenic detection were performed according to Bierwerth et al. (1992).

Data analysis

The fragment pattern of each isolate derived from microsatellite analysis was coded in binary form, i.e., as ones and zeros representing the presence or absence of each amplified fragment. Nei's genetic distance (Nei and Li 1979; a dissimilarity index) was computed from the binary data for all pair combinations of isolates. Data analysis was performed using the software package NTSYS-pc (Rohlf 1993).

Genetic diversity (H) was calculated according to the following equation (Nei 1987):

$$H = [n/(n-1)] \cdot \left[1 - \sum_{i=1} x_i^2\right]$$

where x is the frequency of the i^{th} haplotype in the population, and n is the number of isolates examined.

Results

Microsatellite and RAPD analysis

The resolving power of the microsatellites varied greatly. Out of four oligonucleotides complementary to the microsatellite sequences screened for polymorphism on a sub-sample of isolates, only three revealed polymorphism. Among the informative microsatellites, $(GATA)_4$ had the highest discriminating power, followed by $(GACA)_4$ and $(GGAT)_4$. $(GTG)_5$ did not reveal any polymorphism (data not shown). Among the enzymes tested, *Taq*I gave more polymorphic profiles of restriction fragments with $(GATA)_4$.

Therefore the probe-enzyme combination $(GATA)_4$-*TaqI* was used to genotype the rest of the isolates. The probe-enzyme $(GATA)_4$-*TaqI* found 18 genotypes (Fig. 1) in one genotype (H) of pathotype III (the predominant pathotype in the chickpea-growing regions of Syria). This probe-enzyme combination also resolved the six "races" identified by Reddy and Kabbabeh into five distinct genotypes (Table 1). Races 4 and 6 have the same DNA profile (Fig. 1).

RAPD analysis with the primer OPJ-01 amplified 2–4 DNA fragments from a given template DNA. This primer defined eight genotypes (Fig. 2) and diagnostic DNA profiles for the most predominant genotype of pathotype III.

Genetic distance and diversity estimates

Pair genetic distance (Nei and Li 1979) for the 53 isolates was calculated for all 35 fragments (loci) of the microsatellite and for the six loci amplified by the RAPD marker. The genetic distance for the 53 isolates ranged from 0.00 to 1.25 for the microsatellite marker, and from 0.00 to 1.10 for the RAPD marker (Table 1), indicating clonal to diverse relationship.

Microsatellite analysis classified the isolates into 18 genotypes, whereas RAPD markers could define only eight. The estimate of the overall diversity index for all isolates for the RAPD marker (0.63) was slightly higher than the microsatellite estimate (0.62). However, the resolving power of the pathotypes by microsatellite was better than by RAPD marker. Genotypic groupings of the isolates based on microsatellite analysis contained only a single pathotype, i.e., no more than one pathotype could be seen in one genotypic class. Genotypic groupings based on the RAPD marker contained more than one pathotype. Hence, the microsatellite data were used for calculating genetic diversity estimates within pathotype groupings and within the year of collection (Table 1). The highest estimated genetic diversity within the year of collection was found in 1982 (0.93) and 1991 (0.93), compared to 1992 (0.41) and 1993 (0.47). Within the pathotypic groupings, pathotype I had highest genetic diversity (0.99), followed by pathotype II (0.80), and then pathotype III (0.11).

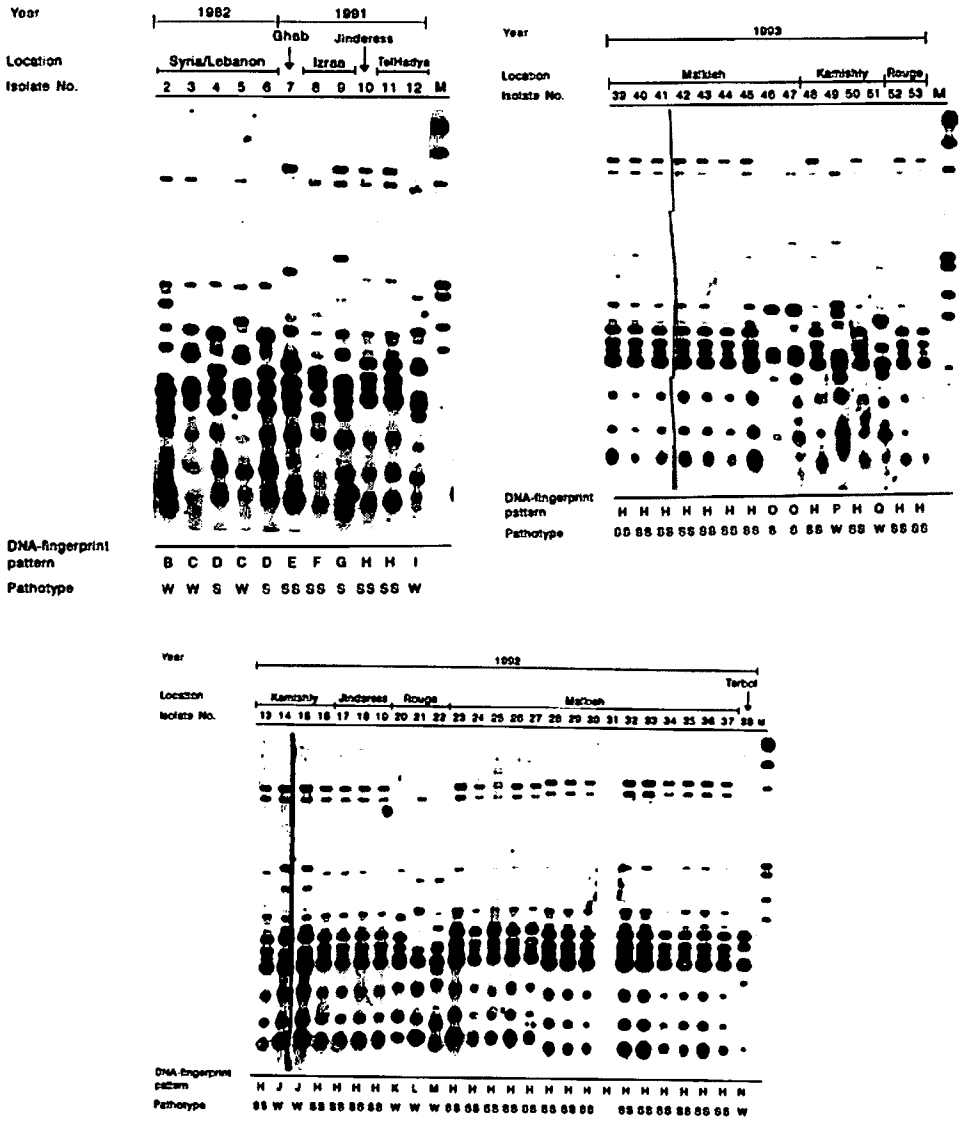

Figure 1. Microsatellite marker analysis of *Ascochyta rabiei* isolates from Syria using the probe-enzyme combination $(GATA)_4$-*Taq*I. Lane M contains molecular weight marker, lambda *Eco*RI-*Hin*dIII DNA digest. W = Pathotype I, S = Pathotype II and SS = Pathotype III.

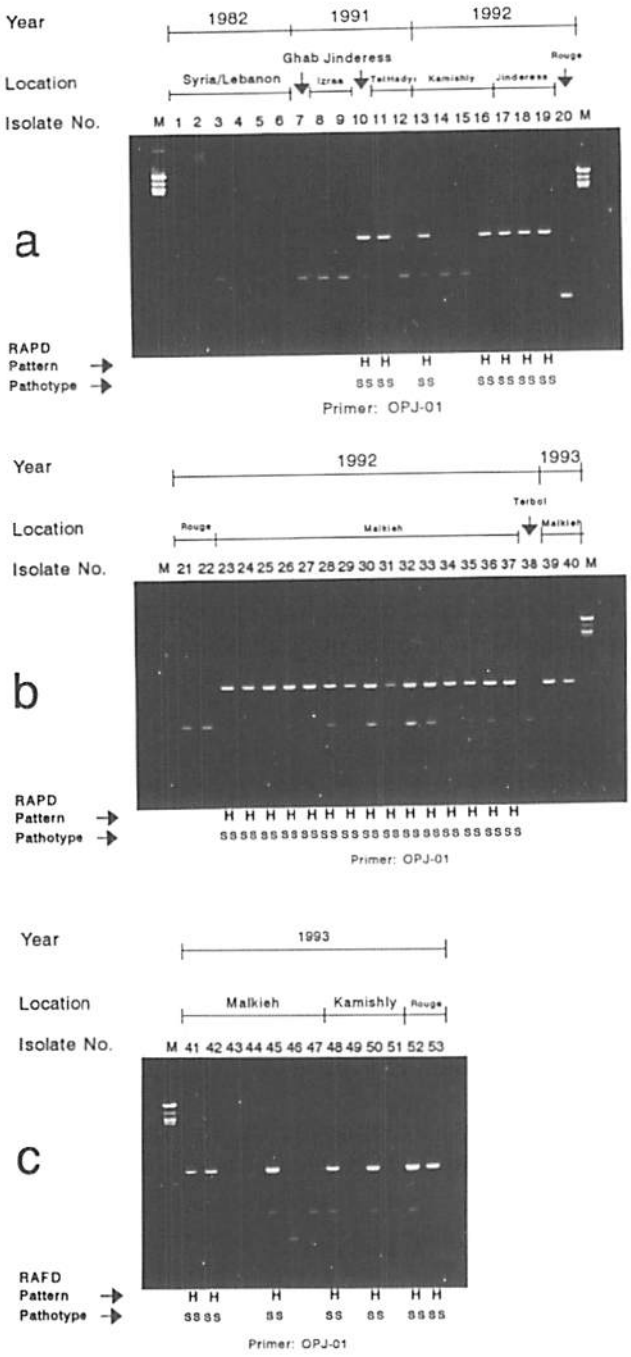

Figure 2. RAPD analysis of *Ascochyta rabiei* isolates from Syria using the primer, OPJ-01. Lane M contains molecular weight marker, lambda *Eco*RI DNA digest. SS = Pathotype III and H = most predominant genotype.

Table 1. Microsatellite marker analysis of *Ascochyta rabiei*.

Collection/ pathotype	Isolate #	Sample size	No. of genotypes	No. of pathotypes	Genetic diversity (H)
All isolates	1–53	53	18	3	0.62
1982	1–6	6	5	2	0.93
1991	7–12	6	5	3	0.93
1992	13–38	26	6	2	0.41
1993	39–53	15	4	3	0.47
Pathotype I	-	13	12	-	0.99
Pathotype II	-	5	3	-	0.80
Pathotype III	-	35	3	-	0.11

Discussion

Our experiments demonstrate that microsatellites and RAPD markers have unprecedented utility for population genetic and phylogenetic analysis of ascochyta blight fungus in chickpea. Since neither method requires radioactive chemicals, they can be used by national programs (NARS) in developing countries, where facilities to handle radioactive chemicals are not available.

Our study also demonstrates that microsatellite markers are more effective in resolving pathotype diversity in *A. rabiei* than RAPD markers. On the other hand, RAPD markers are more effective in identifying the most predominant genotype of the pathogen. Since RAPD analysis is based on polymerase chain reaction (PCR), it is technically simple and requires only small quantities of DNA. Unlike conventional RFLPs, RAPD analysis does not require cumbersome cloning and membrane hybridization processes, and is therefore suitable for the rapid and efficient analysis of large numbers of isolates. The microsatellite marker is also efficient, but the detection process is more cumbersome than with RAPD. Therefore, RAPD markers are more useful for conducting pathogenicity surveys than microsatellites.

The microsatellite marker was diagnostic for the three pathotypes sampled and classified into 18 distinct genotypes. The isolates of the three pathotypes are defined as follows: pathotype I (least aggressive), 12 genotypes; pathotype II (aggressive), three genotypes; and pathotype III (most aggressive), three pathogens, of which one predominants in the chickpea-growing regions of Syria. These genotypic groupings provide a reference base with which to document the emergence of new genotypes (pathotypes) as well as the appearance of new lineages. Future investigation of this information in terms of chickpea cropping patterns will provide valuable insight on the influence of host genotypes (resistance genes) on the population biology of the ascochyta blight fungus.

Our results strongly support the view that *A. rabiei* pathotypes are stable and reliably distinguishable by carefully standardized pathogenicity assays (see Udupa and Weigand, this proceedings). If genotypes (pathotypes) were subject to sudden, continuous, or wholesale changes, it would have been impossible to identify the most predominant genotype (pathotype) on the basis of genetic lineage associations maintained over three years.

Winter sowing of chickpea is a relatively recent practice in Syria (Hawtin and Singh 1984). Cool temperatures and wet weather prevail during winter, favoring the incidence and development of ascochyta blight in chickpea fields (Reddy et al. 1990). Over the years, cultivars with improved levels of resistance have been bred and released for winter cultivation (Singh and Reddy 1993). The most aggressive pathotype evolved through a process of adaptive mutation[1] (Hall 1990; Cairns and Foster 1991) and was selected for over the years, thereby reducing genetic diversity in the recent pathogen population. In addition to periodic strong host selection for specific aggressive genotypes (pathotypes), migration may have played an important role in the evolution of the pathogen. The occurrence of a single genotype over a large area, with increasing frequency, suggests that the aggressive genotype has migrated, most probably through infected seed. Wider geographical distribution and stability of the most predominant genotype further support the view that the genotypes of this fungus are more stable, and that sexual recombination (Kaiser 1992) plays a minor role in the evolution of this pathogen.

Our study demonstrates that microsatellites and RAPD markers reliably index genetic and pathotypic diversity in the *A. rabiei* population of Syria. These markers can further be used to determine the current geographical organization of pathotype diversity and monitor its change over time as well as the dispersal range of specific genotypes. This will improve the process of selecting and deploying more chickpea cultivars with durable resistance for particular chickpea-growing areas. These markers will also facilitate the study of the rate and direction of pathotype evolution in this important pathogen. The definition of genetic variation provided by the markers extends the utility of the ascochyta blight pathogen for clarifying the evolutionary dynamics that underlie host–pathogen interactions.

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[1] Adaptive mutation is a process that appears to produce useful mutations in an organism only in the presence of selection for those mutations and in the absence of cell growth. Unlike random mutation, adaptive mutation is a type of directed mutation. The pathogen can mutate specific genes in response to selection pressure exerted by host plant resistance (host defence system)and adapt itself to the resistant cultivar.

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Genotypic Diversity of Tunisian *Ascochyta rabiei* on Micro- and Macro-geographical Scales

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Abstract

DNA fingerprinting was used to analyze micro- and macro-geographical variations in *Ascochyta rabiei* (Pass.) Lab. populations in the Beja region of Tunisia. One hundred and fifty six isolates were collected from five chickpea fields in 1992. The total genomic DNA of each isolate was restricted with *Hin*fI or *Rsa*I, and hybridized to a set of synthetic oligonucleotides complementary to simple repetitive sequences. On the micro-geographical scale, 12 different fungal genotypes were found at various frequencies within the investigated field (A). In some cases more than one genotype was isolated from the same lesion of a host plant. On the macro-geographical scale, analysis revealed 17 different genotypes in the five fields investigated. Two genotypes were found common to all the fields. TAr1 and TAr6 were the predominant genotypes, representing 38.2% and 22.6%, respectively, of the isolates collected from the five fields. Hierarchical diversity analysis demonstrated that most of the genetic variability was distributed within a single field. The genetic diversity index (H_I) varied between 0.08 at the least variable site to 0.86 at the most variable site, with a mean of 0.77. This study found that: (i) Genotypes were unevenly distributed across the field; (ii) genotypes occurred at highly different frequencies; (iii) different genotypes were observed within a single lesion; (vi) higher levels of diversity were found within rather than between locations; and (v) different genotypes found within a particular location were not obviously related to each other.

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Introduction

Blight, caused by *A. rabiei*, is a destructive disease of chickpea (*Cicer arietinum* L.) in the major growing areas of the world (Nene and Reddy 1987). It has been reported in a continuous belt from Portugal across most Mediterranean countries eastward to South Asia, in North America, and in scattered areas elsewhere, including Australia (Kaiser and Muehlbauer 1988; Porta Puglia 1992). Ascochyta blight was first reported in the northeast of India in 1911 (Singh and Reddy 1983) and is now widespread across the Mediterranean region, where it is considered endemic.

In Tunisia, ascochyta blight is the most important disease of chickpea (Reddey et al. 1980). The blight has caused considerable damage to chickpea crops in sub-humid and superior semi-arid areas (Kassebeer et al. 1976). Although much research has been done on certain aspects of ascochyta blight and its control by various means (Nene and Reddy 1987; Saxena and Singh 1984), attempts to control ascochyta blight have been primarily through the breeding and cultivation of tolerant cultivars. Resistant varieties, however, have provided only temporary control, primarily due to the breakdown of resistance associated with a shift in the variability composition of the pathogen.

Researchers in several countries have studied variability in the morphology (Kaiser 1973; Grewal 1984), pathogenicity (Porta Puglia1992), and toxin production (Alam et al. 1989; Höhl et al. 1991) of *A. rabiei*. Therefore, any identification based on morphology, chemical and/or pathogenic characteristics has been difficult and suffers from several disadvantages. Biological pathotyping is time consuming and labor intensive, and its reproducibility is often poor.

Biochemical and molecular markers have contributed significantly to the understanding of the population biology of many fungi (Fry et al. 1991). Polymorphism at the DNA level has been increasingly used to complement traditional markers in the analysis of genetic identity, variability, and relatedness in fungi. DNA fingerprinting has proved to be a valuable tool in a broad range of disciplines. It relies on the presence of a particular class of repetitive DNA in the eukaryotic genome. This class consists of short motifs arranged in tandem to form long, more or less homogeneous arrays. Depending on the length of the basic repeat unit, these sequences are called minisatellites (Jeffreys et al. 1985) or simple repetitive sequences (SRS; Tautz and Renz 1984). These techniques have already yielded valuable insight into the pathotype diversity, mating systems, and phylogeny of a wide variety of fungal species (Hamer et al. 1989; Levy et al. 1991; McDonald and Martinez 1990, 1991; Weising et al. 1991) and provide a potentially

powerful tool for analyzing population genetic structures in many fungi on micro- and macro-geographical scales (Fry et al. 1991; Xia et al. 1993; Drenth et al. 1993).

We recently reported the successful discrimination of six Syrian *A. rabiei* isolates by DNA fingerprinting with synthetic, tandem repetitive oligonucleotide probes (Weising et al. 1991; Bierwerth et al. 1992) . This technique is more informative than PCR based RAPD analysis for the differentiation of *A. rabiei* genotypes (Kaemmer et al. 1992). However, information concerning the geographical structure of genetic variability in pathogen populations is lacking. An analysis of the genetic structure of *A. rabiei* populations is necessary to determine the influence of migration on the population genetics and the evolution of the *A. rabiei–Cicer arietinum* pathosystem in Tunisia. On the premise that knowledge of the population genetics of fungal plant pathogens may eventually contribute to the development of a more durable disease management strategy, we have initiated such a study for *A. rabiei* (Morjane et al. 1994).

The objective of this research was to demonstrate the validity of the DNA fingerprinting method we have developed with synthetic, tandem-repetitive oligonucleotide probes for *A. rabiei.* We tested the stability of DNA fingerprinting during sporulation and subculturing and estimated the identity and relatedness of individuals in the populations. We also determined the genetic distribution of *A. rabiei* on micro- and macro-geographical scales.

Hierarchical Sampling

The sources of infected chickpea tissue from which *A. rabiei* isolates were obtained are given in Figure 1. All tissue came from naturally infected fields. Isolations were made essentially as described by McDonald and Martinez (1990). The hierarchical sampling scheme was employed to permit partitioning of the total pathogenic diversity into components. The Beja region was subdivided into populations based on the field from which the isolates were collected. Isolates from the same field were considered as coming from the same population. In each field, infected stems were sampled from two to four different, well-separated locations. At each location, two distinct stem lesions were taken from each of five to six plants growing in close proximity. From each lesion, single spores were isolated and germinated as previously described (Weising et al. 1991).

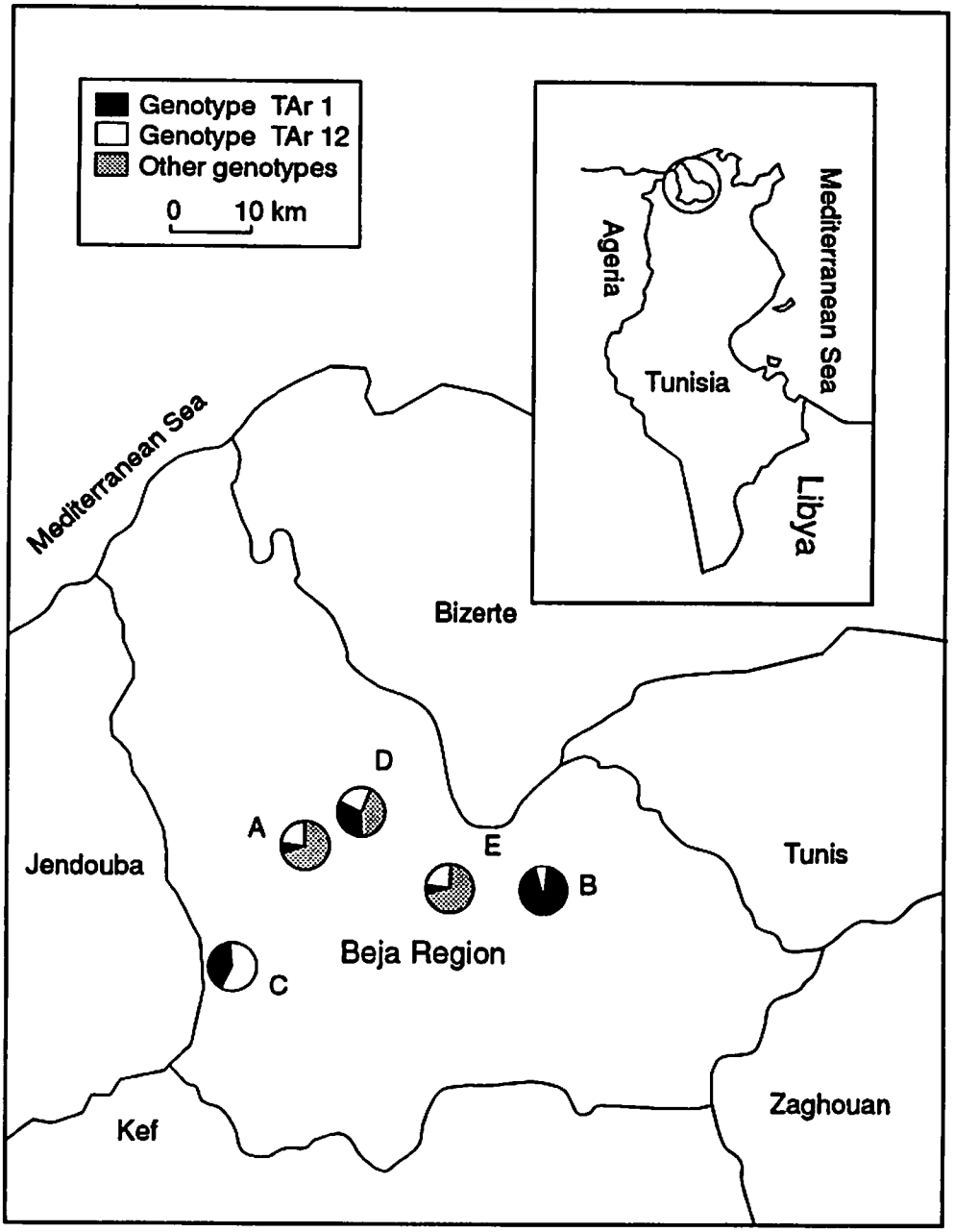

Figure 1. Collection sites of *Ascochyta rabiei* from five fields: Hammam Sayala (A), Oued Zarga (B), Beja/Bousalem (C), Beja (D), and Oued Beja (E). Frequency and distribution of genotypes TAr1, genotype Tar12, and other genotypes in the Beja region in1992.

The objective of this collection strategy was to maximize the amount of diversity and to compare genetic variation at several levels. In total, 156 isolates were collected.

DNA Fingerprinting among *Ascochyta rabiei* Isolates

DNA preparations from *A. rabiei* isolates, digested with *Hin*fI or *Rsa*I, were analyzed in fingerprint experiments with oligonucleotide probes complementary to simple doublet, triplet, or quadruplet repetitive sequences. All of these hypervariable repeats, which have been found in many eukaryotes different from fungi, are obviously present in the genome of all *A. rabiei* isolates. Nevertheless, their organization and relative abundance appear to be somewhat different (Fig. 2). For example, $(CAT)_5$ produced a more or a less homogeneous pattern, which does not discriminate well between the genotypes.

Hybridization with $(CA)_8$ and $(CAA)_5$ resulted in highly polymorphic patterns (Fig. 2). These two probes proved to be highly informative in combination with *Hin*fI and/or *Rsa*I. $(CAG)_5$ detected a highly complex banding pattern with differences in both number of bands and level of informativeness (data not shown). $(GATA)_4$ displayed fewer bands and appeared to be clustered at one predominant locus. In view of these results, $(CA)_8$ and $(CAA)_5$ were selected as the most useful probes for these experiments.

To test for stability during laboratory culturing, two isolates were used. We observed five single-spore cultures from isolates TAr6 and TAr11. The fingerprinting probe $(CAA)_5$ hybridized to many *Hin*fI fragments in isolates TAr6 and TAr11, yielding a different pattern for each isolate (Fig. 3). All isolates had hybridization patterns identical to the parental isolates. The hybridization pattern was stable during vegetative growth. Isolates TAr6 and TAr11 maintained the same patterns after five serial transfers. This indicates that no changes in the hybridization pattern took place during routine subculturing. The oligonucleotide fingerprints were highly reproducible and characteristic for a given genotype. However, by using restriction enzymes sensitive to methylation, the pattern of the two isolates was heterogeneous and some bands disappeared after the third serial transfer (unpublished results).

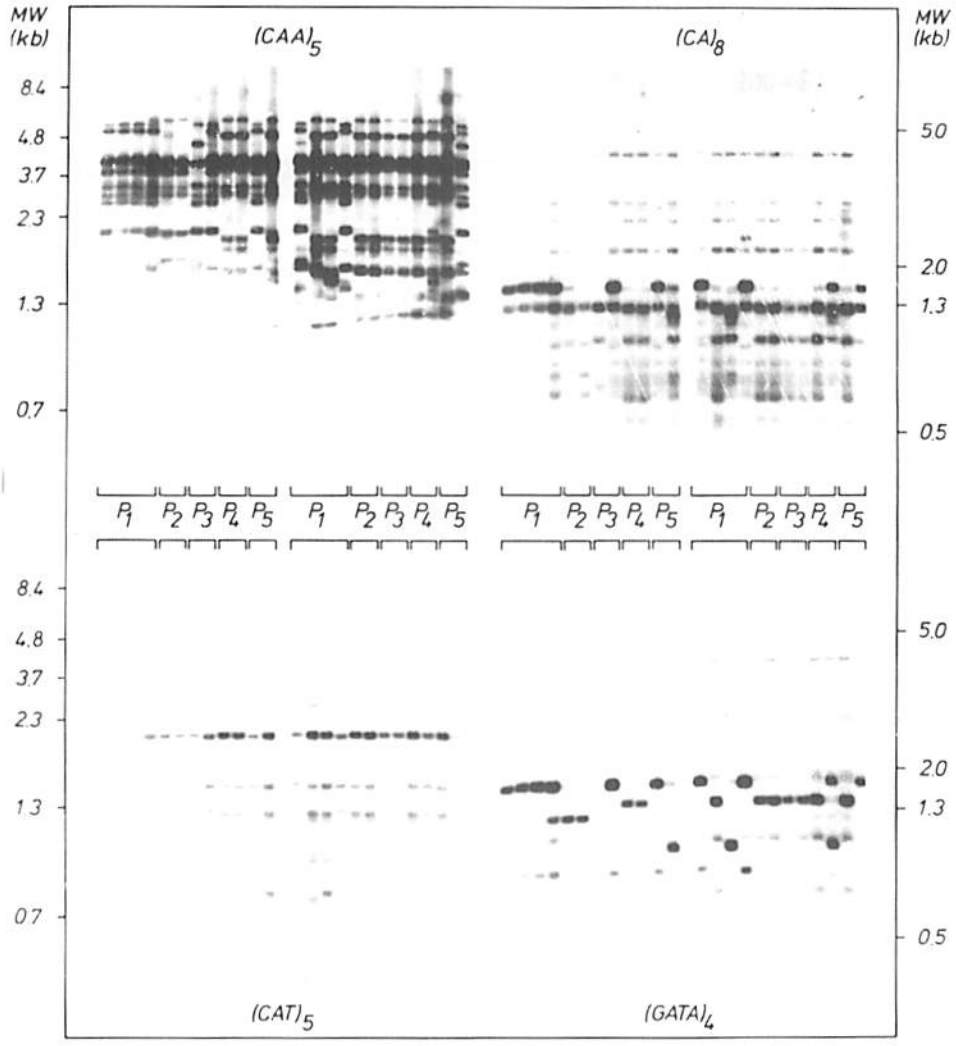

Figure 2. Oligonucleotide fingerprinting of *Ascochyta rabiei* isolates from two locations in a single chickpea field (A). For P1, two isolates were examined from each of two lesions. For individual host plants P2 to P5, one isolate from each of two lesions was examined per plant. Two different sets of molecular weight markers were used (their sizes are indicated in kb).

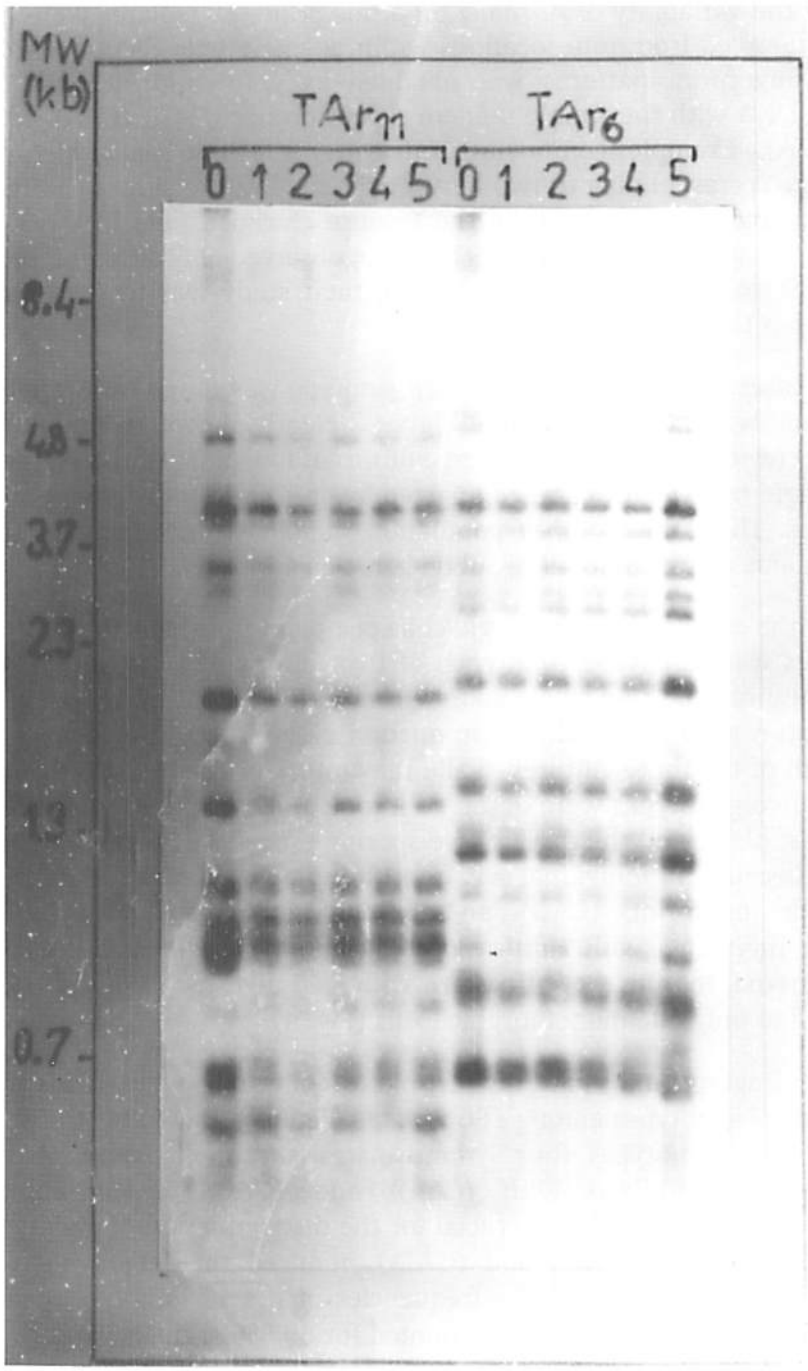

Figure 3. Hybridization of *Ascochyta rabiei* DNA digested with *Hin*fI and probed to $(CAA)_5$. The patterns for isolates TAr6 and TAr11 did not change after five serial transfers.

To study the variability of *A. rabiei* on a fine scale, 50 isolates were hierarchically sampled from four locations within a single field (A). A total of 12 distinct fingerprint patterns was obtained by gel-hybridization of *Hin*fI-digested DNA with the simple tandem repeat probes $(CA)_8$, $(CAA)_5$, $(CAT)_5$, and $(GATA)_4$. Examples comprising *Hin*fI patterns for two sets of hierarchical samplings, corresponding to two locations in field A, are shown in Figure 2. A similar amount of diversity in the Palouse chickpea field in the US was reported by Jan and Wiese (1991), who observed 11 different biotypes among 39 isolates of *A. rabiei*. However, their study was focused on virulence rather than genome characteristics.

In some cases, more than one *A. rabiei* genotype was found on a single host plant, even within a single lesion. Figure 2 clearly shows genetic polymorphisms between all four single-spore cultures of one plant of the hierarchical set. A single host plant can be co-infected by at least four different pathogen genotypes. Thus, the *A. rabiei* population sampled from a single chickpea field contains a large amount of subtle genetic variation.

To study the co-infection of a single chickpea plant in more detail, three single spores were analyzed from each of two lesions of a single host plant. The culture of the three single spores from lesion 1 (L1) were again single spored, after the first conidial reproduction cycle. Three spores were taken from each of the three cultures, while the culture of lesion 2 (L2) was used directly. Two genotypes were observed in one and the same lesion (L1) of a single host plant, and the three single-sporing procedures seemed to be very reliable, because all individual sets from the samples of L1 produced identical genetic fingerprints. $(CAG)_5$, an additional probe, displayed up to 32 hybridizing fragments with *Hin*fI-restricted genomic *A. rabiei* DNA. On the other extreme, the fingerprint pattern with *Rsa*I-digested DNA and $(GATA)_4$ simplified to only one monomorphic locus (Fig. 4).

The DNA fingerprinting patterns of isolates collected in five fields in Beja identified 17 genotypes among 156 isolates. Figure 5 illustrates the patterns of 17 Tunisian genotypes, four Syrian isolates, and one isolate of *Ascochyta fabae* Speg. hybridized with $(CAA)_5$ and $(GACA)_4$. The choice of probe/enzyme combination is crucial for the discrimination of genotypes, e. g. the combination of *Rsa*I and $(GATA)_4$ reduced the fingerprint to one polymorphic locus (Fig. 6). The frequencies of the 17 genotypes were extremely uneven. Two genotypes accounted for 60.8% of the isolates.

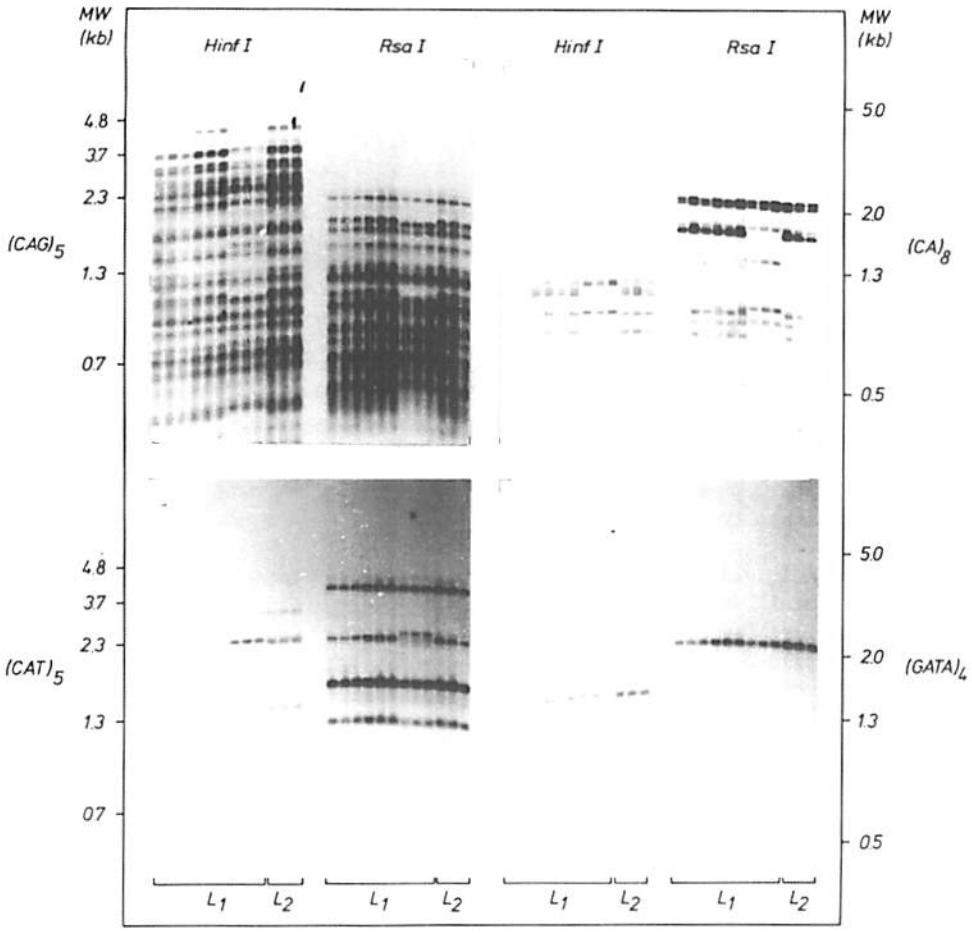

Figure 4. Oligonucleotide fingerprinting of single-spored *Ascochyta rabiei* isolates from two lesions (L1, L2) of a particular chickpea plant from field A. Genomic DNA was digested with *Hin*fI or *Rsa*I. Three isolates per lesion were investigated. For L1, each isolate was again single spored, and three groups of three secondary isolates each were applied to the gel.

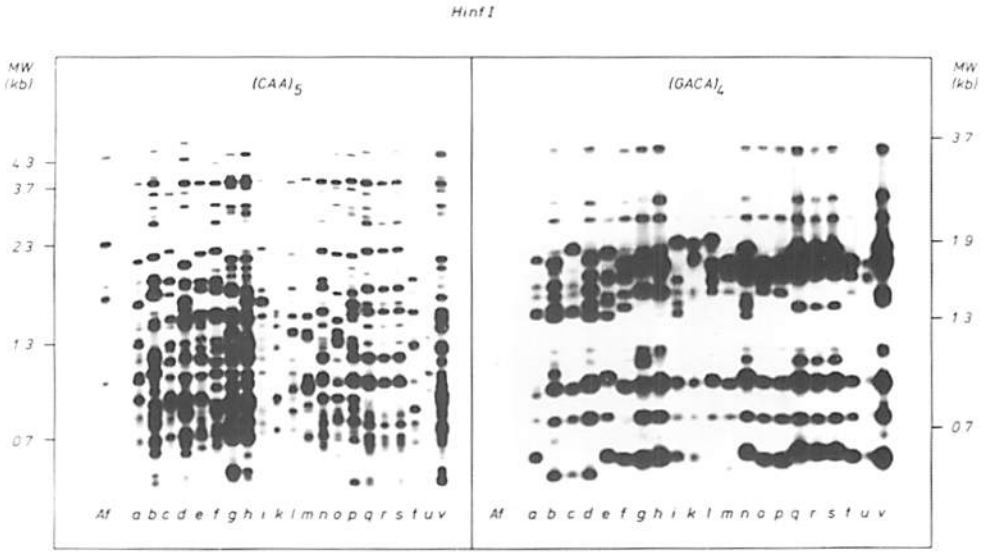

Figure 5. DNA fingerprinting of one *Ascochyta fabae* and 21 *Ascochyta rabiei* genotypes. Total DNA was digested with *Hin*fI and the gel was hybridized to the radio labeled synthetic oligonucleotides $(CAA)_5$ and $(GACA)_4$. Af: *A. fabae*; lanes a, b, c, and d are the Syrian genotypes R1, R2, R3, and R6, respectively, and lanes e to v represent the 17 Tunisian genotypes. Molecular weight markers are indicated in kb.

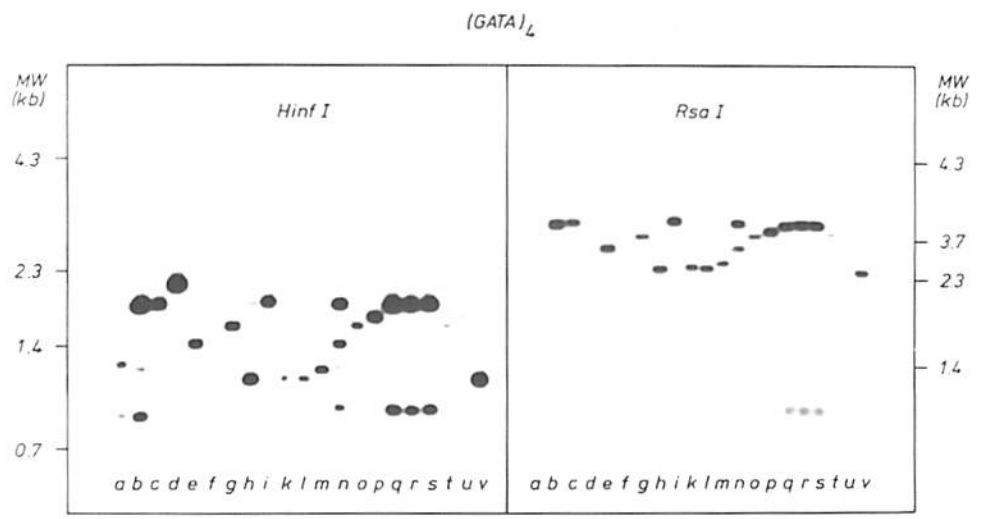

Figure 6. DNA fingerprinting of one *Ascochyta fabae* and 21 *Ascochyta rabiei* genotypes. Total DNA was digested with *Hin*fI or *Rsa*I and the gel was hybridized to the radiolabeled synthetic oligonucleotide $(GATA)_4$. Af: *A. fabae*; lanes a, b, c, and d are the Syrian genotypes R1, R2, R3, and R6, respectively, and lanes e to v represent the 17 Tunisian genotypes. The molecular weight markers are indicated in kb.

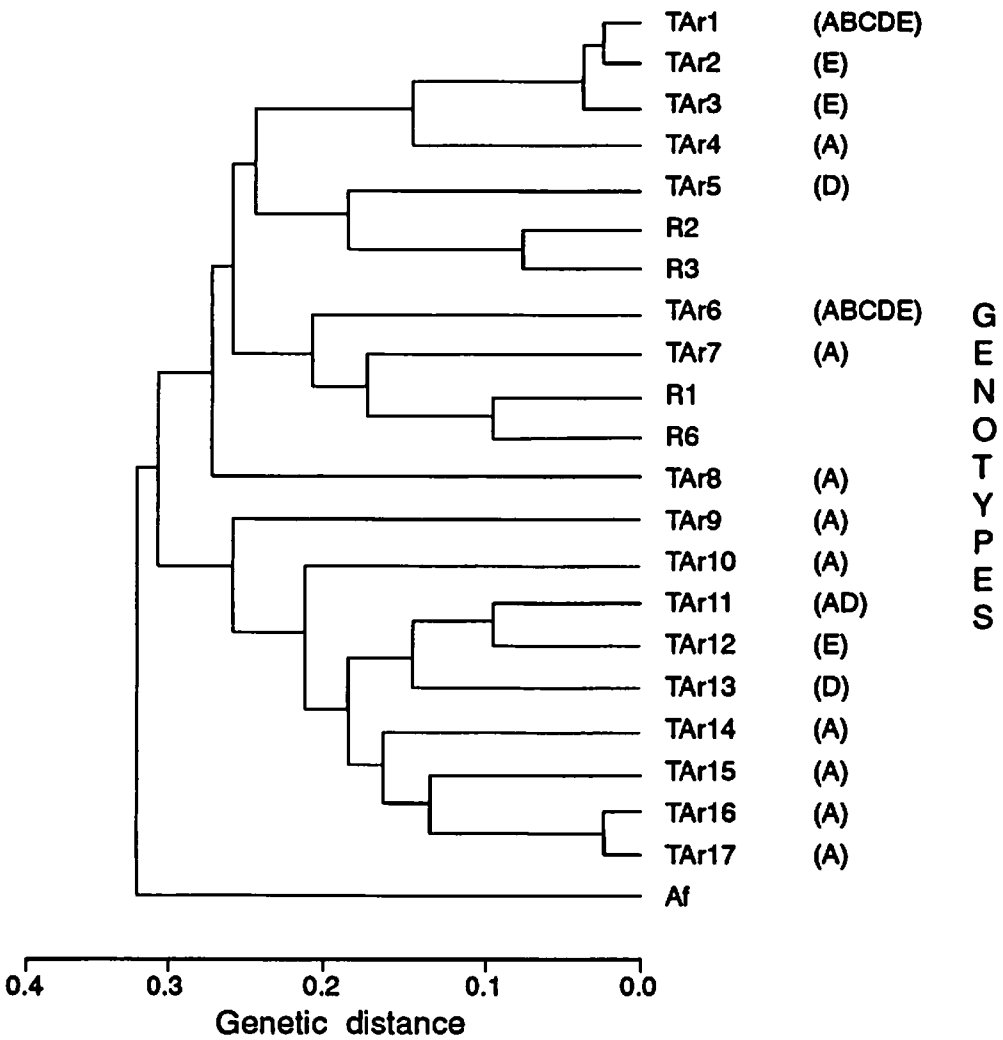

Figure 7. Phenogram based on UPGMA cluster analysis of fingerprint haplotypes of 17 different *A. rabiei* genotypes (TAr1 to TAr17) collected from five fields in the Beja region, four Syrian genotypes (R1, R2; R3 and R6), and one isolate of *A. fabae*. The analysis was based on the presence or absence of bands derived from *Hin*fI-digested DNA and the probes $(CA)_8$, $(CAA)_5$, $(CAG)_5$, and $(GATA)_4$. The occurrence of a particular genotype in fields A, B, C, D, or E is indicated in parentheses.

McDonald and Martinez (1990) conclude that isolates with the same genetic fingerprint are clonally related. Local populations of plant pathogenic fungi are genetically diverse, but may be dominated by one or a few genotypes. This has been detected using isozymes in *Atkinsonella hypoxylon* (Leuchtmann and Clay 1989), vegetative compatibility groups in *Fusarium oxysporum* Schlect em. Snyd. & Hans f.sp. *ciceri* (Padwick) Snyd. & Hans. (Corell et al. 1986) and *Cryphonectrica parasitica* (Anagnostakis and Kranz 1987), and DNA polymorphisms in *Rhynchosporium secalis* (Oud.) J.J.Davis (McDermott et al.1989), *Septoria tritici* (McDonald and Martinez 1990), *Phytophthora infestans* (Mont.) de Bary (Drenth et al. 1993), and *Magnaporthe grisea* (Herbert) Barr (Xia et al. 1993).

To evaluate genetic distances and relatedness between the 17 different *A. rabiei* genotypes, four isolates of *A. rabiei* from ICARDA, and one isolate of *A. fabae*, their fingerprint patterns were converted into a binary 0/1 matrix and subjected to UPGMA cluster analysis using the Treecon program package and a simple matching option (Van de Peer and De Wachter 1993). A phenogram derived from probes $(CA)_8$, $(CAA)_5$, $(CAG)_5$, and $(GATA)_4$ is depicted in Figure 7. All Tunisian genotypes of *A. rabiei* clustered more closely with Syrian isolates than with the *A. fabae* isolate. Values calculated on the basis of UPGMA, and the presence of common restriction fragments among isolates, indicate that oligonucleotide fingerprinting data can be used to reconstruct phylogeny. The similarity tree ordered the isolates into two major branches, in which group one consisted of 12 genotypes composed of three subgroups, and group two consisted of nine genotypes as closely related as the genotypes of group one.

Micro- and Macro-geographical Distribution of *Ascochyta rabiei* Populations

A multi-character genotype was derived for each isolate based on DNA fingerprinting patterns. Genotypes were classified on the basis of the presence or absence of a band, and each band was assumed to represent a single genetic locus (Stephens et al. 1992). Isolates with the same pattern were considered to be identical genotypes.

Nei's diversity index was used to measure genotypic diversity of *A. rabiei* populations (Nei 1973; Xia et al. 1993). Genotypic diversity in each location was calculated as:

$$H_{location} = 1 - \sum_{i=1} x_i^2$$

where x_i is the frequency of isolates with the i^{th} genotype in the field.

DNA fingerprinting diversity was partitioned into components within locations, between locations in a field, and between fields. Isolates from the same infected field were considered to belong to one population. The genetic diversity within each sub-population was calculated for each genotype in each location (H_L). This analysis allowed determination of the amount and distribution of genetic variability in *Ascochyta* spp. populations and estimation of gene flow, natural selection, or host-pathogen co-evolution in plant pathosystems (McDonald and Martinez 1990).

Genotype frequencies among the isolates were highly variable (Table 1). Two genotypes were found in all fields, TAr1 and TAr6, representing 38.2% and 22.6%, respectively, of the isolates in the region. The mean frequency of other genotypes ranged from 0.4 to 12%. In fields B and C, only the two prevalent genotypes were found. In contrast, the isolates collected in the central fields were diverse (Fig. 1). Most of the genotypic diversity was in field A, in which 12 genotypes were found. Nine genotypes were unique to this field. Field D contained five genotypes: TAr15 and Tar16, which were unique, TAr1 and TAr6, which were the prevalent genotypes, and Tar10, which was also found in field A. Field E was infested by five genotypes, three of them unique to the field. Five other genotypes, TAr2, TAr5, TAr6, TAr8, and TAr11, were found only once.

Table1. Genotype frequencies and measure of genotypic diversity in *Ascochyta rabiei* from five fields in the Beja region.

Genotypes	Fields					Mean (%)
	A	B	C	D	E	
TAr1	0.08	0.96	0.46	0.38	0.03	38.2
TAr2	0.02	0.00	0.00	0.00	0.00	0.4
TAr3	0.20	0.00	0.00	0.00	0.00	4.0
TAr4	0.04	0.00	0.00	0.00	0.00	0.8
TAr5	0.02	0.00	0.00	0.00	0.00	0.4
TAr6	0.02	0.00	0.00	0.00	0.00	0.4
TAr7	0.14	0.00	0.00	0.00	0.00	2.8
TAr8	0.02	0.00	0.00	0.00	0.00	0.4
TAr9	0.08	0.00	0.00	0.00	0.00	1.6
TAr10	0.10	0.00	0.00	0.27	0.00	7.4
TAr11	0.00	0.00	0.00	0.00	0.03	0.6
TAr12	0.20	0.04	0.54	0.15	0.20	22.6
TAr13	0.00	0.00	0.00	0.00	0.13	2.6
TAr14	0.00	0.00	0.00	0.00	0.60	12.0
TAr15	0.00	0.00	0.00	0.12	0.00	2.4
TAr16	0.00	0.00	0.00	0.08	0.00	1.6
TAr17	0.08	0.00	0.00	0.00	0.00	1.6
HL	0.86	0.08	0.49	0.73	0.58	0.77

Total genotypic diversity, estimated by Nei's diversity index based on the data obtained with probe $(CAA)_5$, was partitioned into hierarchical components according to location within the field. The within location component was 44.2%, the among location in the field component was 27.3%, and the among fields component was 28.5% (Table 2). Most of the genotypic diversity occurred at the lowest level, within the locations. This high within location component of diversity was largely due to the many unique genotypes in fields A, D, and E. The proportion of the total genotypic diversity due to differentiation among locations presented a moderate level of genetic differentiation.

Table 2. Hierarchical genotypic diversity of *Ascochyta rabiei* populations in the Beja region detected by DNA fingerprinting.

Diversity[†]	Field										Total	
	A		B		C		E		D			
	Cal.	%	Cal.	%	Cal.	%	Cal.	%	Cal.	%	Cal.	%
H_L	0.64	74.4	0.07	96.1	0.12	24.5	0.55	75.3	0.31	53.4	0.34	44.2
D_{LF}	0.22	25.6	0.01	3.9	0.37	75.5	0.18	24.7	0.27	46.6	0.21	27.3
D_{FT}	-	-	-	-	-	-	-	-	-	-	0.22	28.5
H_T	0.86	100.0	0.08	100.0	0.49	100.0	0.73	100.0	0.58	100.0	0.77	100.0

[†] Total diversity was partitioned into components within locations (H_L), between locations in a single field (D_{LF}) and between the five fields (D_{FT}). H_T is total diversity. Cal. = calculated value.

The extent of genetic diversity in fungal populations depends on several factors:

- The presence of a founder effect, in which genetic diversity is a function of the number of founders and the subsequent growth rate in the population (Nei et al. 1975).
- A random genetic drift may reduce genetic variability, while migration increases it.
- Selection over time may either amplify certain genotypes or reduce their presence. Host/pathogen co-evolution may contribute to the maintenance of genetic variability. Three cultivars were used in the Beja region: the susceptible Local Amdoun and two tolerant cultivars, Kasseb (ILC3279) and Chetoui (F83-46C). It is possible that genotypes TAr1 and TAr6 are better adapted to these environmental conditions, and the surrounding cultivars may influence which genotype will predominate.
- The presence of the sexual stage and therefore the occurrence of meiotic recombination affects genetic diversity. It is also possible that an exchange of cytoplasm during a limited parasexual cycle may lead to additional mtDNA and therefore to a more complex genetic diversity (Hintz et al. 1991). Although the sexual stage of *A. rabiei* is not reported in Tunisia,

a relatively high recombination rate may contribute to the high level of genetic diversity in the pathogen populations.

Virulence of *Ascochyta rabiei* Genotypes

Five isolates from the two prevalent genotypes, and one isolate from each of the other genotypes, were selected at random and used in inoculation tests. The reaction of four chickpea cultivars to *A. rabiei* isolates was estimated under controlled conditions. These cultivars were chosen because they are used as sources of resistance to *A. rabiei* in chickpea breeding programs. The set of differentials included Local Amdoun I, ILC3279, ILC482, and F83-46C.

Seed of the four cultivars was sown in 60 × 48 cm plastic trays. Three replications were used. The trays were placed in a growth room and maintained at 22° C with 16 hours of light. Twelve days after sowing, chickpea seedlings were sprayed until run-off with a fresh spore suspension prepared from single spored cultures of *A. rabiei*. The inoculum was standardized at 10^6 spores/ml. The inoculated plants were immediately placed in a dew chamber and incubated for 48 hours, and then returned to the growth room. Three weeks after inoculation the plants were evaluated for disease reaction based on the 1–9 ICARDA scale (Weising et al. 1991) and LII (Linear Infection Index; Riahi et al. 1990).

Virulence diversity of *A. rabiei* is a difficult trait to characterize and is completely dependent on the differential chickpea cultivars and the reaction disease scales used. It is difficult to designate the different genotypes as races. There is, however, strong evidence from several independent studies that races of *A. rabiei* exist (Grewal 1984; Vir and Grewal 1974; Reddy and Kabbabeh 1985). But the host set disease rating scale and severity categories in each of these studies vary, and uniform techniques for race identification in *A. rabiei* are not available (Jan and Wiese 1991).

Different fungal isolates can be discriminated by DNA fingerprinting. This is not possible using conventional techniques such as virulence tests or evaluation of morphologic characters. Indeed, identification of isolates based only on their virulence to a set of host differentials suffers from several disadvantages. The logistic problems of greenhouse tests make it difficult to collect data on the large number of isolates necessary for population studies. That reproducibility is poor was demonstrated by the fact that different investigators give a different order for the virulence reactions of the same *A. rabiei* isolates (Weising et al. 1991; Reddy and Kabbabeh 1985). The characteriza-

tion of isolates based only on virulence data is not necessarily correlated with the amount of genetic variation in the pathogen's genome as a whole (McDonald et al. 1989).

Table 3. Virulence tests of *Ascochyta rabiei* isolates with two rating scales.

Isolate	Genotypes	LII[†]	1–9 scale[‡]
153	TAr15	W	W
1313	TAr5	W	M
181	TAr12	W	M
151	TAr16	W	M
1325	TAr9	W	M
1314	TAr7	W	M
1273	TAr4	M	M
128	TAr14	M	M
122	TAr11	M	M
1322	TAr8	M	M
1313	TAr6	M	M
1131	TAr12	M	M
184	TAr12	M	M
183	TAr12	M	M
1268	TAr2	M	M
1329	TAr17	M	M
1323	TAr10	M	M
1270	TAr3	M	H
1266	TAr1	H	M
176	TAr1	H	M
1133	TAr1	H	M
1319	TAr12	H	M
125	TAr13	H	M
1333	TAr1	H	H
1142	TAr1	H	H

[†] Ascochyta blight rated on Riahi et al. (1990). Isolates causing mean disease rating of 1.0–4.9, 5.0–10.0, and >10 were considered weakly virulent (W), medium virulent (M), and highly virulent (H), respectively.

[‡] Ascochyta blight rated on Weising et al. (1991). Isolates causing mean disease rating of 1.0–2.9, 2.9–5.9, and 6–9 were considered weakly virulent (W), medium virulent (M), and highly virulent (H), respectively.

Conclusion

On the basis of our data we conclude that populations of *A. rabiei* are genetically heterogeneous and this heterogeneity can be precisely and reliably determined by DNA fingerprinting. This technique therefore is of special interest to plant pathologists, because it allows accurate monitoring of the movement of particular genotypes over large geographical areas. Compared to other methods of differentiation between isolates of *A. rabiei*, DNA fin-

gerprinting offers several advantages. It is faster for differentiation than measurement of sporulation, growth rate, and pathogenic and physiological parameters (e.g. toxin production). Compared to molecular approaches such as RFLP (Meyer et al. 1992) or RAPD analysis, oligonucleotide fingerprinting is faster than RFLP and more reliable in reproducibility than RAPD. DNA fingerprinting will also allow a measurement of the flow of distinct virulent genotypes in natural pathogen populations, and thus will be useful in studying host/pathogen co-evolution.

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Towards a Diagnostic PCR Kit for the Rapid Identification of *Ascochyta rabiei* Genotypes

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Abstract

PCR amplifications with microsatellite motifs such as$(GAA)_5$, $(GACA)_4$, $(GATA)_4$, and $(GGGTTT)_3$ as primers were successfully carried out with genomic DNA from *Ascochyta rabiei* (Pass.) Lab. and displayed a high degree of length polymorphism. A polymorphic low molecular weight band, resulting from an amplification with $(GGGTTT)_3$ as a primer, was isolated from an agarose gel and cloned into a sequencing vector. The DNA sequence of the cloned amplification product (isolated from the aggressive Syrian isolate AA6) showed intermingled repetitive motifs throughout the locus. Seventeen distinct microsatellite motifs with different repeat numbers were detected in a 380 bp sequence. Hybridizing the cloned probe to a Southern blot with restriction-digested DNA of *A. rabiei* genotypes from Tunisia, Pakistan, and Syria revealed that the probe creates an RFLP that is present in all investigated genotypes, even those from different geographical regions. Because the length difference of the restriction fragments is in almost all cases rather small (100–200 bp), the polymorphism is probably due to the repeat number itself rather than a gain or loss of a restriction site. This offers the possibility of looking for consensus sequences surrounding this locus in different *A. rabiei* genotypes in order to synthesize unique PCR primers that will allow an *A. rabiei*-specific amplification of this particular polymorphic locus. Distributed as a kit, this technique could be used by national breeding institutes to identify and classify known and unknown isolates using fast and reliable PCR-based diagnosis.

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Introduction

Ascochyta blight, caused by *A. rabiei*, is a devastating fungal disease of chickpea (*Cicer arietinum* L.), an economically important legume crop in the dry areas of West Asia and North Africa. Occurrence of *A. rabiei* has been reported in most chickpea growing countries, with crop loss as high as 100% (Saxena and Singh 1987). This ascomycete is notorious for variability in morphology (Grewal 1984; Kaiser 1973), pathogenicity, and phytotoxin production (Alam et al. 1989; Höhl et al. 1991). Therefore identification based on cultural and/or pathogenic characteristics has been difficult and suffers from several disadvantages. Though biological pathotyping is time consuming and labor intensive, its reproducibility is poor. For these reasons, various investigators have looked for alternative characters to distinguish between genotypes.

One such character, a specific feature of eukaryotic genomes, is repetitive DNA. Sequences repeated in tandem are particularly useful for the detection of DNA polymorphisms, because the numbers of their basic repeat motifs are often highly variable, and blocks of these motifs are generally dispersed throughout the genome of a given species. Hybridization with a suitable probe, complementary to such tandem repeats, with restriction-digested genomic DNA simultaneously detects several polymorphic loci, thereby creating specific banding patterns, the so-called DNA fingerprints (reviewed by Pena et al. 1993). Oligonucleotide fingerprinting (reviewed by Epplen et al. 1991) is a useful technique to distinguish *A. rabiei* genotypes (Weising et al. 1991; Morjane et al. 1994), which reveal large amounts of repetitive DNA compared to other species (Lagercrantz et al. 1993).

Repetitive oligonucleotides such as $(GAA)_5$, $(GACA)_4$, $(GATA)_4$, and $(GGGTTT)_3$ were successfully used as primers in PCR amplifications (Morgante and Olivieri 1993) with genomic *A. rabiei* DNA, obtaining highly polymorphic patterns. The level of informativeness was about 60% of oligonucleotide fingerprinting, and the rate of reproducibility was about 10% lower compared to fingerprinting, which is 100% reproducible when radioactive techniques are used.

A general problem of PCR techniques is that in RAPD analyses (Welsh and McClelland 1990), bands of higher molecular weight (2,500 bp) are not always present in identical reactions. This suggests that specific low molecular weight amplification products should be exploited instead. These are generated with unique primers created from consensus sequences in the flanking regions of a polymorphic locus. Such primers, which are highly specific and do not usually cross-react with other organisms, would allow the establishment of a PCR kit for amplification of a distinct part of the *A. rabiei* genome.

For this reason a 380 bp amplification product, using the genomic *A. rabiei* DNA of the aggressive Syrian isolate AA6 as a template and $(GGGTTT)_3$ as a primer, was excised from an agarose gel and cloned into a sequencing vector. Sequence data revealed intermingled microsatellite motifs throughout the locus. Hybridizing the cloned probe to a Southern blot of genomic *Hinf*I- and *Taq*I-digested *A. rabiei* DNA showed that such a probe creates an RFLP present in Syrian, Tunisian, and Pakistani isolates. Techniques have been established that allow the isolation of clonable restriction fragments homologous to radioactively detected RFLP. A technique for the rapid PCR screening of whole *Escherichia coli* colonies directly from the Petri dish has also been developed. Since the restriction fragment in question carries the amplifiable $(GGGTTT)_3$ motif, PCR screening of transformed colonies is possible. $(GGGTTT)_3$-positive clones representing different *A. rabiei* genotypes can be sequenced and screened for consensus sequences characteristic of this genome, which can be used to synthesize unique PCR primers (Thomas and Scott, 1993) allowing an *A. rabiei*-specific amplification of this particular polymorphic locus.

The practical applications of a PCR kit for the rapid identification and classification of *A. rabiei* genotypes are: (i) fast monitoring of field conditions with only minute amounts of the pathogen; (ii) forecasting of the geographical distribution and movement of certain genotypes; and (iii) screening of chickpea germplasms for *A. rabiei* contamination to prevent seed transmission. Several tests must be done before such a kit is suitable for national breeding institutes. The specificity of the reaction must be tested on all other available *Ascochyta* spp., a representative set of chickpea pathogenic fungi of other species, and the chickpea itself. In all these test organisms there should be no cross-reaction where *A. rabiei* gives a clear amplification signal.

The molecular techniques for constructing a diagnostic PCR kit are as follows:

- PCR amplifications with microsatellites as primers.
- Cloning of a polymorphic PCR band isolated from an agarose gel.
- RFLP analysis with the cloned probe.
- Cloning of microsatellites isolated with their flanking sequences.
- Sequencing of microsatellite-positive restriction fragments.
- Comparison of flanking sequences from different *Ascochyta rabiei* genotypes.
- Construction of unique primers from consensus sequences.
- Establishing the kit.

PCR Amplifications with Repetitive Primers

Since fingerprinting of *A. rabiei* with repetitive oligonucleotides proved successful, microsatellite motifs were also tested as primers in PCR experiments. For example, polymorphic patterns were obtained with $(CAA)_5$, $(GAA)_5$, $(CCTA)_4$, $(GACA)_4$, $(GATA)_4$, $(GTTA)_4$, and $(GGGTTT)_3$ as primers. Stable banding patterns were derived from 200 to 2,500 bp. Especially weak bands of higher molecular weight were not stable in identical reactions under various conditions. The level of informativeness was generally lower than in oligonucleotide fingerprinting, and only one of the primers discriminated all genotypes. Examples are shown in Figures 1–3.

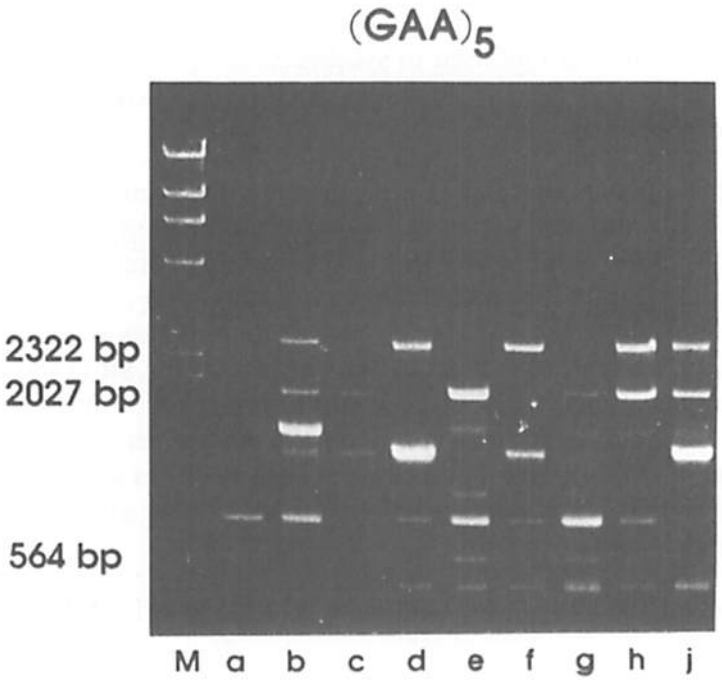

Figure 1. PCR amplifications with a microsatellite as a primer. Lane M = Molecular weight marker (*Hind*III-digested lambda DNA), lane a = Syrian isolate AA6, and lanes b-j eight predominant Tunisian genotypes from the Beja region. The primer $(GAA)_5$ discriminates all genotypes that can also be detected by oligonucleotide fingerprinting, and is so far the most polymorphic.

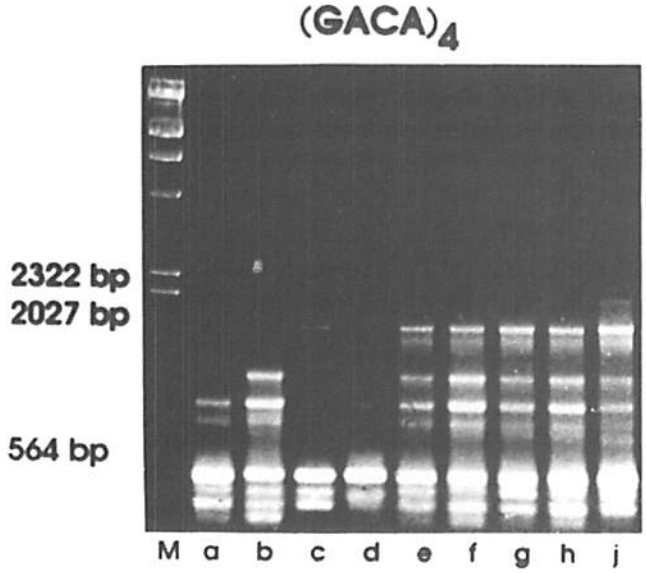

Figure 2. PCR amplifications with a microsatellite as a primer. Lane M = Molecular weight marker (*Hin*dIII-digested lambda DNA), lane a = Syrian isolate AA6 and lanes b-j eight predominant Tunisian genotypes from the Beja region. The primer $(GACA)_4$ displays a rather monomorphic pattern, which is only polymorphic in the very low molecular weight range. It discriminates only four genotypes, while oligonucleotide fingerprinting detects nine.

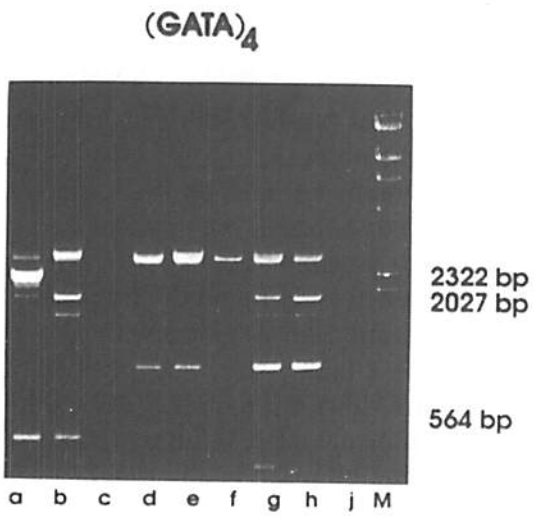

Figure 3. PCR amplifications with a microsatellite as a primer. Lane a = Syrian isolate AA6, lanes b-j = eight predominant Tunisian genotypes from the Beja region and lane M = Molecular weight marker (*Hin*dIII-digested lambda DNA). The primer $(GATA)_4$ displays a polymorphic pattern, but the haplotypes in lanes d and e and in lanes g and h show identical banding. The primer discriminates seven out of nine genotypes that can also be detected by oligonucleotide fingerprinting.

The aggressive Syrian isolate AA6 (lane a) and individuals of eight predominant Tunisian oligonucleotide fingerprint genotypes from Beja (lanes b–j) were used for PCR amplifications. The Syrian isolate always discriminates well from the Tunisian isolates. Conditions favoring the amplification of low molecular weight products were established (20 seconds annealing at T_m [Thein and Wallace 1986] and 20 seconds elongation at 72° C; 50 pmol primer, 150 mmol dNTPs, 50–100ng template, 50 ml, 37 cycles). This allows the use of PCR bands of 2,500 bp as additional characters for the calculation of genetic distances from band-sharing data using cluster analysis.

Much effort has been invested in the transformation of one of the loci amplified by $(GGGTTT)_3$ as a primer into a single locus amplified by unique primers that flank the repetitive region. The amplification of only one specific polymorphic locus of known sequence by PCR combines all advantages of this technique (incomparably fast, non-radioactive, with only minute amounts of the pathogen needed) and eliminates all disadvantages (low reproducibility, separation of many PCR bands by gel electrophoresis, cross-reactions with other organisms). Presented in an elaborate kit, this technique would enable national breeding institutes to identify and classify *A. rabiei* unequivocally within hours, using absolutely harmless molecular techniques (non-radioactive, non-recombinant).

Cloning and Sequencing of a Microsatellite-primed PCR Product

A 380 bp amplification product, derived from a genomic DNA template of the aggressive Syrian isolate AA6 using $(GGGTTT)_3$ as a primer (Fig. 4), was excised from an agarose gel and purified. Since the ends of PCR-amplified DNA stretches are not defined, they cannot be cloned easily into the multiple cloning site of a plasmid vector. Therefore a combined fill-up and ligation reaction is used for cloning PCR fragments. The pSK vector plasmid (Stratagene) was restriction-digested with Eco RV, which sets a blunt-end cut in the multiple cloning site. The purified amplification product was added together with deoxynucleotides (20mmol each), T7 polymerase, and T4 ligase. The T7 polymerase fills the undefined ends with the deoxynucleotides, so they become blunt ends and the T4 enzyme ligates the blunt ends of the vector and PCR product. The Eco RV restriction site is lost during this procedure, but the insert can be cut out from the vector with Pst I and Xho I.

Ten percent of the fill-up ligation reaction (5μl) was used to transform (electroporate) competent cells of the *E. coli* X-LS 1 Blue strain (Stratagene). White colonies were selected from X-gal/IPTG-agar and whole colonies were used for PCR screening. Colonies were picked from the agar, vortexed in

100 ml 50% chloroform/50% TE and centrifuged. About 10% of the supernatant was taken as a template for the amplification with $(GGGTTT)_3$ (annealing 54° C, 50 pmol primer, 150 mmol dNTPs, 50 ml, 37 cycles). A microsatellite-amplification-positive colony was selected. The recombinant fragment stays stable during subculture of the *E. coli* vector and has a constant molecular weight when excised from the plasmid with Pst I and Xho I.

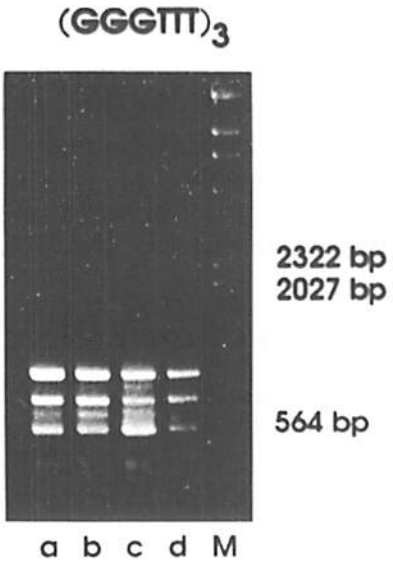

Figure 4. PCR amplifications with a microsatellite as a primer. Lane a = Syrian isolate AA6, lanes b-d = three predominant Tunisian genotypes from the Beja region discriminated by oligonucleotide fingerprinting. Lane M = Molecular weight marker (*Hin*dIII-digested lambda DNA). The single low molecular weight band (380 bp) resulting from genomic DNA of the aggressive Syrian isolate AA6 and $(GGGTTT)_3$ as a primer (lane a) was excised from the agarose gel and cloned into a sequencing vector. Sequence data revealed intermingled microsatellite motifs throughout the whole locus.

The purified recombinant plasmid (5mg) was used for a dideoxy sequencing reaction (Sanger and Coulson 1975). Intermingled repetitive sequences were detected throughout the whole locus. Seventeen distinct microsatellite motifs with different repeat numbers were found in a 380 bp sequence. A partial sequence structure is, for example, 5' -$(GGGTTT)_3$ $(GT)_6$ $(GAGTA)_3$ $(CATTT)_5$ $(ATTTT)_{17}$ $(CA)_8$ $(CACAA)_6$ $(CAA)_5$ $(ATTTC)_7$ $(ATTTCATATC)_5$ ...-3'. The core consensus of telomeric sequences in many organisms (ciliates, yeasts, and some plants)is $(G_{1\text{-}4}T_{1\text{-}4})_n$ and the primer used $(G_3T_3)_3$ matches this motif. Micro- and minisatellites can be abundant in subtelomeric regions. We suggest that a subtelomeric microsatellite island, possibly immi-

grated from telomeric regions, has been isolated from *A. rabiei*. Chromosome ends appear to be very stable in all organisms, because a loss of telomeric sequences destroys the chromosome in a few cell cycles and is therefore lethal.

The sequence itself is potentially polymorphic between different *A. rabiei* genotypes on the basis of its repetitive character and its variable repeat numbers. This is because only one locus of one isolate (AA6) has been sequenced. However, a repeat unit such as $(ATTTT)_{17}$ can easily be different in other genotypes and, for example, display an $(ATTTT)_{15}$ or $_{20}$. Further sequencing of the locus is not necessary to establish the PCR kit, because its polymorphism has already been detected by RFLP analysis. Extended sequencing will be done in the flanking regions of the locus to detect unique consensus sequences for the design of primer sequences from *A. rabiei* isolates from different regions (Syria, Tunisia, and Pakistan).

RFLP Analysis with a Cloned Microsatellite Island

The cloned and sequenced microsatellite island was separated from the vector DNA and radio labeled with ^{32}P dCTP by nick translation. The probe was hybridized to a genomic Southern blot with *Hin*fI-digested DNA from 34 Tunisian and two Syrian isolates (Fig. 5).

Such a highly repetitive probe creates an RFLP, and its target sequence is present in all 36 tested genotypes, even though they originate from widely separated geographical regions.

Under stringent hybridization conditions, one polymorphic single locus was detected in 32 out of 36 genotypes. Only four genotypes showed a second hybridization signal (gain of an additional restriction site or duplication of the locus), and two genotypes displayed a single signal of significantly higher molecular weight (loss of a restriction site). Because the length difference of the restriction fragments in all other genotypes was rather small (100–200 bp), the polymorphism in these cases is probably due to the repeat number itself, rather than a gain or loss of a restriction site. This suggests that the restriction sites flanking this $(GGGTTT)_3$ amplified locus, as well as the surrounding sequences, are highly conserved throughout many *A. rabiei* genotypes.

Isolation, cloning, and sequencing of restriction fragments from different *A. rabiei* genotypes carrying this microsatellite island are expected to reveal consensus sequences characteristic for this particular genome. These will be used to synthesize PCR primers that allow *A. rabiei*-specific amplification of this polymorphic locus.

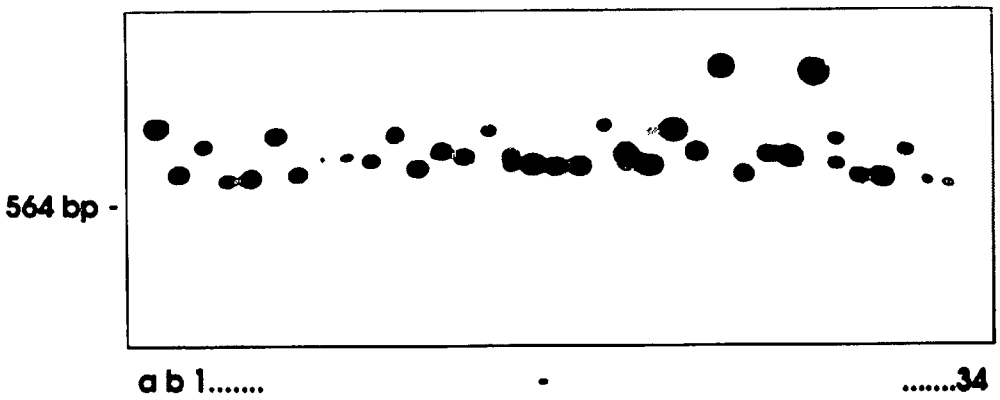

Figure 5. RFLP analysis of various *A. rabiei* isolates from different countries, using the cloned PCR product derived from genomic DNA of the aggressive Syrian isolate AA6 and the microsatellite primer $(GGGTTT)_3$. The repetitive insert was separated from the vector DNA, radio labeled and hybridized to a Southern blot with genomic *Hin*fI-digested DNA from the two Syrian genotypes AA2 and AA6 (lanes a and b) and 34 genotypes from Tunisia, discriminated by oligonucleotide fingerprinting (lanes 1–34). Nine possible band positions were detected. Isolates in lanes 7, 14, 20, and 28 display two hybridizing signals (gain of an additional restriction site or duplication of the locus) and the isolates in lanes 23 and 27 show a signal of significantly higher molecular weight (loss of a restriction site). All other genotypes display a single locus pattern polymorphic between 600 and 800 bp.

Techniques have been established to isolate clonable restriction fragments directly from the blotting gel. A conventional-sized gel bed was filled with a double amount of agarose. Extensively *Taq*I- or *Cla*I-digested genomic *A. rabiei* DNA was applied to the gel and separated by electrophoresis. The gel was cut horizontally with a very fine metal string (.011 mm) and the upper half was blotted onto a nylon membrane, while the lower half was stored at 4° C. The membrane was hybridized to the radioactive probe and a strong signal was obtained. The lanes on the autoradiograph were matched with the ethidium bromide lanes of the stored gel, and agarose blocks, which precisely represent the radioactively-detected restriction fragments, were cut from the gel. The DNA was purified and a small aliquot of each fraction was blotted onto a nylon membrane. Hybridization revealed the positive fractions, which contained the enriched *Taq*I restriction fragments that carry the already cloned and sequenced microsatellite island together with unique flanking DNA. The positive fractions were ligated into the *Cla*I site of the pSK vector plasmid and competent cells of the *E. coli* X-LS1 Blue strain were

transformed (electroporated). White colonies were picked from X-gal/IPTG-agar and prepared for colony hybridization.

Four isolates were selected for this experiment, two from Syria (AA6 and AA17), one from Tunisia (T28) and one from Pakistan (P3). *Taq*I and *Cla*I were selected as restriction enzymes because their cut restriction sites are easily clonable into sequencing vectors. Signals resulting from *Cla*I-digests in the 15kb range were much too large for the construction of a PCR kit. Signals resulting from *Taq*I-digested DNA occurred at a molecular size of about 1,400 bp, an excellent size for cloning, and one which also promises to contain enough non-repetitive unique DNA to find consensus sequences. No RFLP could be detected between the two Syrian isolates AA6 and AA17. When isolates from different countries were compared, a polymorphism was visible between all three of them. The Tunisian isolate (T28) was selected because it shows two hybridizing bands with *Hin*fI as a restriction enzyme (Fig. 3). The same holds true for TaqI, were a double band appears in the 1,400 bp range, suggesting that duplication is more likely than appearance of an additional restriction site.

Both bands were saved from the gel and cloned to compare the sequences. The two Syrian and the Pakistani isolates displayed a single locus pattern. For colony hybridization white colonies from blue/white screening were pitched onto fresh plates and incubated. Colonies were lifted onto a nylon membrane and hybridized at 68° C with a hot probe generated from the cloned repetitive PCR fragment. After a stringent washing step, membranes were exposed to an X-ray film. Strong signals appeared. About 300 colonies were screened per tested isolate: for S6, two, for S17, ten, for T28, four, and for P3, three positive colonies were detected and prepared for a DNA sequencing reaction.

Establishing a Diagnostic PCR Kit for Practical Applications

Since positive transformations of the *E. coli* vector with the enriched fractions of the microsatellite island containing restriction fragments have already been achieved, and colony hybridization has also been successful, only sequencing remains to be carried out. Isolates from North Africa (Tunisia), the Middle East (Syria), and Asia (Pakistan) have been selected to obtain the highest possible variability of *A. rabiei* genotypes. Consensus sequences shared by all of these investigated haplotypes will make it possible to identify and classify *A. rabiei* genotypes from around the world on the same molecular basis.

Even if consensus sequences are detected, several tests must be carried out before the kit is ready for national breeding institutes. The specificity of the reaction for *A. rabiei* has to be tested with several primer sets before the optimal primer combination can be determined. Test objects include all other available *Ascochyta* species (*A. pisi*, *A. fabae*, etc.), a representative set of pathogenic fungi of Leguminosae (*Fusarium*, *Botrytis*, and *Phoma* species), and chickpea. There should be no cross-reaction with the primer set under conditions where *A. rabiei* DNA gives a clear amplification signal. The composition of the bases in the primer sequence should be chosen from available consensus sequences, so that the highest possible annealing temperature can be used for the reaction, and so that there are no primer/primer interactions or secondary structures of the oligos. The annealing reaction of the primers with genomic *A. rabiei* DNA should be specific across a broad temperature range (5° C) to avoid difficulties that can arise by transferring the detection kit from one PCR machine to another.

A diagnostic *A. rabiei* detection kit should contain:

- Unique primers in an optimized concentration.
- *A. rabiei* control DNA (positive control).
- Chickpea and *Fusarium* spp. DNA (negative controls).
- 10x buffer with optimized $MgCl_2$ concentration.
- dNTPs in an optimized concentration.
- Taq-polymerase in an optimized concentration.
- DNA length standards that identify the resulting amplification products .
- A manual including a standard suggested thermal cycle program.
- A list of thermal cyclers on which the kit was tested.
- A manual for the DNA extraction from the smallest specimen of *A. rabiei* with the desired template-end concentration indicated.

Aside from radioactive methods, the PCR technique represents the most sensitive detection system so far developed. Until now, diagnostic detection kits (for example, to identify pathogens) have been based largely on immunoassays, such as Western blot techniques (Duncan and Torrance 1990). Antibody production and protein isolation are time consuming, labor intensive, and require large amounts of the tested organisms as extensive technical equipment. PCR overcomes these problems. With a primer pair of 18–25 bases each, which finds a 100% matching sequence in the *A. rabiei* genome and amplifies a region of known molecular size, only a few cells of the pathogen are needed for detection. Neither the culturing of *A. rabiei* over long periods of time nor difficult isolation techniques (protein or DNA) are

necessary. Crude cell extracts (for example, a simple chloroform extract) are usually sufficient, if primers of the highest possible specificity are used. Until now, at least one month of hard work has been required just to calculate a rough number of *A. rabiei* genotypes in a single chickpea field. PCR with specific primers can reduce the length of this procedure to only one day: collecting lesions from chickpea plants in the morning, PCR from whole lesions at noon, running the gel in the afternoon, and learning the result in the evening.

A multitude of applications can be imagined for such a fast and sensitive detection method. Screening of chickpea germplasms also seems possible, but may require a more labor-intensive isolation procedure, since *A. rabiei* is not present on the seed surface but on the embryo, covered by the cotyledones. Evaluation and forecasting of conditions can most probably be performed with whole lesions.

The classificatory potential of the method has not yet been fully explored. Based on RFLP analysis, nine RFLP groups have been detected among 36 oligonucleotide fingerprint genotypes. Once the identification is based on a unique primer pair, resolution will be much higher, depending on the electrophoresis matrix used. Polyacrylamide or gel matrixes especially developed for PCR applications (e. g. Visigel™, Stratagene) can display a much finer classification scale. The primers probably do not reveal any relationship to pathogenicity of a certain identified isolate (because they detect one polymorphic locus on only one chromosome) unless a duplication of this particular locus or the whole chromosome (partial diploidy) is involved in pathogenicity. Nevertheless, this work is exemplary, because once pathogenesis-related loci are identified, a kit could be constructed to evaluate the aggressiveness of an upcoming pathovar at a very early stage.

Acknowledgments

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Host–Plant Resistance

Studies on Inheritance of Ascochyta Blight Resistance in Chickpea

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Abstract

The literature on the mode of inheritance of resistance to ascochyta blight in chickpea presents a wide range of study results. There are cases of monogenic, digenic, oligogenic, and polygenic control, and resistance is described by different authors as qualitative, quantitative, vertical, and horizontal. This has created a need to coordinate inheritance studies, combine and link the results, and arrive at an integrated concept. The quantitative nature of disease resistance and the evidence of a significant vertical component are acknowledged. We conclude that for breeding purposes gene pyramiding should be attempted, with diverse germplasm and diverse pathogenic isolates used. Lower mean environmental disease ratings will enhance effective selection for resistance.

Introduction

Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Lab. is a serious foliar fungal disease of chickpea, but only in some of the world production areas. The disease poses a serious problem to cultivators and researchers alike in West Asia and North Africa, where attempts are being made to replace spring sowing with winter sowing. Not only is the variability of the pathogen very great (Reddy and Kabbabeh 1985; Jan and Wiese 1991; Porta Puglia 1992), but the varietal response of chickpea to the disease also varies over the full range of the often used 1–9 scale (Reddy and Singh 1990). Review of the literature on the mode of inheritance of resistance to ascochyta blight raises pertinent questions: is resistance horizontal or vertical, is its control monogenic or polygenic, and is it qualitative or quantitative (Reddy

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et al. 1992; Gowen et al. 1989; Malik 1990)? This report will review the literature, discuss results of recent studies in India, give a synthesis of current knowledge, and propose strategies for genetic control of the disease.

Inheritance of Resistance to Ascochyta Blight

Review of literature

The literature on the inheritance of resistance to ascochyta blight in chickpea varies greatly. Hafiz and Ashraf (1953) were probably the first to report on the inheritance of resistance to the disease. Their hypothesis was that the resistance was monogenically controlled. However, their classification of resistance and susceptibility was arbitrarily set at the 50 percent index point, and the data gave evidence of a more complex control system (Table 1).

Table 1. Number of plants, grouped by disease index representing chickpea reaction to the ascochyta blight fungus as observed by Hafiz and Ashraf (1953).

Cross	Classes of disease indices (%)				Total Population
	A 0–15	B 16–50	C 51–90	D 91–100	
P_7	0	0	8	92	100
F_8	100	0	0	0	100
$F_1(P_7 \times F_8)$	6	0	0	0	6
$F_1(F_8 \times P_7)$	8	0	0	0	8
$F_2(P_7 \times F_8)$	115	25	28	16	184
$F_2(F_8 \times P_7)$	382	84	100	60	626
Total F_2	497	109	128	76	810

Other authors—Vir et al. (1975), Singh and Reddy (1983), and Singh and Reddy (1989)—also report simple Mendelian inheritance of ascochyta blight resistance. Here again an arbitrary division by susceptibility and resistance, influenced by environment, seems to have affected the hypotheses. Table 2 presents a hypothetical case of decagenic inheritance, where 10 genes have 2 alleles each that contribute equally and independently to resistance in an additive fashion.

Usually, ascochyta blight damage is scored on a 1–9 scale, where 1 = no symptoms and 9 = plant killed (Nene et al. 1981; Reddy and Singh 1990). In our hypothetical case, we assumed that each resistance allele reduces the disease score by 0.4. Where all resistance alleles are present, the disease rating is 1, and where only susceptibility alleles are present, the disease rating is 9. The F_2 population will show a "monogenic dominant" 3:1 ratio for resistance:susceptibility if we classify as resistant all plants with scores

≥5.4, and as susceptible all plants with scores >5.4. But if we classify resistance at ≤4.2 and susceptibility at >4.2 the ratio will be a "monogenic recessive" 1:3. This example is given to stress the danger of classifying plants in distinct groups for traits that are continuous in nature, as in the case of ascochyta blight resistance.

Results of international chickpea screening nurseries reported by Reddy et al. (1992) give the impression that resistance against ascochyta blight is qualitative and vertical in nature because of the classification into resistant and susceptible, and the reversals from location to location. Singh (1993a and 1993b) conclude that resistance to ascochyta blight was commonly considered monogenically controlled, with either a single dominant or recessive gene conferring resistance. But he also observes that varieties have shown different reactions at different locations, suggesting that more genes play a role. Tewari and Pandey (1986) recorded clear cut Mendelian ratios in their inheritance studies of ascochyta blight resistance, supporting the hypothesis of one recessive and two dominant genes controlling resistance. They conducted their studies under field conditions at Pantnagar, India, an environment conducive for botrytis gray mold incidence in chickpea but not for ascochyta blight. In a recent study Dey and Singh (1993) identified one recessive and five dominant genes for ascochyta blight resistance, but they also conclude, using generation mean analysis, that the genes did not follow the simple Mendelian inheritance pattern but were influenced by inter-allelic interactions. Pieters and Taheri (1986), while breeding for horizontal resistance in Morocco, observed that the percentage of chickpea pod infection by ascochyta blight remained "fairly constant" over three years. All tests were done at Rabat, but each year the isolate was from a different place. We found that the correlation coefficients between the scores for their 11 tested varieties varied from $r = 0.80$ to $r = 0.97$. The isolates, however, may not have differed in virulence. The authors concluded that the control of resistance is oligogenic and additive. Gowen et al. (1989) observed ranking stability for chickpea varieties exposed to different isolates of ascochyta blight and concluded that resistance is controlled polygenically. Their observation that spread of ascochyta blight isolates on resistant cultivars caused rapid increases in pathogenicity (which they thought could possibly explain why the most aggressive isolates come from areas of intensive chickpea cultivation where disease pressure is greatest) is alarming. This is especially worrisome for the change from spring sowing to winter sowing over larger areas when and where the conditions for the pathogen are particularly favorable. However, Strange (1994) feels that the issue of whether races of the pathogen really exist has not yet been resolved.

Table 2. Hypothetical F_2 population of a decahybrid cross with 10 independent genes for disease reaction[1]

	Number of susceptible alleles							
	0	1	2...	8...	11...	18	19 →→→	20
	20	19 ←←←	18...	12...	9...	2	1	0
	Number of resistant alleles							
Disease rating	1.0	1.4	1.8	4.2	5.4	8.2	8.6	9.0
Expected plants	1	20	190	125970	167960	190	20	1
Total: 1,048,576								
	Ratio Res: Susc.							
1. Susc: >5.4< ←784626(Resistant)→ : ←263950(Susceptible)→ (2.973:1)								
2. Susc: >4.2< ←263950(Resistant)→ : ←784626(Susceptible)→ (1:2.973)								

[1] Genetic constitution of resistant parent: AABBCC....JJ, disease rating 1; genetic constitution of susceptible parent: aabbcc....jj, disease rating 9; all genes are additive and of equal effect; each resistance allele reduces the disease rating by 0.4; distribution of alleles is binomial and the smallest complete population, with all combinations of alleles represented, contains 2^{20} = 1,048,756 plants.

Malik (1990), in his study on the inheritance of resistance in chickpea to ascochyta blight, attempts to fit simple and more complicated Mendelian models to his extensive data sets, but no generalization can be made, and based on his biometrical models he concludes that the genetic control of the quantitative variation of resistance is complex. Jimenez-Diaz et al. (1993) close their paper on genetics of resistance to ascochyta blight by remarking that "whether resistance to ascochyta blight is inherited qualitatively or quantitatively has important implications in breeding strategies for resistance to the disease. Therefore, further genetic studies are needed to understand better the inheritance of resistance to blight." Van Rheenen and Haware (in press), analyzing data sets of multilocational ascochyta blight screening trials and plant growth room experiments with chickpea, conclude that resistance is quantitative, and that significant horizontal and vertical components of resistance play a role in the crop–disease pathosystem. From the observation of "loss" of resistance in varieties such as C 12/34, C 235, C 727, and CM 72 (Nene and Reddy 1987; Singh and Reddy 1989; Hari Chand, personal communications) they conclude that major genes have indeed been of importance in blight control. They also found a significant negative correlation between the mean locational disease rating and the corresponding standard deviation, indicating that varietal differences are obscured by higher locational disease pressure. A synthesis of the above observations leads to the conclusion that resistance to ascochyta blight is quantitative and its control "pseudomonogenic" (Vanderplank 1984), or rather polygenic, with important horizontal and vertical components. This also implies that races with differences in virulence really exist.

Strategies for Genetic Control of Ascochyta Blight

Malik and Rahman (1992) review the options for ascochyta blight resistance breeding. They include the development of multilines and breeding for horizontal resistance. Although this approach was attempted at ICARDA, it was abandoned because good results were not achieved (Singh et al. 1992). However, we feel the option should remain open. Another avenue to be explored is gene pyramiding, as described by Van Rheenen et al. (1992). To a certain extent this is being done by crossing resistant varieties of different origins. But by increasing the number of different varieties and isolates, the base of the pyramid can be broadened, and by using only "medium" disease pressure the test material can express more subtle resistance differences (Van Rheenen and Haware, in press). The identification of molecular markers for resistance genes would greatly support pyramiding efforts and other resistance gene deployment strategies.

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Experience with Pyramiding of Ascochyta Blight Resistance Genes in Kabuli Chickpea[1]

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Why Worry about Ascochyta Blight?

Chickpea (*Cicer arietinum* L.) is the most important food legume in the Indian subcontinent and the Mediterranean region. Between 1990 and 1992 it was grown on 10.1 million ha, producing 7.1 million tonnes (FAO Production Yearbook 1992). It fits in well with the farming systems, synthesizes atmospheric nitrogen, and is a source of protein in the diet. Despite its importance, the per hectare productivity of 700 kg ha^{-1} is low. Although there are other reasons for this low yield, diseases cause heavy losses. Of the 50 diseases effecting chickpea that have been reported in the world (Nene and Reddy 1987), ascochyta blight—*Ascochyta rabiei* (Pass.) Lab.—is the most destructive.

Ascochyta blight has been reported in 32 countries (Nene et al. 1989). It is highly damaging to chickpea crops in the Mediterranean, northwest India, Pakistan, eastern Europe, and North America. In years in which the disease is epidemic, 100% losses in yield have been reported in many countries (Nene and Reddy 1987). Blight has assumed an even greater significance since the introduction of winter sowing in the Mediterranean (Singh 1990). If ascochyta blight can be effectively controlled through the use of resistant cultivars, chickpea production will double in this region.

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What Have We Done so Far?

Efforts to identify sources of resistance began in the 1920s, shortly after the disease was discovered to be destructive. One of the first resistant lines was 4F32, identified in France, and renamed F8 in India by Luthra et al. (1938). This was the first line released as a cultivar in India. C-12/34 was the first cultivar developed through hybridization between F8 and Pb1, and was released in the 1940s in India. Subsequently, a large number of resistant sources and cultivars were developed throughout the world (Nene 1984; Saxena and Singh 1984; Singh 1987; Singh and Reddy 1991; Jimenez-Diaz et al. 1993).

Much of the earlier work was based on screening sources of resistance during natural epidemics, and as such was not based on planned research. After ICARDA's development of a reliable large-scale field screening technique, planned screening for resistance began (Singh et al. 1981). ICARDA has evaluated nearly 20,000 germplasm accessions and identified five resistant (ILC 200, ILC 6482, ICC 4475, ICC 6328, and ICC 12004) and nine tolerant (ILC 187, ILC 3856, ILC 5913, ICC 3606, ICC 4286, ICC 4828, ICC 8540, ICC 8566, and ICC 9584) lines (Singh and Reddy 1993). These and other lines form the basis of resistance breeding, and have been used in breeding programs throughout the world, resulting in the breeding of lines with resistance to ascochyta blight and other desirable characters. At ICARDA alone, over 2,500 lines have been bred, and have been shared with many national programs. Over 50 cultivars have been released in 17 countries (ICARDA 1994).

Breakdown of resistance to foliar diseases is common. This is partly due to the emergence of new races. Line C-12/34 lost its resistance around 1950. ILC 482 lost its resistance in Morocco in 1991 and northeast Syria in 1993. This pattern is exactly the same as with any other disease. But the search for durable resistance should continue. We do not believe that permanent resistance is possible, but we can breed lines with a longer life span. To help meet this goal, pyramiding of genes for resistance has been initiated at ICARDA. Results are described below.

Some of the lines found to be resistant to multiple races at multiple locations are listed in Table 1. Crosses are shown in Table 2. Some of these lines have a higher level of resistance than others, which may be due to the combination of resistance genes. This led to the idea that crosses between two resistant parents of diverse origins would increase the chances of combing the genes for resistance. It was also thought that the resistance of such crosses might be more durable.

Table 1. Kabuli accessions identified as resistant to ascochyta blight from the world collection.

Genotype	Resistant to individual races	Reaction to a mixture of six races		Resistant at % in international testing
		Field	Greenhouse	
ILC 72	1,2,3,4	R	T	73
ILC 200	NT	R	R	66
ILC 201	1,2,4,5	R	T	62
ILC 202	1,2,3,4,5	R	T	73
ILC 2506	1,2,4,6	R	T	58
ILC 2956	1,3,5,6	R	T	64
ILC 3279	1,3,4,5	R	T	67
ILC 3856	1,2,3,4,6	R	R	59
ILC 5928	1,2,4,5,6,	R	T	59

R = resistant; T = tolerant; NT = not tested

Table 2. Lines developed through the pyramiding of genes for resistance to blight.

Genotype	Blight reaction in the field	Cross number	Parentage
FLIP 83-48C	3	X80TH176	ILC 72 × ILC 215
FLIP 84-79C	3	X80TH176	ILC 72 × ILC 215
FLIP 84-145C	3	X81TH105	ILC 72 × ILC 484
FLIP 85-4C	3	X82TH66	ILC 2593 × ILC 3279
FLIP 85-42C	3	3X82TH66	ILC 2593 × ILC 3279
ILC 72	4		
ILC 215	7		
ILC 484	7		
ILC 2593	4		
ILC 3279	4		

Scale: 1 = free from damage; 9 = all plants killed.

In 1989, a project to pyramid resistance genes to ascochyta blight was initiated, with two approaches. First, a few resistant sources were chosen, namely ILC 183, ILC 200, ILC 201, ILC 202, ILC 215, ILC 482, ILC 2506, ILC 2548, ILC 2956, ILC 3279, ILC 3346, ILC 3856, ILC 4421, ILC 5586, ILC 5921, and ILC 6188. These were crossed with a common susceptible parent. The idea was to identify parents with different genes for resistance and cross them with lines with diverse genes for resistance. Second, resistant parents of diverse origin were crossed in the hope that they would have different genes for resistance.

The second approach was pursued more vigorously. The crosses were made during the main season at Tel Hadya. The F_1s were grown in the off season at Terbol, Lebanon. The F_2 bulks were grown in the blight-disease nursery,

where the disease was created artificially in epidemic form following the method described by Singh et al. (1981). Plants were scored when the susceptible check cultivar (ILC 263) was killed. Only those plants which showed higher resistance than the resistant check (ILC 3279) were selected. Like F_2, F_3, F_4,F_5, and F_6 generations were handled. During 1994, plants with a rating of 3 were selected in F_5 and F_6 generations (the original resistant parent was rated 4–5 in the nursery). These lines were then evaluated in the greenhouse for the Biotechnology Section pathotype III. Four promising lines (S 94605, S 94621, S 94633, and S 94685) were identified (Table 3). Thus, pyramiding of genes has helped in the development of lines with greater resistance. Only time will tell whether or not this resistance is durable.

Table 3. Lines developed through pyramiding of genes for resistance at Tel Hadya (1994).

Genotype	Field[†]	Greenhouse[‡]	
		Seedling	Adult plant
S 94605	3	2	5
S 94621	3	2	5
S 94633	3	2	5
S 94685	3	2	5

[†] Evaluation against a mixture of six races.

[‡] Evaluation against Biotechnology Section pathotype III.

Where Do We Stand Today?

We know now that there are six races of *A. rabiei* in Syria (Reddy and Kabbabeh 1984) and 13 races in West Asia and North Africa (Reddy et al. 1992). We have lines that are resistant to a mixture of the six races, but we do not have lines that are resistant to the 13 races. There is difficulty in obtaining all the races from WANA due to quarantine regulations.

At Tel Hadya, we have developed over 2,500 lines resistant to existing races, utilizing resistance sources. Some of them have been successfully introduced and extensively grown in many countries.

Where Do We Go From Here?

Despite the reports of races for the ascochyta blight pathogen, doubts have been expressed by some scientists about their existence. Therefore, the existence of races must be established. After that is accomplished (as we have no doubt it will be), we should proceed with the identification of races throughout the world. A beginning has been made at the University of

Bonn, Germany, with the participation of three countries. We should ensure the continuation of this program, and aim for coverage of the entire world.

Identification of genes for resistance to different races is another important task. The published work in this area through 1991 was reviewed by Singh and Reddy (1991). After the identification of genes, efforts should be directed at pyramiding of genes for resistance for their use in breeding programs.

In view of the frequent emergence of new races, which leads to the breakdown of resistance in existing cultivars, it is advisable to use tools such as RFLP or PCR to develop durable sources of resistance.

For effective control of blight, it is necessary to release several cultivars (with different genetic backgrounds for resistance) in a given area at any given time. This would allow for withdrawal of the line if it becomes susceptible.

To keep the disease under control, the cultivation of susceptible cultivars should be banned.

The epidemiology of the disease is not fully understood. Efforts made to understand it will help to effectively combat the disease. This problem can be facilitated through international cooperation among scientists.

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Generation of Simple Repetitive Sequence-mediated DNA Markers for the Analysis of Chickpea Populations

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Abstract

Genetic diversity in cultivated chickpea accessions is low. However, considerable variation is detectable by oligonucleotide fingerprinting with simple repetitive sequence (SRS) motifs, which can be exploited as genetic markers. Design of second generation markers is possible with microsatellite-PCR techniques. We demonstrate techniques developed for the fast generation and characterization of SRS-containing loci of chickpea, which are especially useful as co-dominant markers in segregating populations.

Introduction

The degree of polymorphism in cultivated chickpea as revealed by morphological or isozyme markers is low (Kazan et al. 1993). Therefore, the potential of DNA markers to detect genetic diversity must be explored. In recent years, some DNA marker systems have been developed and used for the analysis of animal, plant, and fungal populations. Among these, the restriction fragment length polymorphism (RFLP) technique is most commonly used (Tanksley et al. 1989). This technique is based on the presence or absence of a restriction endonuclease recognition sequence at a particular locus of the genome of related DNA. Digestion with a particular restriction enzyme will produce genomic fragments that vary in length and can be separated by agarose gel electrophoresis, transferred to a solid support, and probed with a radio labeled nuclear DNA probe.

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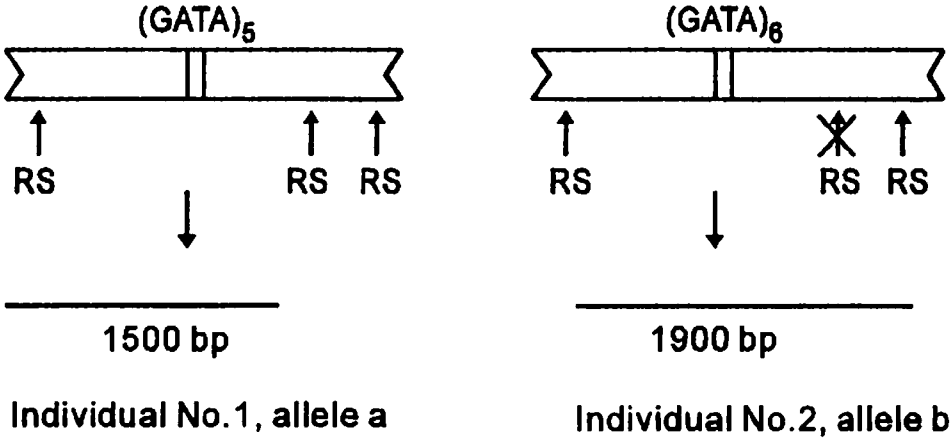
RFLP probes are generated by cloning of either genomic DNA or cDNA, with each clone hybridized to target DNA. This approach requires many screening cycles to generate probes that detect polymorphisms between, for example, accessions, and depends on genetic variability between analyzed individuals and populations. In chickpea, genetic variability as revealed by RFLP studies is rather narrow (Udupa et al. 1993) and other marker systems must be considered. Weising et al. (1989, 1991, 1992) found considerable variation in *Cicer arietinum* L. by using synthetic oligonucleotides complementary to SRS, or the so-called "microsatellites." We extended this approach by using all practically useful di-, tri-, and tetrameric repeat motifs for the analysis of chickpea accessions (Sharma et al. 1994).

The term microsatellite describes the repeated organization in tandem of these short di-, tri-, and tetranucleotide motifs shared with satellite DNA and minisatellites. The different types of repeat sequences, however, differ in many aspects. For example, satellite DNA is usually located in heterochromatin, mainly in centromeric parts of chromosomes (Tautz, 1993), whereas minisatellites cluster mainly around telomeres, at least in human and mouse DNA (Julier et al. 1990; Wells et al. 1989). Microsatellites, on the other hand, are not clustered in the genome. They are detected within and adjacent to coding regions of genes (Morgante and Olivieri 1993), and are also associated with minisatellite motifs in plant genomes (Broun and Tanksley 1993).

The application of such microsatellites as molecular markers is immediately evident via two approaches, depicted in Figure 1. First, simple sequence repeat motifs can be in-gel hybridized to restriction endonuclease-digested genomic DNA, revealing a multilocus polymorphic fingerprint pattern (Weising et al. 1989). Second, they can be utilized as single-locus markers due to their repeat length polymorphisms (sequence-tagged microsatellite sites; Beckmann and Soller 1990). In contrast to simple-sequence repeat oligonucleotides, this marker type is only applicable after construction of a genomic library, cloning of repeat-containing DNA-fragments, DNA sequencing, and synthesis of primers flanking the respective microsatellite repeat. The length polymorphism of a particular locus can then be analyzed by PCR (Weber 1990). The application of co-dominant markers such as repeat length polymorphic microsatellites or RFLP probes results in more information than dominant markers such as multilocus or random amplified DNA (RAPD) probes: they detect the homo- or heterozygous state of an allele.

A. Polymorphism as a result of loss or gain of a restriction site adjacent to an SRS locus.



B. Polymorphism as a result of different repeat lengths at an SRS locus.

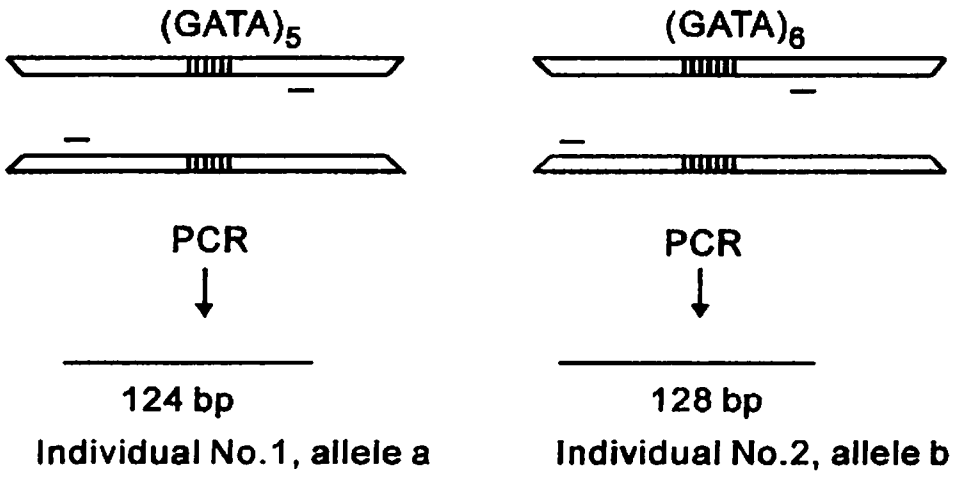


Figure 1: Simple repetitive sequence loci as molecular markers in chickpea breeding. Simple repetitive sequences can be exploited as molecular markers by oligonucleotide fingerprinting (A) or by PCR analysis (B). The molecular basis of polymorphism is illustrated.

The objective of our research was to clone an SRS-containing locus of chickpea linked to *Ascochyta rabiei* resistance. Since no such SRS locus detectable by DNA fingerprinting was available, and to gain experienced with SRS cloning, research focused on: (i) the characterization of genomic loci detected by SRS motifs; (ii) analysis of repeat-containing fragments to evaluate their potential as RFLP probes; and (iii) the establishment of a cloning technique that allows us to trace any specific SRS-containing fragment linked to an interesting agronomical trait.

The Cloning of SRS: $(GATA)_n$ as a Test System

Simple repetitive DNA sequences are ubiquitous components of all eukaryotic genomes investigated so far (Beckmann and Weber 1992; Weising and Kahl 1990). In prokaryotic organisms this class of repetitive sequences is absent, but the reason for this distinction is not yet clear.

Early experiments were aimed at categorizing genomic fragments, such as the synthetic oligonucleotide $(GATA)_4$ probed with DNA, that give strong hybridization signals to SRS motifs (Weising et al. 1989). Therefore, to analyze the structure of SRS loci yielding strong hybridization signals, we constructed a genomic DNA library for chickpea. The cloning process involved TaqI-digested DNA from ILC 3279 that was ligated into an appropriate plasmid vector and transformed into *Escherichia coli* K12 SURE. Colony hybridization to $(GATA)_4$ produced more than 100 positive clones with hybridization signals of widely varying intensity. These recombinant bacterial colonies were selected for the isolation of the respective genomic fragments. Unexpectedly, after two screening cycles, only 23 out of 100 tested recombinant clones still contained $(GATA)_4$-positive genomic fragments. They showed a clear bias towards small fragments, and were thus generally smaller than expected from the $(GATA)_4$ fingerprint pattern of TaqI-digested chickpea DNA. Nevertheless, the isolated fragments produced a strong signal with radio labeled $(GATA)_4$ and were chosen for sequencing and RFLP analysis.

Sequencing data for some of the selected genomic fragments is shown in Figure 2. Longer $(GATA)_n$ repeat units within these strongly hybridizing genomic fragments are found only occasionally. For example, in clone CaT24, the $(GATA)_n$ repeats are intermingled with $(GACA)_n$ repeats: $(GATA)_3$ TAGATA $(GACA)_2$ $(GATA)_3$ $(GACA)_2$. Interestingly, in clone CaT27, an interrupted $(GATA)_n$ repeat is flanked by an $(AT)_{15}$ and a $(GT_{3\text{-}6})_{11}$ repeat. This is similar to the situation found in cloned mouse $(GATA)_n$ repeats, where additional repetitive sequence motifs flank the $(GATA)_n$ repeat. In genomic fragments of mouse these motifs differ from the GATA element by only one or

two bases, such as $(TATA)_n$, or $(GGTA)_n$ (Studer et al. 1991). Moreover, the instability of $(GATA)_n$ fragments and other repeat motifs during the cloning process has been demonstrated in detail: internal repeat length reduction, loss of repetitive DNA without changes in the flanking sequences, and insertion/duplication events involving the flanking vector sequences and thus changing the integrity of the cloned fragment (Levinson and Gutman 1987; Studer et al. 1991; Zischler et al. 1991). This was found in clone CaT3, where the TaqI genomic restriction fragment could not be recovered. Such cloning artifacts might contribute to the loss of a majority of recombinant GATA element-containing fragments.

Clone CaT 2

5'-GAGACCGAACGACAAACCAAACGACAAATAGACAGACAAACCAACAAACAGACA
AACAGATAGATAGATAGACAGACCGACAGATAGAAAAACAAACAAATCGA-3'

Clone CaT 24

5'-TATTGATAGAATAGACAGACAGACAAACAATAGATAGATAGATA
TAGATAGACAGACAGATAGATAGATAGACAGACAGATCGA-3'

Clone CaT 27

5'-AAATTAGATTAAGTACGGTGAATCTTTTGTCTAAGAATAATTTATTTATGTTACGAAG
AATTTAATTCCGGGAAGTTTTGTTAAAAACATCTCGGGATGCTGAAAATTGCATTTGTCAA
TTGAGTCGCGACGAGAGTATATTTTTATCAAAAATCCTTGAGAATATGGTGAATGGTATT
GATAGATATCTAGATATTTATATATATATATATATATATATATATATATTGAGAAAA
AAGAAAAGAAAAGAAAGAAAAGAAAGAAAGAAAAAGAAAGAAAGAAAACTGA
AAGAAAACGTTTTTCTTCCTCCCCGATTGTCACACGAATCCTCCATCTTCTTC
CTCCTTCTTCATTTTTGTTTACTTCCTTTTTCTTTAAGAATTTTTCCCAAGGC
TTGGTGGGTAAGAAAGCAAGGAACCCCCAGCTTCATTCTTCTTCTCGGATTCGA-3'

Figure 2: Examples of $(GATA)_n$-containing SRS-loci in ILC 3279 DNA. $(GATA)_n$-containing DNA clones of chickpea were selected for sequencing by the Sanger chain termination method. $(GATA)_n$ and other SRS motifs (e.g. $[AT]_n$) are underlined.

Several fragments were evaluated for their potential as RFLP markers. DNA of different chickpea accessions was digested with several restriction endonucleases and hybridized to a radio-labeled genomic probe. In all cases except one, the hybridization pattern was perfectly monomorphic, again confirming the low genetic variability in chickpea when analyzed with genomic probes.

The Cloning of a Distinct $(CAA)_n$ Repeat Linked to a Susceptibility Locus

As detailed by Bünger and colleagues (in preparation) we applied SRS motifs to a segregating population of chickpea, i.e. an intraspecific cross of ILC 1272 (susceptible to *A. rabiei* race 6) and ILC 3279 (resistant to race 6). Motifs detecting polymorphisms between accessions were probed in an F_2 population, where the level of resistance to race 6 was rated. Linkage analysis (LINKAGE-1; Suiter et al. 1983) showed the correlation between the presence of one specific restriction fragment detected by $(CAA)_5$ and phenotype susceptibility.

Although the linkage to a putative susceptibility-mediating locus is not very close (still 20 centiMorgan), cloning of the respective fragment is useful for several reasons. First, transformation of this dominant marker into a codominant marker will facilitate the detection of its counterpart in the genome of ILC 3279 and prove or disprove the linkage detected by DNA fingerprinting. Second, it will be possible to analyze other crosses between susceptible and resistant partners more quickly, and to predict the adult phenotype at the seedling stage (although with limited probability). Third, a marker of this type can be used as a starting point for "walking" towards the susceptibility locus, either by chromosome jumping or chromosome walking techniques.

For the identification of such a fragment in a cloning process, the following characteristics of the marker locus must be present: (i) the fingerprint signal on the autoradiograph must be detectable in TaqI-digested parental ILC 1272 DNA and in approximately 75% of the F_2 population, but not in parental ILC 3279 DNA; (ii) the molecular weight of the locus must be approximately 1,300 bp; and (iii) the marker locus must contain at least one $(CAA)_5$ repeat motif.

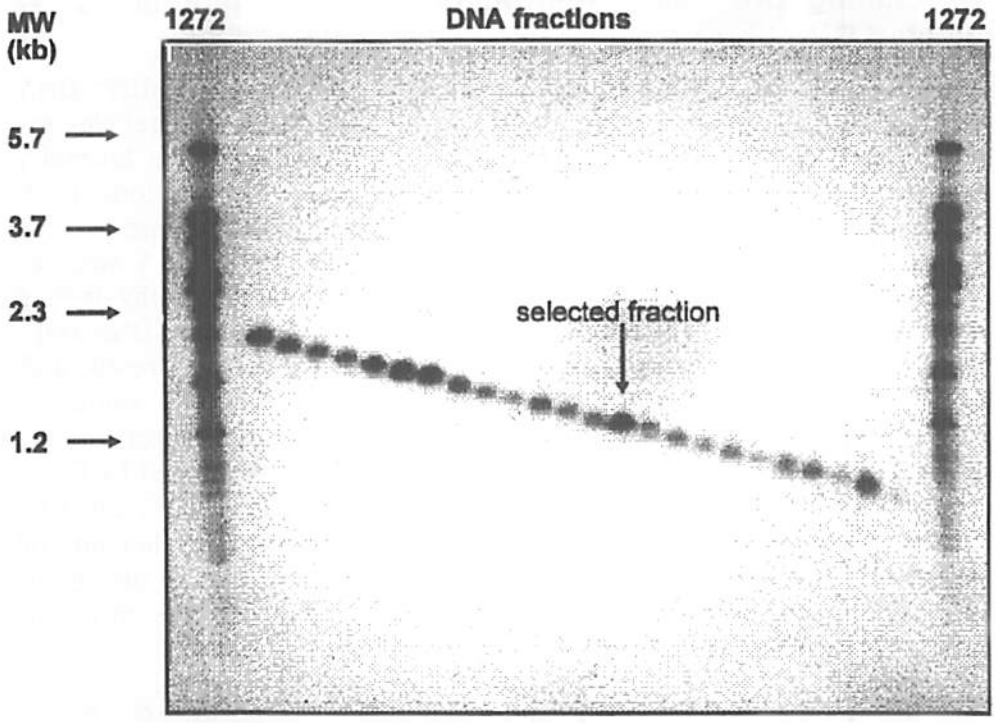


Figure 3: Size selection of $(CAA)_n$-containing SRS loci of TaqI-digested ILC 1272 DNA. TaqI-digested ILC 1272 DNA was separated on low melting agarose, stained with ethidium bromide, and sliced into fractions with a modified "egg cutter." DNA fragments were recovered from agarose slices by agarase treatment. From each fraction an aliquot was re-electrophoresed (1.2% agarose in 1xTBE buffer; TBE = 89 mM Tris pH 8.3, 49 mM boric acid, 2mM EDTA) and in-gel hybridized with ^{32}P end-labeled $(CAA)_5$. The DNA fragment harboring the fragment linked to *Ascochyta* susceptibility was selected for cloning (indicated by arrow).

The cloning process: construction of a subgenomic size-selected DNA library

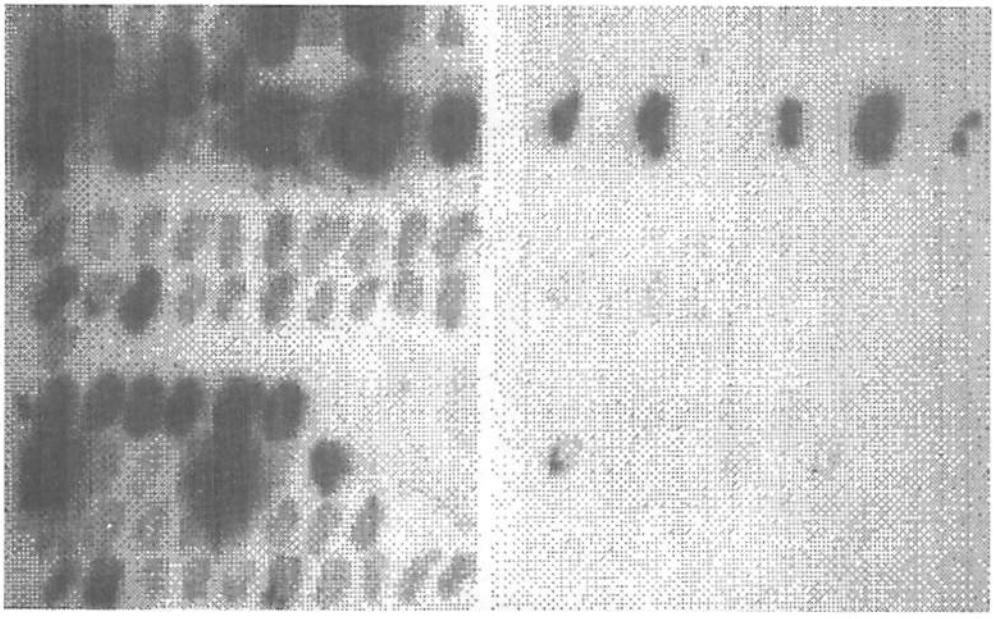
The first step in the cloning procedure was the restriction of ILC 1272 DNA with TaqI. The DNA was then separated by low melting agarose gel electrophoresis. After brief staining of the digested DNA with ethidium bromide, the gel was sliced with a modified "egg cutter" into many sections. DNA fragments harbored in the agarose slice were recovered by digestion with agarase to release the DNA. An aliquot of the recovered DNA was re-electrophoresed and hybridized to $(CAA)_5$. After autoradiography it was possible to exactly determine the fraction containing a specific DNA fragment (Fig. 3). Alternatively, TaqI-digested DNA was electrophoresed, and one half of the gel was used for the hybridization experiment, while the other half was stored at 4° C. After DNA fingerprinting, the gel region harboring the fragment of choice was excised and the DNA recovered by DNA-binding glass beads. After electroporation of this DNA into an *E. coli* K12 SURE strain, a subgenomic plasmid library was established. Either one or two replicas were produced and hybridized to $(CAA)_5$. As a result, about 100 positive colonies were detected, even under extremely stringent conditions.

There are two possible reasons for this high number of positive clones: (i) the subgenomic library contained each $(CAA)_5$-positive fragment approximately five times or more; and (ii) the hybridization parameters—for in-gel hybridization on the one hand, and for analysis of genomic libraries on the other—are not comparable, because the chickpea genome may contain many more $(CAA)_n$-containing fragments than revealed by a DNA fingerprint, but detectable by screening a chickpea genomic library. The diversity of cloned fragments and their repeat length has to be kept in mind, because neither sequence nor hybridization analysis of so many clones is feasible. Therefore, we developed a series of techniques that allow fast discrimination between DNA fragments that altogether carry $(CAA)_n$ repeats.

Establishing criteria for selective hybridization

If the standard hybridization conditions for oligonucleotides (e.g. $[CAA]_5$: T_m = 40° C in 6 × SSC) are applied to blots containing recombinant bacterial colonies, discrimination between positive signals is not possible. Therefore we drastically changed the protocols: first we stripped the DNA-containing blots for 3 × 20 minutes in 3× SSC at 40° C. We then removed one replica filter, while the other was further incubated at 60° C, 1× SSC for additional 45 minutes (Fig. 4). Although far beyond the T_m value predicted by the standard formula used for oligonucleotides (T_m = 40° C in 6× SSC),

we could clearly discriminate between clones, and identify differences in the intensity of the hybridization signals between clones as an indication of the repeat length in each fragment.



Filter A	Filter B
Washing conditions: 3 × SSC at 40°C for 3 × 20 min.	Washing conditions: 3 × SSC at 40°C for 3 × 20 min. 1 × SSC at 60°C for 3 × 15 min.

Filter A and filter B contained identical bacterial colonies yielding strong hybridization signals with $(CAA)_5$ under standard conditions.

Figure 4: Selective colony hybridization with $(CAA)_5$. In order to clone the $(CAA)_n$-repeat carrying fragment indicated by the arrow in Figure 3, the respective ILC 1272 DNA fraction was used to construct a sub-genomic library. Colony hybridization was performed with a ^{32}P end-labeled $(CAA)_5$ oligonucleotide. Identification of the clone carrying the desired target fragment was possible by drastically increased post-hybridization washing stringency.

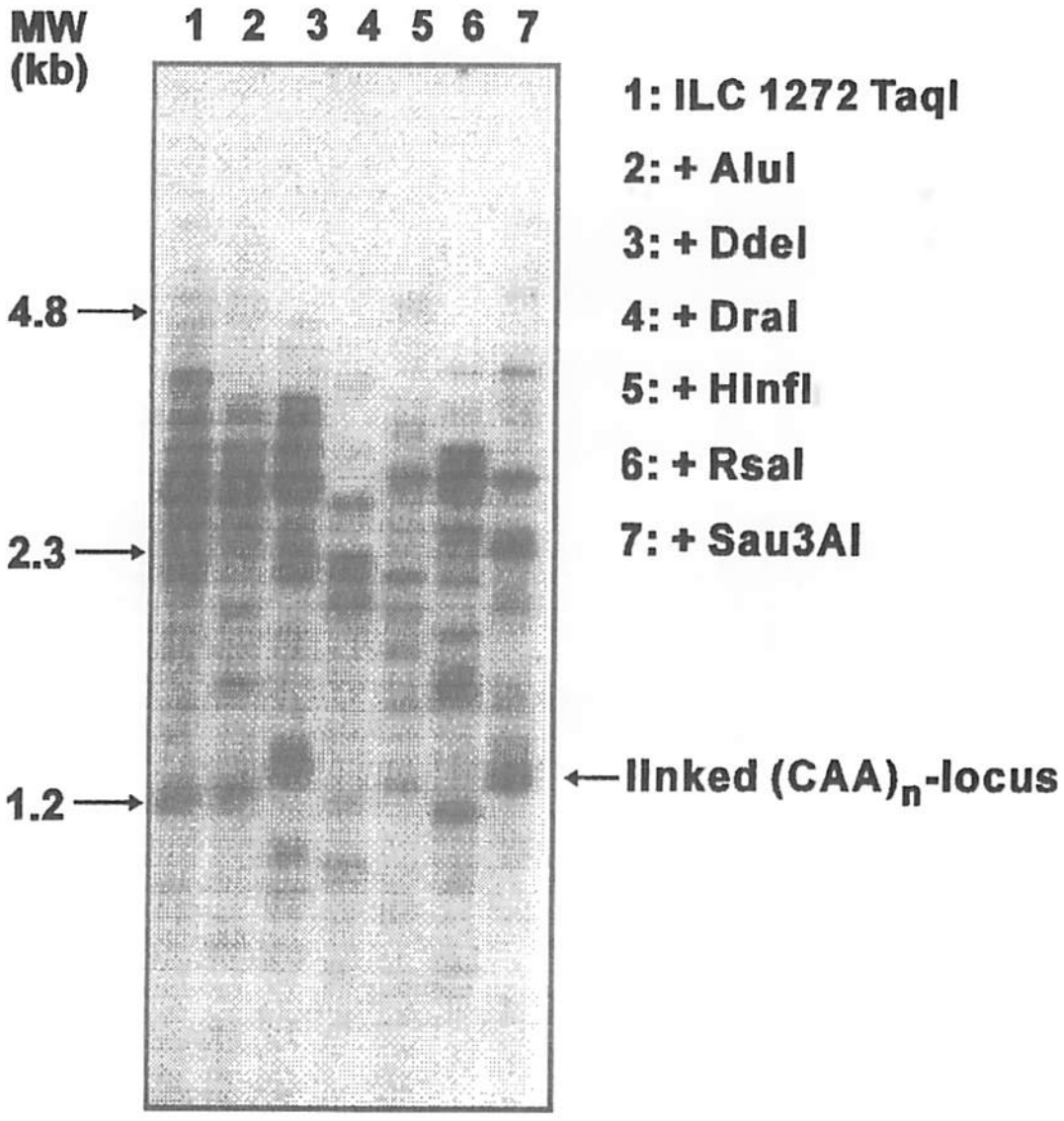


Figure 5: Restriction analysis of $(CAA)_n$-loci in ILC 1272. $(CAA)_5$ fingerprints of ILC 1272 DNA, resulting from double-digestion. Lane 1 represents TaqI-digested DNA, while TaqI plus an additional enzyme was used for lanes 2–7. Samples were electrophoresed in 1.4% agarose and 1xTBE buffer, and in-gel hybridized to ^{32}P end-labeled $(CAA)_5$. The $(CAA)_n$-containing DNA fragment linked to ascochyta blight resistance has additional recognition sites for *Dde*I, *Dra*I, and *Rsa*I.

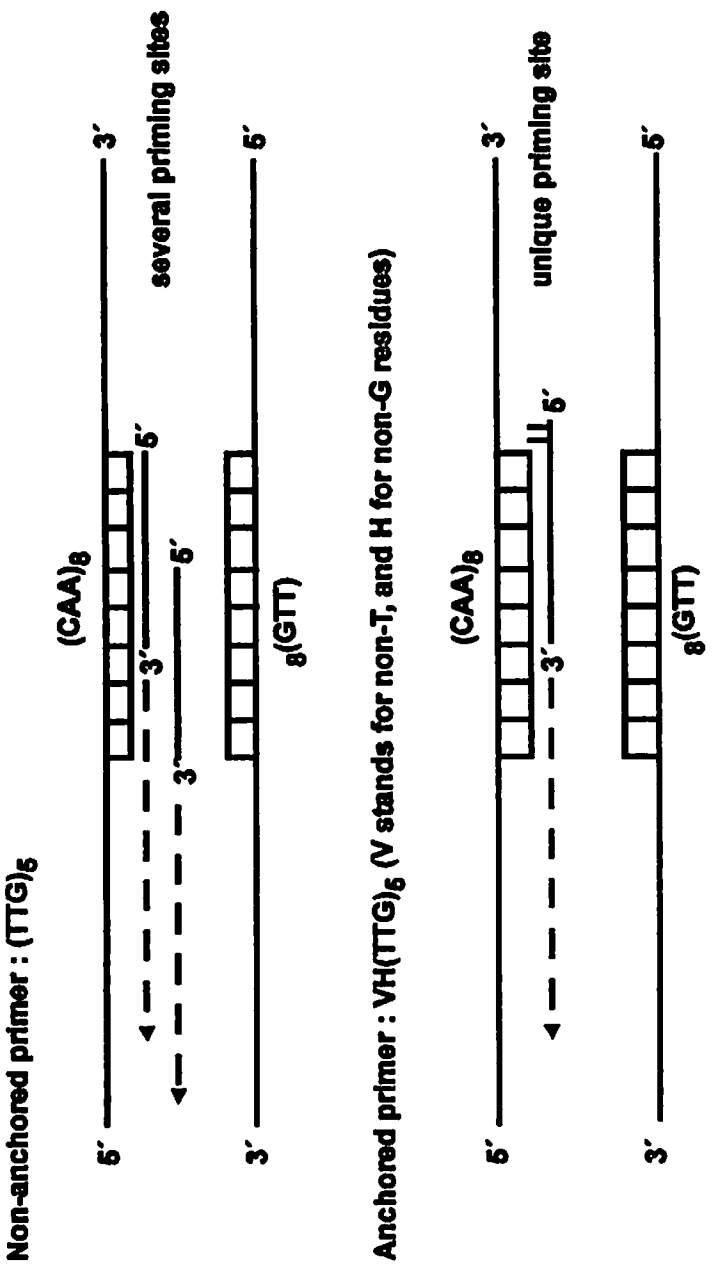


Figure 6. Determination of SRS flanking sequences using degenerate primers. $(CAA)_n$-repeat flanking sequences were determined by using degenerate $(CAA)_n$ and $(TTG)_n$ motifs as sequencing primers. The degeneration at the 5′ end anchors the primers to the end of a microsatellite locus and provides a unique starting site for the DNA polymerase. This technique was applied to several cloned $(CAA)_n$-containing fragments (approximately 1,300 bp long).

Restriction analysis of SRS loci

DNA fingerprint analysis of TaqI-digested ILC 1272 DNA revealed a $(CAA)_5$-positive fragment, approximately 1,300 bp long, linked to a susceptibility locus. A set of restriction endonucleases with an AT-rich recognition sequence was selected to analyze restriction sites within the fragment. We choose the endonucleases *Alu*I, *Dde*I, *Dra*I, *Hin*fI, *Rsa*I, and *Sau*3AI for renewed restriction of *Taq*I-digested DNA. After gel electrophoresis and hybridization to $(CAA)_5$, we were able to identify the enzymes cutting within the linked fragment: *Dde*I, *Dra*I and *Rsa*I (see Fig. 5).

Using this additional information of restriction sites harbored by the TaqI-fragment, we analyzed cloned genomic fragments to select clones, following the same restriction pattern. Several clones were tested, but no clone was found that perfectly fit the pattern revealed by restriction analysis of chickpea DNA. Note that we have to consider sequence rearrangements during the cloning process, as found, for example, with GATA-, CAC-, or CA-elements (Studer et al. 1991; Weissenbach et al. 1992). Therefore, restriction sites found in chickpea DNA may change during the cloning process of the respective fragment in *E. coli*.

Rapid determination of sequences flanking $(CAA)_n$ SRS-loci

To achieve the direct and fast generation of DNA sequence information of regions that flank the SRS, we used degenerate (CAA) and (TTG) microsatellite motifs as sequencing primers (Fig. 6). The degeneration at the 5′ end anchors the sequencing primers to the end of a microsatellite locus, presenting a unique start site for the polymerization reaction catalyzed by DNA polymerase. Although the performance of such primers is inferior to that of unique repeatless sequencing primers, we were successful in determining the flanking regions of several fragments without any subcloning steps (which are usually required when working with a fragment approximately 1,300 bp long. The flanking sequence can be directly utilized for the construction of primers bracketing the SRS motif, which will allow evaluation of length polymorphisms between different individuals in a PCR experiment. In this case, individual No. 1 may carry a $(CAA)_7$-containing PCR product, while at the same locus individual No. 2 harbors $(CAA)_8$. This can serve as a co-dominant marker between individuals in a breeding program. Note that ambiguous data may arise if more than one microsatellite of the same type is present in a given clone, although we found this to be the exception rather than the rule.

Conclusion

With these techniques we were able to distinguish given DNA fragments from counterparts with identical molecular weight harboring a $(CAA)_n$ motif. Analysis (now in the final stages) of DNA fragments that are putatively linked by (CAA)-loci to the susceptibility region is based on the following criteria:

1. Such a fragment should contain a high $(CAA)_n$ repeat number, because it is detectable by DNA fingerprinting only in ILC 1272. Therefore we specifically select clones that reveal strong hybridization intensities even under stringent conditions. Although many fragments can be detected in cloned TaqI-digested ILC 1272 DNA with a molecular weight of approximately 1,300 bp, the resulting fingerprint band cannot result from an additive effect of many $(CAA)_n$-containing fragments. This is because it does not segregate in a Mendelian mode in the F_2 population.
2. Double digestion with restriction enzymes will be used, yielding a specific restriction pattern for each fragment. A fragment that fits into the pattern will be selected for sequencing, though it might not be reliable enough due to cloning artifacts (Zischler et al. 1991). Sequencing with degenerate (CAA) and (TTG) primers will be used to produce the flanking sequence of the $(CAA)_n$ locus. After deduction of primers (primer bracket), respective SRS loci will be analyzed by PCR. The assumption that the fingerprint band detecting the linked locus is due to length polymorphism of the $(CAA)_n$ repeat was confirmed by DNA fingerprinting of ILC 1272 and ILC 3279 individuals, where a fingerprint band approximately 1,300 bp long could be detected in both accessions but clearly differed in hybridization intensity when probed to $(CAA)_5$.

Perspectives

This article reports on experiences with the cloning of: (i) randomly selected DNA fragments containing the $(GATA)_n$ SRS motif; and (ii) a size-selected $(CAA)_n$-containing SRS motif of chickpea. These motifs can be cloned in a prokaryotic host such as *E. coli*, but the danger of cloning artifacts (for example, the loss of restriction sites or sequence rearrangements) cannot be ignored (Studer et al. 1991; Zischler et al. 1991). These changes affecting SRS-containing fragments may be due to slipped-strand mispairing (probably the basic process involved in the evolution of SRS). (Levinson and Gutman 1987; Tautz 1989; Schlötterer and Tautz 1992.) Therefore, identification of a specific DNA fingerprint band linked to a specific trait by DNA cloning involves the risk that the fragment is either not clonable at all in *E.*

coli, or prone to intramolecular changes. All in all, identification of an SRS locus might be faster and more reliable using other techniques, for example PCR-based approaches.

To summarize, we developed a set of reliable techniques for the identification of SRS-motif-containing DNA fragments detectable in the chickpea genome. The practical impact of this research is the fast establishment of SRS PCR markers. These co-dominant markers are especially useful in the analysis of segregating populations by allowing the discrimination of homo- or heterozygotic states of an allele, increasing the information content per locus. Therefore, this marker is superior to oligonucleotide fingerprint markers, which can only reveal information such as "fingerprint band detectable" (the homo- *or* heterozygous status of the allele containing the respective SRS), or "fingerprint band not detectable" (indicating homozygous absence of the allele). Besides the higher information content of PCR microsatellite markers (co-dominant marker system), these markers are characterized by greater polymorphism information (PIC; Botstein et al. 1980) than RFLP and even minisatellite markers (Weissenbach et al. 1992). The PIC value is a parameter for the likelihood of revealing polymorphisms between unrelated individuals; high PIC values for microsatellites are also observed in plants. Studies on PCR microsatellites in soybean have revealed hypervariability. In a cross between two *Glycine max* plants, 15 out of 17 microsatellites detected two alleles per locus, while one out of five single copy probes was polymorphic (Morgante and Olivieri 1993). The number of SRS sequences in plants was calculated using database analysis by Lagercrantz et al. (1993), yielding an estimate of one SRS every 29 kb, given a pure repeat length of 20 bp or more. A rough calculation for chickpea (genome size: 1C = 740 Mb [Arumuganathan and Earle 1991]) is 25,000 SRS. Therefore this marker type can serve as a nearly undepletable reservoir for new polymorphic DNA markers.

Compared to the generation of synthetic oligonucleotide probes for DNA fingerprinting, the time required for generation of a high number of SRS markers is very long. In studies of chickpea populations both marker types can complement each other, especially in segregation analysis of crosses.

Acknowledgments

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Development of a DNA Marker for Fusarium Wilt Resistance in Chickpea

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Introduction

Chickpea (*Cicer arietinum* L.) is a staple crop in the Middle East, North Africa, Ethiopia, and the Indian subcontinent. In these areas, chickpea is affected by numerous diseases, of which ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Lab., and fusarium wilt, caused by *Fusarium oxysporum* Schlect emend. Snyd. & Hans f. sp. *ciceri* (Padwick) Snyd. & Hans. are the most serious (Jimenez-Diaz et al. 1993). Fusarium wilt has been reported throughout the world and has caused yield losses estimated at 10% annually in India (Singh and Dahiya 1973) and Spain (Trapero-Casas and Jimenez-Diaz 1986). The disease is also present in the states of Sonora and Sinaloa in Mexico, and California in the USA (personal observation). The possibility that the disease will spread to additional areas of the USA prompted our efforts to determine the genetics of resistance and develop resistant cultivars.

The use of resistant cultivars is considered the most effective and economical means of controlling the disease. Germplasm lines resistant to fusarium wilt were recognized as early as in the 1930s and employed in breeding programs (reviewed by Jimenez-Diaz 1992). A large number of wilt-resistant germplasm lines are now available for breeding, and breeding programs in a number of countries have developed resistant cultivars. Screen-

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ing of segregating populations in "wilt-sick" plots is considered the most efficient means of identifying wilt resistant selections (Jimenez-Diaz et al. 1993). Plants are grown from seed under pathogen-free conditions and then transplanted to pots or beds in which the pathogen is maintained; the plants are then monitored for signs of wilt symptoms that can take weeks to develop. A decade-long screening program undertaken at ICRISAT has identified resistance in 160 out of more than 13,500 accessions tested (Haware et al. 1992).

The genetic basis of resistance in germplasm lines appears to be controlled by a single gene with incomplete dominance, or by a single recessive gene (Kumar and Haware 1982). However, in some cases a good fit to expected ratios is not obtainable, suggesting that resistance may involve multiple genes (Kumar and Haware 1983; Singh et al. 1987a, 1987b, 1991; Upadhyaya et al. 1983a, 1983b; reviewed in Singh and Reddy 1991). In some crosses it appeared that resistance is controlled by at least three independent loci, designated H-1, H-2, and H-3 (Upadhyaya et al. 1983b; Singh et al. 1987, 1988).

Wilt-resistance screening and inheritance analysis would be more efficient if genetic markers closely linked to resistance genes could be identified. Marker-assisted selection in breeding programs has been envisaged since the discovery that genes are linked and transmitted to progeny in blocks. The use of easily identifiable morphological traits as tags for genes is feasible, but not often possible due to insufficient morphological variation, the lack of close linkage, and, more importantly, the deleterious effects of such traits on progeny.

Identification of molecular "tags" for resistance genes could lead to quicker assessment of susceptibility, allow the screening of seed or seedlings, and eliminate the need for maintaining and culturing pathogens. Isozyme electrophoresis could provide relatively quick and easy access to useful and reliable molecular markers. However, the total number of isozymes that can be readily analyzed in plant tissue is usually less than 50—and often much less. Moreover, isozyme electrophoresis of chickpea has revealed insufficient polymorphism to be useful in screening for tags for wilt resistance (A. Tullu, unpublished data).

A potentially much larger number of markers can be resolved using restriction fragment length polymorphisms (RFLPs). RFLP technology has been used to establish high density gene maps for an increasing number of crop species (O'Brien 1993). A low-density gene map including RFLPs has been developed for lentil (Havey and Muehlbauer 1989) and RFLPs have been added to the chickpea gene map (Simon and Muehlbauer in preparation).

RFLP markers are examined through a laborious and somewhat difficult procedure and often requires the use of radioactive labeling to visualize banding patterns, although the use of non-radioactive labeling is increasing.

Random amplified polymorphic DNA (RAPD) markers represent a source of genetic variation that is easy to access and often abundant within populations (Williams et al. 1990; Welsh and McClelland 1990). RAPD markers are universal—the same primers may be used on any organism. Moreover, there is virtually no limit to the number of primers that can be designed for use in RAPD reactions. RAPD analysis is also much faster than RFLP analysis, which can involve transferring DNA from gels to filters, producing and labeling cloned probes, and hybridizing probes to filters. Furthermore, the number of potential RAPD markers can be several orders of magnitude larger than those for morphological or isozyme markers, leading to a much higher level of marker saturation on genetic maps. Despite their newness, RAPDs have already been widely employed for mapping genomes (Carlson et al. 1991; Foolad et al. 1993; Halward et al. 1992; Kiss et al. 1993; Quiros et al. 1991; Torres et al. 1993; Uphoff and Wricke 1992; Williams et al. 1990; 1993) and for tagging resistance genes (Barua et al. 1993; Haley et al. 1993; Martin et al. 1991; Paran et al. 1991; Paran and Michelmore 1993; Penner et al. 1993; Yu and Pauls 1993).

In this paper we discuss our approach to studying the inheritance of wilt resistance in chickpea, and our efforts to tag and map resistance loci on the chickpea map (Simon et al., in preparation) using RAPD markers. We argue that the establishment of recombinant inbred lines from a cross between well-characterized parents is the most efficient way to reveal patterns of linkage and segregation among genetic characteristics. Furthermore, we suggest that RAPDs may provide a superior breeding tool; they have nearly unlimited potential for tapping into polymorphisms between genomes, and can serve as precursors to markers with high specificity.

Materials and Methods

Chickpea culture and DNA extraction

A germplasm line (WR-315) resistant to fusarium wilt race 1 was crossed to a late-wilting susceptible line (C-104). The difference between the two cultivars in response to the wilt fungus was apparently controlled by a single recessive gene (Kumar and Haware 1982). The cross was made at ICRISAT. F_3 families were then provided to the USDA, ARS Grain Legume Genetics and Physiology Research Unit at Pullman, Washington by Dr Jagdish Kumar. We then advanced 100 F_2 lines to F_6 by single-seed descent.

DNA was isolated from vegetative bud and leaf tissue using the "microprep" method of Simon and Weeden (personal communication). Samples (3–4 buds) were ground in 1.5 ml microfuge tubes with a pestle mounted onto a variable speed hand motor (Dremel model 732). The procedure yields up to 100 μg of DNA of more than adequate purity for RAPD analysis.

Culture and inoculation of the fusarium wilt pathogen

Samples of *Fusarium oxysporum* f. sp. *ciceri* were obtained from J.M. Kraft, USDA Plant Pathologist. A small amount of infected soil was sprinkled on PDA; the culture was single-spored on 2% water agar and increased on fresh PDA under white fluorescent light with a 16 hour photoperiod. Wild colonies were selected to produce liquid culture following the procedures of Roberts and Kraft (1974), Westerlund et al. (1974), and Bhatti (1990). Conidial concentrations were adjusted to 1×10^6 spores/ml using a hemacytometer.

Ten seeds from each of the 100 F_6 recombinant inbred lines were planted in single rows in coarse perlite in a greenhouse (21–25° C). The experiment was replicated twice. When the plants reached the third node stage of growth, they were removed from the perlite and inoculated by cutting the roots and dipping them in the spore suspension (Nene and Haware 1980; Bahatti 1990). After 3–5 minutes in the suspension, inoculated plants were transplanted back into the perlite bed. A control replicate was grown in a separate greenhouse and inoculated with sterile water (Nene and Haware 1980; Bhatti 1990).

Resistance/susceptibility scoring was carried out over the next two months. Data were compared to the non-inoculated controls in order to minimize escapes and verify scoring. The greenhouse data were further corroborated by taking stem cuttings from the sixth node of each plant and placing them onto fresh PDA, and subsequently scoring for the presence or absence of fungal growth.

RAPD analysis

Preliminary wilt tests were made on portions of the F_3 families so that the genotypes of the F_2-derived lines could be inferred. We then chose 12 F_2 lines for the RAPD survey: six suspected to be homozygous for susceptibility and six suspected to be homozygous for resistance. We employed 370 10-mer oligonucleotide primers (J. Carlson, Univ. of British Columbia, Vancouver) in separate reactions to screen the DNA samples; test gels were examined for DNA bands that were unique to either the resistant or the susceptible lines. The polymerase chain reaction (PCR) protocol followed a modifi-

cation of Williams et al. (1990). The PCR products were separated electrophoretically in a 2% agarose gel and banding patterns were revealed by staining with ethidium bromide and inspection under ultraviolet light.

After we had identified the primers yielding markers apparently linked to resistance, we screened all 100 F_5 lines. Portions of the F_6 families were screened for resistance to fusarium wilt, allowing inference of the genotype of the F_5 parents. The genotypic data were then used to determine the degree of linkage between the two markers and their positions relative to *Fw* locus (Mayer et al., in preparation).

Once diagnostic RAPD markers were identified, we wanted to ensure reliable, unambiguous, and rapid screening for the resistance allele in germplasm resources. We constructed allele-specific associated primers (ASAPs; Weeden et al. 1993) so that PCR involving DNA that contained the allele for resistance to wilt would result in a single amplification product. We excised the RAPD marker fragments from the agarose gel for cloning and sequencing. With this information, we designed 18–24 bp ASAP primers that included the RAPD primer sequence and the next 8–14 bases interior to it (Mayer et al., in preparation). Presence or absence of the allele is revealed through agarose gel electrophoresis or by the post amplification concentration of DNA in the PCR cocktail, which can be measured by absorbency at 260 nm.

Results and Discussion

Ninety-nine of the 100 F2-derived lines were tested for wilt resistance using portions of the F_6 families, with one line lost due to failure of seed set. Forty-eight of the lines were resistant to fusarium wilt, 46 were susceptible, and five lines were still segregating (Mayer et al., in preparation). These data closely fit a 1:1 ratio and support the contention that a single locus controls the difference in wilt response between cultivars C-104 and WR-315.

Well-characterized populations of recombinant inbred lines are a prerequisite for effective RAPD analysis and for the identification of markers closely linked to resistance loci.

One of the 370 10-mer RAPD primers amplified a DNA fragment linked to resistance to fusarium wilt (UBC 170_{550}); another primer amplified a DNA fragment linked to susceptibility (CS 27_{700}). There was 5% recombination between the loci corresponding to UBC CS 27_{700} and 170_{550}, and 7% and 8% recombination, respectively, between these loci and *Fw*, the locus that con-

trols resistance to fusarium wilt (Mayer et al. in preparation). These data suggest that the two markers are located on the same side of *Fw*, rather than in flanking positions on the chromosome.

The lack of additional markers and the overall scarcity of polymorphism among the F_2-derived lines reflects the lack of divergence of the parental lines (C-104 and WR-315). This paucity of genetic polymorphism was a considerable handicap to the project. Fortunately, given enough time and RAPD primers, we were able to discover good markers. Low levels of genetic variation may in fact be a common characteristic of cultivated chickpea germplasm. Several recent surveys failed to find substantial polymorphism at loci coding for soluble enzymes (Tuwafe et al. 1988; Gaur and Slinkard 1990; Kazan and Muehlbauer 1991). Future searches for molecular-based markers in chickpea must consider whether or not the "width of the cross" is compatible with the sensitivity of the assay being used. If time and funding are not limiting, RAPDs represent a good alternative, because an unlimited number of primers can be designed for the technique, allowing access to virtually any polymorphism present between two genomes.

There is a growing number of cases in which RAPD markers have been used to develop either allele-specific or locus-specific primers (e.g., Paran and Michelmore 1993; Williamson et al. 1994). We successfully constructed a pair of allele-specific associated primers (ASAPs) for CS 27_{700}, the marker for susceptibility to fusarium wilt (Mayer et al., in preparation). Moreover, we detected a few recombinants among the F_2-derived lines that were resistant to wilt but produced the CS 27_{700} band through amplification with the ASAP primers. Therefore, we now have resistant inbred lines in which the resistance is either positively linked or negatively linked with amplification of CS 27_{700}. This kind of germplasm resource will be valuable to breeding programs in which the potential for amplification of CS 27_{700} in the recurrent parent is unknown.

Consistent resolution of UBC 170_{550} was difficult due to poor priming or to variation in the PCR cocktail—problems we hoped to eliminate by constructing allele-specific primers. Moreover, a slightly slower fragment that all but co-migrates with UBC 170_{550} was amplified in all the samples (Mayer et al., in preparation), sometimes making interpretation of banding patterns difficult. Creating allele-specific primers will not only enhance the reliability of PCR results, but will eliminate bands that could confuse diagnoses of resistance or susceptibility.

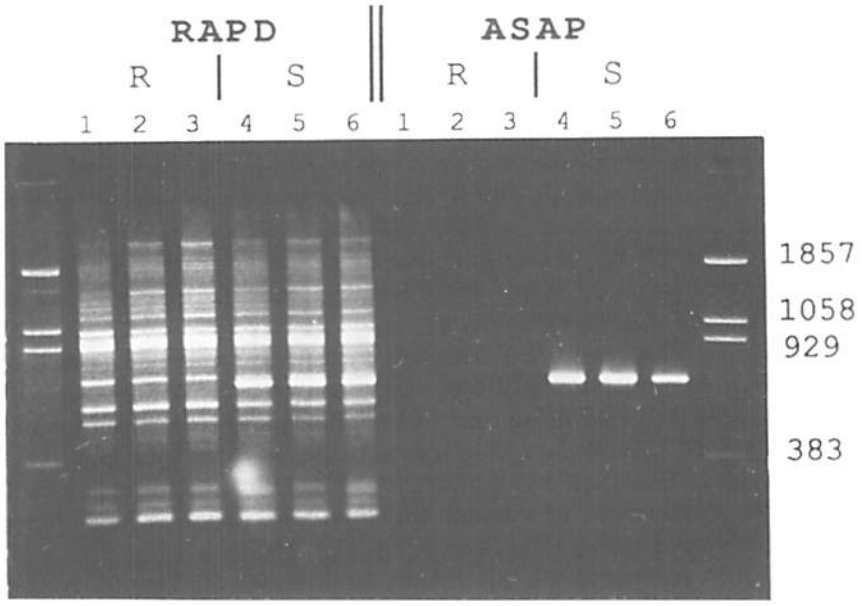

Figure 1. Comparison of products resulting from PCR using RAPD primers (left) and ASAP primers (right) on DNA samples from resistant lines (lanes 1–3) and susceptible lines (lanes 4–6).

The effectiveness of the ASAPs for the fragment UBC 170_{550} was limited due to the faint amplification of a fragment of similar size in susceptible lines (Mayer et al., in preparation). This may occur because the length of the new primers may enable them to tolerate minor mismatches with genomic sequences, in contrast with shorter RAPD primers. These ASAPs will still be valuable, however, if used to introgress resistance from a cultivar such as WR-315 into a recurrent cultivar that does not produce a fragment similar to UBC 170_{550}. Preliminary screening will enable the breeder to select the resistant line and corresponding ASAPs that will be appropriate to the cultivar targeted for introgression of disease resistance.

The effective use of RAPD analysis to identify markers closely linked to genes of interest is best approached through the use of well-characterized populations of recombinant inbred lines, preferably in the F_6 or later generations. Inoculation and evaluation of the RI lines must be completed with care to avoid any possibility of misclassification.

Although the use of arbitrary oligonucleotide primers is still in its infancy, it has already facilitated gene tagging, genome mapping, genetic fingerprinting, assessment of levels and distribution of genetic variation, and identifi-

cation of hybridization. Clearly, PCR has revolutionized molecular biology and will continue to serve as the foundation for new procedures, such as RAPD analysis. The RAPD technique represents a simple and flexible application of PCR, a first step towards greater characterization of DNA polymorphisms via tools such as ASAPs.

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Development and Use of Molecular Markers for Chickpea Improvement

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Abstract

Chickpea breeding, especially resistance breeding, will benefit from the application of DNA markers for the development of superior, resistant cultivars. This paper discusses the generation of such markers, including the choice of suitable test populations, rating conditions, and marker types. A rudimentary genetic map of the chickpea genome based on microsatellite markers is presented. Breeding applications for DNA markers, including a marker linked to Ascochyta resistance, are discussed.

Introduction

Chickpea breeding aims at the improvement of the crop using gene stock already present in cultivated species or wild relatives. Lines displaying the desired traits are crossed, and the offspring tested for the presence of the desired new gene combinations in several rounds of selection from F_2 to F_8. This time consuming and costly process can be speeded up considerably by the use of DNA markers. These enable the breeder to characterize breeding lines, construct horizontal resistance (i.e. pyramiding of resistance genes), and detect the presence of recessive genes in the heterozygous state. All this makes the development of DNA markers a prerequisite for successful crop management.

The development and the practical use of DNA markers are two separate processes. Whereas in marker development highly sophisticated technology, experienced manpower, specially designed crosses, and artificial test situa-

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tions are used, for application in the field methods have to be robust and reliable. In fact, crosses that display high levels of genomic and phenotypic polymorphism may be useless for the breeder because of poor performance of plants in the field. Markers that are easy to develop are not applicable in routine tests, because of their complicated handling. Therefore, the aim of marker development is not only to find tightly linked markers for agronomically important traits, but also to design them in a way that allows their application in the field.

Development of DNA Markers

The introduction of DNA markers into breeding programs requires careful planning of test crosses. The best marker type for each purpose must be chosen. The steps necessary for the development and use of DNA markers for plant improvement include:

- Choice of suitable parents for test crosses.
- Design of test populations (F_2, IF_2, BC, F_3, NIL, or RI lines).
- Design of tests for a special trait.
- Choice of the best suited marker types.
- Generation of a genome map.
- Linkage analysis and identification of marker couples with the trait.
- Conversion of markers into sequence-tagged sites for routine use.
- Evaluation of markers on normal crosses and routine field tests.
- Generation of "donor lines" for trait combinations or gene pyramids.

Choice of parents

Chickpea is, genetically, a relatively homogenous species, which shows only a few polymorphisms with most DNA markers. When crosses are planned for marker development, special emphasis should therefore be laid on finding parents with the highest degree of genomic polymorphism. This can be tested on the DNA of siblings from selected parents with multilocus markers such as microsatellites. Whenever possible, one of the parents should be selected from the wild species, *Cicer echinospermum* P.H. Davis and *Cicer reticulatum* Lad., especially when a high-density genetic map is desired. These crosses will provide the most informative DNA polymorphisms, due to the evolutionary distance of the species.

Furtheron crossing partners should be selected from lines that show the greatest difference in the trait for which a linked marker is sought. For in-

stance, in a cross designed for the detection of an *Ascochyta rabiei* resistance gene, partners should represent the most susceptible and resistant cultivars or wild species, as outlined below in recommendations for rating. This rating system is followed for the selection of parents to ensure clear-cut differences in the offspring and to draw maximum information from linkage analysis.

In test crosses for marker development, little or no attention should be paid to the possible agricultural value of a cross if it conflicts with the polymorphism of the selected parents. Crosses for development of molecular markers on the one hand, and for conventional breeding for chickpea improvement on the other, differ considerably from each other, as shown in Table 1.

Table1. Crosses for conventional and molecular marker-assisted breeding.

Conventional breeding for plant improvement	Application of molecular markers in plant improvement
Two parental lines (cultivars) chosen for combined agricultural value	Several parental lines chosen for greatest detectable molecular and phenotypical polymorphism (cultivars and wild species)
Bulked pedigree breeding	Single plant descent
Repeated cycles of bulked selection for desired traits (F2 to F8)	Analysis of individual F2 or F3 plants for the desired mono- or polygenic traits
Identification of plants with desired traits	Identification of molecular markers
Combination of new traits by new crosses and selection (F2 to F8)	Combination of new traits by analysis of F2 or F3 for combination of molecular markers

Design of test populations

The design of a well-suited test population is a crucial step in the development of markers for agronomical traits. Theoretically, a large F_2 generation is the most informative population for genome mapping (Melchinger 1990), especially if a map is in an early stage and only a few markers are mapped. For practical use in marker development for agronomically interesting traits, an F_2 has three major drawbacks: (i) the same individuals that are tested for the trait also have to be used for linkage analysis (this can cause serious problems, since after extensive testing, with, for example, a pathogenic fungus such as *Ascochyta rabiei* (Pass.) Lab., some of the plants will be too affected to provide enough DNA for linkage analysis); (ii) after completion of their life cycle, plants die and the best pheno- and genotypically characterized individuals will no longer be available for backcrosses or further genetic analysis; and (iii) most multilocus markers such as RAPD, mini- and microsatellites are dominant markers, whose homo- or heterozygous state cannot be determined. We cannot distinguish between these two possibilities in F_2, and so a lot of information is lost.

To circumvent problems concerning the use of F_2 for mapping and linkage analysis, plants are either grown as shoot cultures (Ganal et al. 1992), or an immortalized F_2 (IF_2) is generated by pooled random mating of individual F_2 families in the F_3 and subsequent generations (Gardiner et al. 1993). The latter strategy is too time consuming for a self-pollinating crop such as chickpea, and tissue culture is too expensive. An interesting idea is to use pooled (Havey and Muehlbauer 1989) or individual F_3 DNA of individual F_2 descent for linkage analysis, because this will not only allow testing for the hetero- or homozygous state of dominant multilocus markers (Landry et al. 1987), but can also serve as a long-lasting source of DNA. This population could then be further genetically fixed by several rounds of selfing to give nearly homozygous (F_8: 99.2%) recombinant inbred lines (RI; Burr et al. 1988; Reiter et al. 1992). For more advanced studies with very tight linkage between markers and trait—which is a prerequisite for pyramiding of resistance genes (outlined below)—nearly isogenic lines (NILs) derived from several rounds of backcrossing and selfing are being used with high efficiency in several species (Sarfatti et al. 1989; Bentolila et al. 1991; Paran et al. 1991), and have already led to the map-based cloning of a resistance gene in tomato (Martin et al. 1993).

Use of wild species

For the creation of a genetic map of chickpea, crosses of wild *Cicer* species with cultivated lines are clearly preferable, because of the high degree of morphological, isozyme, and DNA polymorphisms available in the genus. A map based on morphological and isozyme markers already exists for crosses between *C. arietinum*, *C. reticulatum*, and *C. echinospermum* (Kazan et al. 1993), into which a DNA marker map could be easily integrated. The most useful population for the generation of a high-density map might be an F_3 or an RI line derived from the F_2 of the cross *C. arietinum* × *C. reticulatum*.

This population could also provide the basis for fine-mapping of polygenes responsible for quantitative traits such as: time of flowering, plant stature, number of seeds/plant, seed weight, biological yield, seed yield (Singh and Ocampo 1993), and cold and nematode resistance (Singh et al. 1993 a). This presupposes that individuals are selected that represent the most extreme parts of the distribution curve for each trait. These individuals can then be tested for the highest proportion of donor parent genomes by oligonucleotide fingerprinting. Individuals containing more than 80% of the genome of the recurrent parent (i. e. 20% or 150 Mbp of the donor) would be backcrossed, resulting in BC_1S_1 individuals containing an average of only 75 Mbp of the donor genome. These would include individuals carrying the genes responsible for the best (and worse) performance for every trait,

which could be identified in a second round of tests. If their genomes were analyzed with DNA probes, tightly linked markers for the traits could be established using computer software for the statistical analysis of quantitative trait loci (QTL; Lander et al. 1987). Because only 5% of the population is investigated, the number of individuals probed with DNA markers could be reduced to 250–300 per trait, down from the nearly 800 normally required for the analysis of a QTL (Beckmann and Soller 1986). These numbers may seem large, but the calculation was done for a QTL with only 5% impact on the trait and a marker distance of 5 cM, and therefore the numbers are quite conservative.

A second round of marker-assisted backcrosses, selfings, and selections would result in individuals carrying the QTLs of interest on a residual part of the donor genome of only 15 Mbp. Genomes should be fixed as NILs, to reach more than 99% homozygosity in the BC_2S_8 generation (Burr et al. 1988). A set of 100 individuals/trait would be sufficient to determine the genomic regions carrying the respective genes on intervals of 150 kpb. This is close enough to clone the genes, using yeast artificial chromosomes (YACs) or other molecular approaches (Winter and Driesel 1991). As this strategy is rather time-consuming, especially if one has to start from the beginning, existing crosses between cultivated and wild species already in an advanced generation (F_6 to F_8), should be considered for use (Giovannoni et al. 1991). The use of an existing F_8 population for the detection of QTL is schematically presented in Figure 1.

Quantitative trait loci, represented by the black bars in the genome of the donor parent and by white bars in the genome of the recipient parent, are transferred by crossing the donor and recipient parents to the recipient parent and selfing the F_2 individuals up to a nearly homozygous F_8 generation. One thousand F_8 individuals are then tested for the presence of QTL and the 50 best performing individuals—containing the QTL from the donor parent together with a portion of its surrounding chromosome regions—are selected. These individuals are subjected to linkage analysis with a set of DNA markers derived from the flanking genomic regions. Marker brackets of the most closely linked markers, M1/M2 and M3/M4, are defined and used for marker-assisted accumulation of QTL in further breeding programs.

A single cross between two lines is usually insufficient to generate markers for a desired trait, since in different cultivars different genes may be responsible for the same trait. For example, Bünger et al. (unpubished results) demonstrate linkage of a fingerprint marker to a dominant susceptibility locus for *A. rabiei* in the susceptible line ILC 1272, using a cross with the resistant parent ILC 3279. In order to accumulate resistance genes, it has to be

proven that different genes are responsible for susceptibility in other lines, which could not be exchanged by the putative allelic resistance locus in ILC 3279. This can be tested by analyzing crosses between ILC 3279 and other susceptible lines such as ILC 1929. In a "reciprocal" set of crosses between ILC 1272 and several resistant lines (mentioned by Singh et al. 1993 b) results should show whether or not the same locus is responsible for susceptibility in all combinations.

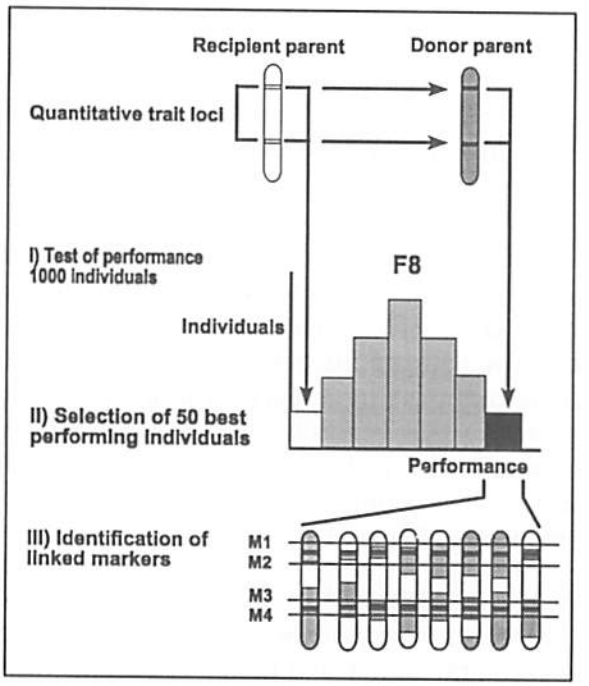

Figure 1. Detection of QTL in existing F_8 populations (Giovannoni et al. 1991).

Design of tests for desired traits

The catalogue of kabuli chickpea germplasm lists the ratings of several thousand kabuli cultivars and wild *Cicer* species for agronomically important traits (Singh et al. 1991). Ratings were taken in the field using several plants from one cultivar, thus averaging the effect of test conditions. Though probably sufficient to give a first hint of the overall performance of a cultivar, this method does not exploit the full potential of available screening methods for the elucidation of different gene effects on the behavior of a plant in the field. If tests for these aspects are performed separately as illustrated, taking *Ascochyta rabiei* resistance as an example, DNA markers have the potential to tag different genomic loci that play a role in plant performance.

In a recent review, Barz and colleagues (1993) listed a set of eight major mechanisms involved in resistance and susceptibility of chickpea towards fungal attack. These mechanisms include:

- The complex of hypersensitive response reactions (HR).
- Plant inhibitors for fungal cell-wall hydrolases.
- Induced phytoalexin production.
- Susceptibility of isoflavone and ptenrocarpan synthesis to fungal suppressor.
- Pathogen-induced enforcement of plant cell walls (HRGPs, phenolic compounds).
- Pathogen-related (PR) proteins.
- Secretion of hydrolases (chitinases, glucanases) for fungal cell walls.
- Sensitivity to fungal solanapyrone toxins.

The above-mentioned authors strongly recommend that researchers "investigate, individually, the impact of pathogen-derived molecules" such as suppressors, toxins etc. on the reaction of resistant and susceptible cultivars and use these individual reactions as "resistance markers."

For the highly desirable construction of pyramidal resistance in chickpea, a detailed knowledge of these mechanisms in different cultivars and wild species is essential, as these cultivars with different reactions (listed above) will provide the sources for the genes involved in resistance gene pyramids. If *Ascochyta* spp. resistance really depends on "gene-for-gene" interactions with considerable additive, dominant, and epistatic gene effects as suggested by Harrabi and Halila (1989), resistance has to be treated as a quantitative trait including one or several major dominant genes. Additive effects may also influence the plant's reaction at different stages of its life cycle, since some cultivars are more resistant as seedlings, others at the pod stage (Khoury 1993).

A combination of biochemical and field test conditions with DNA marker analysis will reveal the most useful genomic loci for the accumulation of resistance genes. This should include multiple loci for:

- Lowest level of induction of fungal toxins.
- No response to the toxins.
- Highest level of HR.
- Maximum growth inhibition of the fungus.
- Overall fitness of the host in the presence of the fungus.

If different fungal pathotypes are used in the tests, it may well turn out that different chickpea cultivars and wild species do not react the same way to various fungal pathovars (Reddy and Kabbabeh 1984). In this case different loci will be relevant for different gene pyramids. This has to be taken into account when "donor lines" for pyramidal resistance genes are designed.

Choice of DNA markers

Various DNA marker types are available. They differ significantly in terms of the time and cost that must be invested for their development and use.

For the elucidation of a polymorphic locus and its placement on the chickpea genomic map, virtually any type of marker that displays a polymorphism is suitable. For practical purposes, however, markers should be designed to:

- Test large numbers of individual offspring quickly.
- Allow the selection of individuals in an early stage of development.
- Require only limited amounts of DNA
- Give reliable, clear-cut results.
- Display polymorphisms between many cultivars.
- Be inexpensive.

These requirements are fulfilled only by PCR-based markers, which depend on primers derived from flanking sequences of polymorphic loci—so-called sequence-tagged sites (STS; Olson et al. 1989)—or, even better, from sequence-tagged microsatellite sites (STMS; Beckmann and Soller 1990). As STS and STMS are co-dominant markers, they enable the breeder to determine the allelic state of a particular locus already in the F_2 generation. All other markers will have to be converted into ST(M)S by cloning and sequencing before application in the field (Table 2).

For the development of DNA probes, the repertoire of markers listed in Table 2 is readily available—though not all are suitable for chickpea, as far as our experience is concerned. For example, easy-to-use multilocus markers, such as random amplified polymorphic DNA (RAPD; Williams et al. 1990) are not practical with chickpea DNA, because results are not reproducible. An alternative, worth testing with chickpea, is the use of semi-random RAPD markers (Weining and Langridge 1991; Rogowsky et al. 1992), where one primer is fixed to a dispersed repeat or splicing consensus sequence and the other primer binds randomly. This technique combines the advantages of RAPD with those of STS. However, their use requires information about repetitive DNA in the chickpea genome, which is not available at present.

Repeats could also play a useful role in crosses between wild and cultivated chickpea. These species vary in the amount and sequence of repeats, a fact which in this case can be used to tag genomic regions derived from one or the other parent (Jung et al. 1992). Other multilocus probes, such as minisatellites (Jeffrey et al. 1986; Dallas 1988), displayed only low levels of polymorphism in our tests, even at low stringency hybridization conditions, though they perform well in other organisms. Single locus probes such as RFLPs are normally very useful co-dominant markers. Unfortunately, in a genetically homogenous species such as chickpea, the high amount of DNA and the time needed for the separation of polymorphic from monomorphic probes make their use prohibitive. The development of random STS is technically demanding, because every locus has to be sequenced individually, and locus-specific PCR primers must be designed. On the other hand, STS and STMS will be the most useful probes for the breeder, and therefore should also be considered as first generation markers.

Until now, the best results have been obtained with synthetic oligonucleotide probes that detect polymorphic microsatellite loci in the chickpea genome. These markers are highly polymorphic, reliable, and require only limited amounts of DNA. They are also useful to the breeder because they allow the estimation of the distribution of parental genomes in offspring or the identification of cultivars (see below). Microsatellite loci are also used for the generation of STMS (Hüttel and colleagues, this proceedings) and have become the most useful single locus probes for routine use. STMS from tri- and tetranucleotide microsatellites are preferable to mono- or dinucleotide repeats, due to the clear banding pattern they provide (Hearne et al. 1992).

Table 2. Possible DNA markers for use in chickpea breeding.

DNA marker	Loci detected per experiment	Total loci per genome	Development time per locus	Efficiency of DNA use	Efficiency in use for breeding
RFLP	1–10	1/100bp (1)	long	low	low
Minisatellites	10–60	>1,000 (2)	short	high	low
Microsatellites					
a) Fingerprints	1–20	<200 (3)	short	high	(high)
b) ST(M)S	1	>25,000 (4)	medium	high	high
RAPD	1–10	infinite (5)	short	high	low
Semirandom PCR	10–20	infinite (6)	short	high	low
Repeats	<10,000	>100,000 (5)	short	high	low

Numbers of loci detected per experiment and per genome are estimates from comparable genomes, as exact numbers are not generally known, except for microsatellite fingerprints in chickpea. Estimations are from: (i) Soller and Beckmann (1983); (ii) Hillel et. al. (1990); (iii) Sharma et al. (1994 and this proceedings); (iv) Hüttel, personal communication; (v) my

own calculations; and (vi) Weining and Langridge (1991). Fingerprint markers listed in Table 2 are useful for the breeder only for specialized purposes such as the estimation of the number of parental genomes in F_2 or backcross generations and the evaluation (high) is therefore put into parentheses.

Towards a DNA marker map of chickpea

A genome map based on polymorphic markers provides a framework that makes it possible to find linked molecular markers for particular genomic regions involved in the expression of a trait using a limited set of markers scattered all over the genome. In a second screening cycle, more densely spaced markers from that particular region are used to tag the locus responsible for a trait. The most reliable information is provided by the pair of markers or "marker bracket" (Melchinger 1990) that flank the locus. Only one or two markers are used to indicate the presence of the locus in every subsequent cross.

The usefulness of a genome map is determined first by the density and even distribution of markers, and second by the marker's polymorphic information content (PIC). This means that a marker has to be polymorphic not only in the cross for which it was developed, but also in crosses between very closely related cultivars, crosses performed to increase the agricultural value of the crop. As this is not the case even with markers of very high information content, such as STMS, alternative markers that slightly increase the desirable marker density for the same locus should be to hand.

For the first screening to reliably localize the genomic region responsible for a trait, a marker spacing of 15 to 20 cM is sufficient. The chickpea genome contains about 750 Mbp of DNA, which is slightly less than the well-characterized tomato genome (950 Mbp; Arumuganathan and Earle 1991). If the same recombination rates are assumed for both crops, 1 cM should equal 500 kbp physical distance, so that 70–100 evenly distributed markers are sufficient for the detection of any particular locus in the chickpea genome. To cover 98% of a genome of 1,000 cM (such as the chickpea genome) with randomly distributed markers of 20 cM spacing, 178 probes are necessary to start with (Beckmann and Soller 1986). A genetic distance of 15–20 cM between marker and locus means that 15–20% of the offspring will have the adverse genotype of that indicated by the marker. As this error also occurs in subsequent generations, failure rates increase. For practical applications, such a high starting error cannot be tolerated (Soller and Beckmann 1983).

The same is true with gene pyramiding, where error rates increase with the number of genes involved, so that for a pyramid of four genes, with each defined by a marker 20 cM away, prediction of genotypes has an error rate that might be called chance. To overcome this problem, more tightly-linked markers for each locus, which can be detected by screening with a set of markers derived from the region of interest, must be used. Three to five markers are sufficient to find one within 3–5 cM of the desired locus. These calculations show that a genomic map of 300–400 evenly spaced markers is dense enough for every practical purpose. To further increase the marker density of the map, an exponentially increasing number of probes is required (Beckmann and Soller 1986). If, in a specific genomic region, more densely-spaced markers are needed, chromosome jumping should be used (Winter and Driesel 1991).

There is an apparent problem, concerning a desired map resolution of 3–5 cM, which is connected with the population size used for mapping. For a first screening cycle with 70–100 markers spaced at 15–20 cM, small F_2 populations of 40–50 individuals are sufficient and easy to handle. This tiny population is too small to allow a resolution of 5 cM, which is the upper limit for marker-assisted pyramiding of genes. This is because, statistically, a crossover occurs in the genomic region under observation only once in 20 individual meioses, which is much less than necessary to determine the most tightly linked marker with any certainty. For this purpose, either very large F_2s (1,000 individuals; Messeguer et al. 1991), NILs, or, as has been suggested for other crops (Giovannoni et al. 1991), advanced (F_6 to F_8) mapping populations can be used as outlined in Figure 2.

A set of 20 synthetic oligonucleotides is sufficient to reveal more than 70 polymorphic microsatellite loci between chickpea cultivars, as has been shown by Sharma and colleagues (1994 and this proceedings). Four of the 20 probes have been used by us for the detection of a dominant susceptibility locus for *A. rabiei* in the susceptible cultivar ILC 1272 (Bünger et al., unpublished). The obvious suitability of these markers for linkage analysis suggests their use for the generation of a basic linkage map in chickpea. First efforts to construct linkage groups from microsatellite fingerprint bands were successful, as shown in Table 3.

Table 3a. Linkage groups in ILC 3279.

Linkage group	Band number	Band name	Recombination fraction	Lod score	Genetic distance
1	16	gaatp2p2	0.17	2.44	21.5
	18	gaatp2s2	0.06	5.01	6.2
	38	catp2e1	0.0	7.73	0.01
	1	ctgtp2c2	cluster size		27.7
2	2	caap2h2a	0.14	3.31	16.31
	9	gatap2u2	cluster size		16.31
3	8	gatap2r2	0.2	2.65	25.0
	7	gatap2q2	0.2	2.94	26.3
	15	gaatp2n2	cluster size		51.4
4	12	gaatp2j2	0.17	2.44	21.1
	17	gaatp2q2	0.16	3.17	19.2
	10	gatap2w2	cluster size		40.3
5	11	gaatp2i2	0.14	3.31	16.31
	13	gaatp2l2	cluster size		16.31
Unlinked	7				152.2
Total	21				

Table 3b. Linkage groups in ILC 1272.

Linkage group	Band number	Band name	Recombination fraction	Lod score	Genetic distance
1	32	gaatp1g1	0.1	2.93	11.2
	29	gaatp1a1	0.09	4.18	9.8
	25	gatap1d1	0.0	9.17	0
	20	ctgtp1a1	0.05	5.90	5.9
	34	catp1a1	cluster size		26.9
2	30	gaatp1b1	0.17	2.44	21.44
	22	caap1g1	0.17	2.44	21.44
	33	ArS1	cluster size		42.5
3	31	gaatp1f1	0.07	5.89	8.11
	36	catp1c1	cluster size		8.11
Unlinked	10				
Total	17				77.51

Linkage analysis was performed with the program "Mapmaker" (Lander et al. 1987). Lod scores >3 are indicated by bold letters, as is the dominant susceptibility locus ArS1 and the linked marker band caap1g1.

Microsatellite marker bands were assigned to five linkage groups in ILC 3279 and three in ILC 1272, comprising 152 and 77 cM, respectively, of the chickpea genome. Taking into account the great number of unlinked loci, and that only five of the 20 useful synthetic oligonucleotides were used,

more linkage groups are expected to be found as experiments proceed. It is evident that microsatellite loci are distributed evenly over the genome, which turns them into useful markers in their own right for purposes such as prediction of the percentage of a parental genome in F_2 and BC individuals.

The fact that for each parent a separate linkage map has to be drawn exemplifies the drawbacks of multilocus dominant markers for linkage analysis. As it is not known which of the bands of one parent corresponds to a specific band of the other parent, or if the parent displays any band at all, no correlation can be constructed between the two maps until at least one of the markers from each linkage group is converted into a co-dominant STMS by cloning and sequencing.

On the other hand, the existence of many unlinked markers, which will fall into linkage groups when more markers are applied, shows the potential of the method for the generation of a basic map of the chickpea genome.

Unfortunately, the preliminary map displayed above cannot be integrated into already existing morphological and isozyme maps of chickpea (Gaur and Slinkard 1990a, b; Kazan et al. 1993), which were constructed on crosses between the cultivated chickpea *C. arietinum* and its wild relatives *C. reticulatum* and *C. echinospermum*. It would help if researchers could agree to use a common reference population for their linkage analysis, designed along the lines discussed for the use of wild species in the "design of population" section. This population could be provided by one of the international research institutions, as the Centre Ètude de Polymorphism Human in Paris does for the human genome.

Routine Use of DNA Markers

DNA markers can be used for chickpea improvement in two ways:

1. For the management of germplasm, elimination of false F_1 individuals, and estimation of the percentage of parental genomes in F_2 and backcross generations, only polymorphisms need to be known. The exact location of visible markers, or homo- or heterozygosity of loci, is not required. These markers are already developed in the form of microsatellite fingerprints and ST(M)S (Hüttel et al., this proceedings), and await evaluation in field tests.
2. For introgression and accumulation of genes responsible for disease resistance, stress tolerance, yield, and other quantitative traits, markers have to be much better characterized. Information about their map position is essential. They reveal their full potential only in a genome map of chick-

pea. For every application a set of a few STS or STMS markers should be designed, which, due to the power of the PCR, can be used in the very early stages of cultivar improvement on large numbers of young plants.

As the generation of this kind of marker, as well as a genome map of chickpea, is still in its infancy, further discussion takes the shape of a proposal rather than a recipe, and should be accepted as a glimpse into the future of marker-assisted breeding in chickpea.

DNA preparation methods for routine use with DNA markers

The preparation of DNA is a limiting step in the screening of large amounts of individual plants with DNA markers, a fact which could seriously affect the use of DNA markers in the field. However, fast, robust, and reliable methods are available that are yield sufficient DNA for all practical applications. Three of these techniques are discussed below:

1. Squashes of plant tissue as substrate for PCR (Langridge et al. 1991). This method, schematically presented in Figure 2, is designed for the preparation of many samples from individual plants for use with PCR-based markers such as STS or STMS. A board is covered with Whatman 3MM paper and Hybond N nylon membrane, both presoaked in 1M NaOH. The pack is covered with a clear, robust, plastic screen (overhead foil), in which 0.5 cm diameter holes have been punched. A 1 cm leaf segment is cut from the plant and crunched onto the membrane with a stainless steel rod, leaving a green circle on the filter. The membrane is then rinsed several times in a neutralization solution, after which it can be directly used for PCR or dried and stored for later use. Before use, the membrane segment with the green circle is cut from the membrane and half of it is incubated for 5 minutes with 30 μl of water at 94° C in an Eppendorf tube. Twenty microliters of the solution is sufficient for a PCR reaction. The method does not require any centrifuge and a hundred samples can be prepared per person per day.
2. Preparation of pure genomic DNA from isolated nuclei (Winter and Sharma, unpublished. This method is a shortcut to a method originally developed by Willmitzer and Wagner (1981) and generates high molecular weight DNA, suitable for all purposes, including cloning. The technique is easy, fast, and reliable and works especially well with chickpea leaves. Up to 24 individual samples can be prepared per person per day. As the method has not been published, it is added as an appendix to this article.
3. Another rapid and useful technique is based on the extraction of plant tissue with cetyl-trimethylammonium bromide (CTAB) and was originally developed by Saghai-Maroof et al. (1984). This technique works with vir-

tually any material, including dried leaves, fungal hyphae, roots, and shoots but sometimes needs subsequent CsCl gradient centrifugation to yield digestible DNA. The method has been used routinely for fingerprinting in chickpea (Weising et al. 1991).

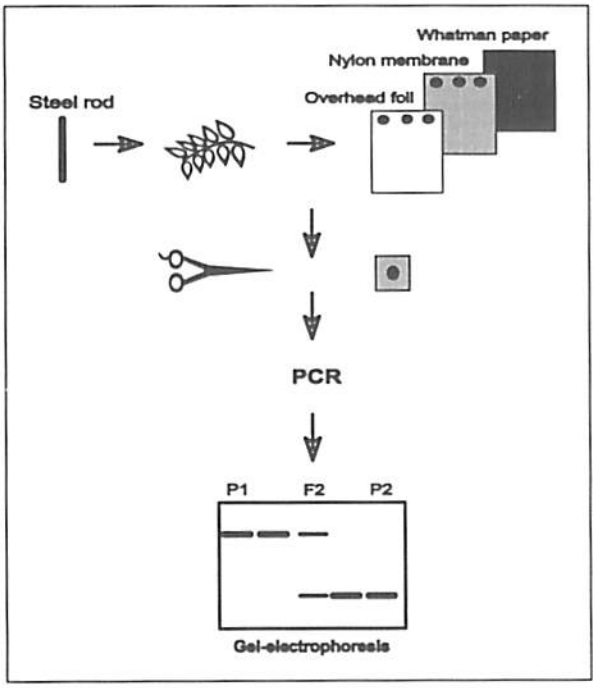

Figure 2. Squashes of plant tissue as substrate for PCR.

Characterization of landraces, cultivars and breeding lines

Large germplasm collections such as ICARDA's contain thousands of different cultivars, many of which cannot be distinguished phenotypically. To characterize and manage these lines, as well as to protect breeders' rights, microsatellite fingerprinting with synthetic oligonucleotide probes is ideal (Weising et al. 1991, 1992; Sharma et al. 1994; this proceedings). This is because many loci can be made visible at the same time, only small amounts of DNA ($<10\ \mu g$) are needed, the technique is highly reproducible, and fingerprint data can be digitally stored and handled using a computerized data management system. As fingerprinting in chickpea is possible using non-radioactive techniques (Bierwerth et al. 1992), it can be used in any laboratory without special safety precautions or health hazards and does not necessitate a supply of radioactive phosphate.

Elimination of selfed individuals from F_1 populations

Chickpea is a self-pollinating crop and needs emasculation before pollen can be applied to the stamina. As this is not always successful, or carried out at the right time, some of the F_1 will be derived from selfing instead of crossing, and, in the absence of morphological markers, will contaminate the offspring with pure parental genotypes.

A real F_1 individual contains both parental genomes in equal portions. The presence of only one DNA marker from the male is therefore sufficient to indicate a real cross. This can be tested by performing PCRs, with one or two ST(M)S markers for which the parents are polymorphic, on DNA from tissue squashes of one leaf of each individual (see above). A set of 96 individuals can be tested per day, using a 96-well PCR machine. The low amount of DNA needed allows this screening to be done on very young plants, saving space and resources for real crosses.

Identification of offspring carrying desired genes by marker-facilitated selection

The identification of desired genes in F_2 or subsequent generations with DNA markers is based on the availability of a tightly linked marker and the knowledge of its phenotype (i. e. band size) in both parents. Because marker loci with the highest information content are not only polymorphic between accessions, but also within accessions (Sharma et al. 1994; this proceedings), a control DNA from the individual parents used in the cross—or at least from their siblings—should always be included to assign a desired gene without ambiguity. In principle, the marker type and the identification process are the same as for the identification of false F_1 individuals. The difference is that here the locus should be well defined and, whenever possible, a "marker bracket" (Melchinger 1990) of two flanking markers should be applied to minimize error rates. When squashes of plant tissue are used, enough F_2 plants can be screened by one person to determine the genotype of the whole population from a cross for one locus (300–400 individuals) within a week. If more loci are to be screened, in, for example, the detection of QTL, this number has to be reduced. On the other hand, it may be possible to combine several primers for different loci in one PCR reaction, which could increase the throughput and reduce cost.

Selection of optimal F_2 individuals for backcrosses: Estimation of the amount of parental genomes in offspring

Often, the breeder wishes to introduce only one gene—for instance, a resistance gene—into an otherwise favorable variety. This necessitates the reduc-

tion of the donor parent's genome as much as possible, saving only the introgressed gene. This is conventionally done by several rounds of backcrossing, selfing, and testing for the trait. The use of molecular markers can shorten the time needed from more than six to only three generations, until—after one and a half instead of five years—nearly 100% of the recurrent genome is achieved (Beckmann and Soller 1986; Tanksley et al. 1989). This calculation is based on an average marker spacing of 20 cM, which is approximated by the 70 or more polymorphic microsatellite fingerprint bands detected on average by 20 category I oligonucleotide probes. Thus, it is currently possible to perform this selection.

Plants that are homozygous for the gene for which the cross is performed can be identified at an early stage by STS or STMS markers, before application of fingerprint markers. Only these plants are grown and subjected to microsatellite fingerprinting. In a backcross program to recover at least eight carriers of the desired gene after BC_3, approximately 100 plants are tested if a marker bracket of 10 cM distance is applied, 80–90 plants are tested for a 5 cM marker bracket. If direct screening methods are used, only 76 plants are tested. However, at BC_3, marker-selected individuals will have gained nearly 100% of the recurrent genome, compared to 87.5% without selection (Melchinger 1990). Therefore, the economic benefit of backcross marker selection for introgression of single genes is based mainly on speed.

Prospects for Marker-assisted Accumulation of Resistance Genes

The introgression of different resistance genes from various sources into one cultivar has been assumed to confer durable resistance for different *Ascochyta rabiei* pathotypes (Anonymous 1989). Therefore, one of the major aims of chickpea breeding is the detection of suitable resistance genes and tightly linked markers that are a prerequisite for the construction of polygenic resistance in chickpea.

The first linkage of markers to resistance loci for fungal diseases of chickpea such as fusarium wilt and ascochyta blight are described by Muehlbauer, Bünger and colleagues (this proceedings). Identified loci can now be used to discriminate between different resistance loci. Suitable crosses, test conditions, and a densely spaced linkage map should allow us to tag additional loci for a whole set of plant reactions with, ideally, PCR-based markers such as STS or STMS.

As only two partners can be crossed at one time, a generation of "donor lines" will be necessary for the construction of polygenic resistance. The design of these lines will depend on genetic models that—at least in the case of *Ascochyta rabiei* resistance—must still be evaluated. At present, existing DNA markers for resistance loci to ascochyta blight and fusarium wilt can already be used to generate donor lines and to transfer both traits into susceptible cultivars, thus serving as a model system in marker-assisted breeding for chickpea improvement.

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Appendix: Isolation of Chickpea DNA from Isolated Nuclei (Winter and Sharma, unpublished)

Two grams of fresh chickpea leaves are homogenized, using Ultrathorax (Bükler, Tübingen, Germany), in small plastic beakers for 30–60 seconds at full speed in 20 ml ice-cold homogenization buffer consisting of 0.25M sucrose, 10mM NaCl, 10mM morpholinoethane sulphonic acid (MES), 5mM EDTA, 0.15mM spermine, 0.5mM spermidine, 20mM-mercaptoethanol, and 0.1% bovine serum albumin (BSA). Mercaptoethanol and BSA are added after the components are dissolved in sterile double distilled water and sterile filtration. The homogenate is filtered through four layers of miracloth and centrifuged for 10 minutes at 2000xg at 4° C. The pellet of chloroplasts, nuclei, and starch is suspended in 10 ml cold 5× homogenization buffer, containing 0.6% Nonidet P40, by repeated suction into and release from a 5 ml pipit to destroy the chloroplasts. The suspension is centrifuged as above, the supernatent discarded, and the procedure repeated. If the pellet is still contaminated by green debris, it is re-suspended and filtered through miracloth. The white pellet is gently re-suspended in 500 μl TE (10mM Tris-HCl, pH 7.5, 1mM EDTA), then 500 μl NDS buffer (0.5 M EDTA, 1% sodium dodecyl sulfate) is added and the mixture incubated with 50 g/ml proteinase K at 56° C for 3–4 hours. After standard phenol/chloroform extraction, DNA is precipitated with 2 vol. of cold ethanol and either fished out with a cut-end pipit tip or pelleted by centrifugation at 5,000xg for 10 minutes.

The DNA is washed once in 1ml 70% ethanol, and the ethanol residue is evaporated without allowing the DNA to become totally dry. Finally, the DNA is rehydrated in 100 μl TE and directly subjected to restriction. The method yields 40 g DNA or more from 2 g of leaves.

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Expanding the Repertoire of Molecular Markers for Resistance Breeding in Chickpea

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Abstract

The use of molecular markers has assisted plant breeding in a variety of areas, including resistance breeding. This paper deals with different types of molecular markers (isozymes, mini-and microsatellites, RFLPs, and RAPDs) and their use for tagging of genes for disease resistance.

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume in the world, after dry bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.). Despite many efforts during the last three decades to breed for increased yield in chickpea, strides in this direction have been less impressive than for many other important crops such as wheat, rice, and soybean. There are two main reasons for this situation: (i) limited genetic variation (van Rheenen *et. al.* 1993); and (ii) susceptibility to many diseases (Nene and Reddy 1987) and pests. The first problem can be addressed by expanding the existing gene pool by non-conventional breeding approaches such as mutation breeding and interspecific hybridization. To overcome the second problem, the most desirable and effective strategy has been to incorporate disease resistance genes into commercially acceptable cultivars. This approach is continuously used for breeding resistant chickpea cultivars (Singh *et* al. 1992). However, this approach is time-consuming and breeding for pyramidal resistance is difficult.

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In recent years, the use of molecular markers has speeded up and assisted practical plant breeding for a variety of objectives, including resistance breeding (Melchinger 1990). The establishment of molecular marker technology and the generation of a genetic crop map is also desirable for marker-assisted selection and gene tagging, leading to positional cloning of genes for economically important traits including disease resistance. This approach holds great practical promise for resistance breeding, since the products are not known for either host resistance genes or pathogen virulence genes.

Plant genetic maps can be constructed using phenotypic or molecular markers. Phenotypic markers include traits such as leaf or flower morphology, plant size, and pigment biosynthesis. Genetic maps based on phenotypic markers are now termed "classical maps" to distinguish them from "molecular maps," which are based on protein or DNA markers. However, integrated maps based on phenotypic and molecular markers are also now available. Molecular markers have several advantages over morphological markers: numerous markers can be identified in a single breeding population; a relatively large number of alleles are available for one marker; most molecular markers exhibit a co-dominant mode of inheritance; molecular markers are generally silent in their effect on phenotype and are not influenced by the environment; and they can be scored at a very early development stage, allowing early population screening. An ideal DNA marker should be highly polymorphic, detectable with a non-radioactive, simple, cheap, and reproducible assay.

Protein markers, generally isozymes, are fast and inexpensive, but have limited usefulness because of their low frequency (Helentjaris et al. 1986). DNA markers arise from variations in DNA sequences, either base changes or insertions/deletions. These changes are termed DNA polymorphisms. In the past, molecular markers have been used to map loci for qualitative and quantitative traits and to generate linkage maps in a variety of crop plants such as wheat (Hart and Gate 1990), tomato, potato (Tanksley et al. 1992), lentil (Havey and Muehlbauer 1989; Muehlbauer et al. 1989), soybean (Palmer and Kiang 1990), lettuce (Kesseli et al. 1990), maize (Coe et al. 1990), and barley (Graner et al. 1991; Heun et al. 1991).

In chickpea, several spontaneous and induced mutations affecting traits such as leaf morphology, number of flowers per peduncle, polycarpy in flowers, seed protein content, and nodulation have been reported (for references see Kazan et al. 1993). Muehlbauer and Singh (1987) review the genetics of several traits in this crop and report linkages among several morphological markers. A rudimentary genetic map for *Cicer* species has been

created, which includes 26 isozymes and three morphological loci (Gaur and Slinkard 1990). Kazan et al. (1993) reaffirm many linkages of this map and propose a linkage map containing 23 molecular (isozyme) and five morphological loci. It has been suggested that pea, lentil, and chickpea have several common linkage groups consisting of homologous genes. Thus, linkages found in one genus can be used to predict similar linkages in related genera.

Following, a brief account of various molecular markers for the preparation of genetic maps and for tagging of genes—particularly for disease resistance—is presented.

Isozymes

During the past two decades, techniques have been developed for the analysis of allelic variants coded for specific enzymes (isozymes) in plant tissue (Tanksley and Oston 1983). Isozymes, or multiple molecular forms of enzymes, recognize a common substrate but differ in electrophoretic mobility because their amino acid sequences vary. Proteins in crude tissue extracts are separated by electrophoresis on starch or polyacrylamide gels and subsequently made visible by staining the gel for a specific enzyme activity. Different enzyme systems often require specific laboratory procedures. Some enzymes can only be assayed at a certain developmental stage or in specific tissues. Data retrieved from electrophoretic gels consist of the number and relative mobility of various enzyme products. Using appropriate genetic analysis, this data can be transformed into single or multilocus genotypes. Once established, isozyme methods are fairly simple and can be employed on a large scale. Isozyme systems have been applied to more than 30 crop species (Tanksley and Oston 1983). Although in some species (e.g. *Zea mays* and *Secale cereale*) 40 or more isozyme systems are known, only 10–20 polymorphic isozyme loci are commonly found in most breeding populations (Stuber and Goodmann 1983).

Many studies on isozymes in chickpea have been conducted (Gaur and Slinkard 1990a, b; Kazan and Muehlabuer 1991; Kazan et al. 1991, 1993; Oram et al. 1987; Tuwafe et al. 1988). These studies show that a majority of isozyme loci are monomorphic. Out of the 27 loci studied only four were polymorphic, suggesting limited variability of isozyme loci in cultivated chickpea. Of these polymorphic loci, one had three alleles. However, the level of polymorphism detected at inter-specific levels was much higher, since 26 of the 27 loci were found to be polymorphic (Oram et al. 1987). No attempts have been made to use information on isozyme polymorphisms to detect linkages with disease resistance traits in chickpea. Nevertheless, a

number of studies have shown linkage of isozyme markers with disease resistance in other crop plants. For example, the locus for the plastid-specific form of phophoglucomutase (Pgm-p) is closely linked to the gene for resistance to bean yellow mosaic virus (Mo) in pea (Weeden et al. 1984). Similarity, an alcohol dehydrogenase locus (Adh-1) has been linked to a gene for resistance to pea enation mosaic virus (En; Weeden and Providenti 1987).

A close association was also identified between an endopeptidase locus and stem breaker foot rot resistance in wheat (McMillin et al. 1986), and an acid phophatase locus and nematode resistance in tomato (Rick and Fobes 1974). Feuerstein et al. (1990) have used *Hordeum vulgare* L. subsp. *spontaneum* backcross-divided lines to identify isozyme loci (Est 2, Acp 3, and Dip 2), which are linked to rust resistance genes (*Puccinia hordei* Otth) in barley. Wagner et al. (1992) have devised a procedure for the estimation of linkage in cases where distorted segregations are observed, a situation often observed with isozyme and molecular markers during segregation.

Due to the low polymorphism detectable with isozyme markers, and other inherent disadvantages such as low allelic number and the need for a specific developmental stage for certain enzymes, this technology has had only limited use for tagging genes for disease resistance—particularly in chickpea, where little intraspecific polymorphism is available.

Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs detect differences in the length of specific DNA fragments after digestion of genomic DNA with sequence-specific endonuclease (Botstein et al. 1980). These differences result from base pair changes or other rearrangements (e.g. translocation and inversion) at the recognition site of the restriction enzyme, or from internal deletion/insertion events. The restricted fragments are separated according to size by agarose gel electrophoresis, and polymorphism is detected by Southern blot analysis. The DNA sequences used as hybridization probes are generally pre-selected to contain unique or low copy number DNA sequences and labeled with ^{32}P or biotin (Winnacker 1987). Such probes can either be cloned DNA from specific genes, cDNAs, random genomic DNAs, or specifically synthesized oligonucleotide (Beckmann and Soller 1986). A survey of the different types and sources of probes as well as the different techniques to detect RFLPs is given by Landry and Michelmore (1987).

Since RFLPs directly reflect differences in DNA sequences, they are independent of gene expression and can be determined at any stage of develop-

ment and in all tissues, with few exceptions. Moreover, laboratory methods for detecting RFLPs are generally the same, irrespective of the species or markers investigated. One of the foremost attributes of RFLPs—compared to isozymes—is the detection of substantially greater polymorphism in breeding material (Beckmann and Soller 1986). RFLP and isozyme markers have another advantage: a large homology shared by related taxa, for example, tomato, potato and pepper (Bonierbale et al. 1988; Tanksley et al. 1988), pea, lentil and chickpea (Kaznan et al. 1993). However, RFLP assays require expensive laboratory supplies and are more time consuming than isozyme analysis.

RFLP markers have frequently been used to generate linkage maps in a variety of animal and plant species. During this process a number of qualitative and quantitative traits, including disease resistance, are tagged. For example, Landry et al. (1987) have established a linkage map of lettuce (*Lactuca sativa*) that includes five downy mildew resistance genes. Similarity, RFLP loci linked to disease resistance have been reported in maize (McMullen and Lovie 1989), tomato (Sarfatti et al. 1989, 1991; Young et al. 1988), and barley (Hinze et al. 1991). There are other examples for tagging disease resistance genes. Recently, Churchill et al. (1993) described a pooled sample approach to identify markers (e.g. RFLPs) closely linked to a locus of interest in a large segregating population. The feasibility of this method is demonstrated by the high resolution mapping of a region on chromosome 5 of tomato that contains a gene regulating fruit ripening.

RFLPs have been repeatedly tried in chickpea without success, since not much polymorphism could be detected in the cultivar (R.P. Sharma, personal communication; van Rheenen et al. 1993). However, before discarding RFLPs for mapping purposes in chickpea, more enzyme probe combinations need to be explored. The use of a segregating population following interspecific crosses involving *C. arietinum.* and *C. reticulatum* Lad. or *C. echinospermum* P.H. Davis may also be helpful. However, resistance breeding will not be helped much, as the crossable wild *Cicer* species are generally susceptible to major diseases.

Minisatellites or VNTRs

These so-called hypervariable markers detect high levels of restriction fragment length polymorphisms due to tandem repeat arrays in the genome. More specifically, RFLPs arise from variations in the number of repeat units in the tandem arrays, hence the acronym VNTR—variable number of tandem repeats.

Minisatellite markers were first discovered in humans by Wyman and White (1980) and Jeffreys et al. (1985a, b). The first study involved a random cloned sequence, whereas subsequent reports were based on studies with genes of medical interest such as the insulin gene, globin genes, and the HrasI oncongen. The repeat unit is 9–65 bp long, reiterated 10–300 times at each locus (array range from 0.1 to 20 kbp). There may be up to 1,000 minisatellite loci in the human genome (Jarman and Wells 1989; Jeffreys et al. 1985b; Wahls et al. 1990). Different human minisatellite arrays share a 10–15 bp conserved core sequence (see Wells et al. 1989). Therefore, Southern hybridization with a probe containing the core sequence will lead to a complex banding pattern, which constitutes a DNA fingerprint because of the inherent variability of some minisatellites.

The variation in the number of repeat units can be detected after DNA digestion with enzymes that do not cut within the repeat units but do cut outside the minisatellite array. Various mechanisms have been suggested to account for the variation in repeat unit number, including unequal crossovers, replication slippage, and insertion/deletion of repeat units (Jarman and Wells 1989; Jeffreys et al. 1985a). Analysis of recombination involving markers flanking the minisatellite array suggested that new alleles do not arise from unequal crossover between homologous chromosomes (Wolff et al. 1989). However, sister chromatid exchange at mitosis or meiosis was not ruled out. A skewing towards smaller alleles suggested that larger alleles were comparatively less stable (Jarman and Wells 1989).

Southern hybridization of minisatellite markers derived from one species has revealed DNA fingerprinting patterns in other species. Surprisingly, an apparently unrelated sequence from M13 (*Escherichia coli* phage) has also revealed DNA fingerprinting patterns in a wide range of organisms, including plants and prokaryotes (for references see Gepts et al. 1992). Cultivated plants in which M13 has detected fingerprinting patterns include alfalfa, apple, *Arabidopsis thaliana*, avocado, blackberry, clover, common bean, flax, lettuce, *Lolium perenne* L., maize, pea, *Pinus radiata* (D. Don), plum, and raspberry.

Minisatellite markers have also been used for a variety of other purposes to take advantage of their hypervariability, including mapping, where they have been particularly useful because of their high polymorphism information content (Donis-Keller et al. 1986; Jeffreys et al. 1985a; Nakamura et al. 1987). However, the preferential localization of variable M13-related sequences near the chromosome ends (Royle et al. 1988) suggests that this class of markers is suitable for mapping of chromosome ends in conjunction with telomeric sequences (Armour et al. 1989) rather than general map-

ping. Despite their highly polymorphic nature, with many allelic forms, minisatellite markers are cumbersome, as they involve the use of Southern hybridization. We attempted fingerprinting chickpea accessions using nine VNTR markers (isolated by Nakamura and his colleagues) without any success (our unpublished results).

A more practical alternative involves the use of PCR—if primers can be designed that flank the tandem repeat array, and the size of the arrays does not exceed the amplification capability of the technique.

Microsatellites or Simple Sequence Repeats (SSRs)

Another class of hypervariable markers, similar to the minisatellite, is the microsatellite, except that the repeat unit consists of di, tri-, or tetranucleotides such as $(CA)_n$, $(GTG)_n$, and $(GATA)_n$. In the human genome, the number of dinucleotide repeats is 15–40. The repeat-containing sequences are much more common than VNTR sequences ($4x10^4$), and apparently randomly distributed, showing high polymorphism with six or more alleles (Love et al. 1990). These so-called simple sequences, or microsatellites, are ubiquitous components of all eukaryotic gnomes analyzed so far (Tautz and Renz 1984; Greaves and Patient 1985; Epplen 1988, Weising et al. 1991). A survey of published DNA sequence data for the presence, abundance, and ubiquity of all types of dinucleotide and trinucleotide in higher plants shows that such microsatellites are frequent, and widely distributed, with a frequency of one every 50 kb (Morgante and Olivieri 1993).

Simple sequences are a major source of genomic variation (Tautz et al. 1986) and therefore it is not surprising that probes complementary to simple motifs could be successfully applied to DNA fingerprinting for more than 150 animal, plant, and fungal species (Epplen et al. 1988). In-gel hybridization with labeled oligonucleotide probes offers several advantages over the conventional blotting technique: it is faster, prehybridization steps can be omitted, a 100% transfer efficiency is obtained, and non-radioactive probes can be used.

Simple sequence motifs originate mainly from slipped-stand mispairing during replication (Levinson and Gutman 1987). They are also, however, generated by telomerase activities (Zakian 1989). Once a stretch of simple sequences is present, heterogeneity may be created by slipping combined with point mutations, unequal crossing over, or other recombinational events (Tautz and Renz 1984; Tautz et al. 1986; Levinson and Gutman 1987).

Although multilocus DNA fingerprinting using minisatellite probes has already served to produce linkage maps in humans (Wells et al. 1989), animals (Julier et al. 1990; Georges et al. 1990), and fungi (Romao and Hamer 1992), fingerprinting with simple sequence repeat probes for detecting linkages in plants has been attempted in chickpea only by Thomas Bünger (unpublished results). Weising et al. (1992) and Sharma et al. (1994) have shown that high levels of intraspecific polymorphism in chickpea can be generated by employing a variety of these probes.

Table 1. Numbers of within- and between-accession polymorphisms detected by oligonucleotide fingerprinting of *Taq*I- and *Dra*I-digested DNA of three chickpea accessions with polymorphic probes.

Enzyme	Accession	Number of probes tested	Number of polymorphic bands			
			Total	Min. Per probe	Max. per probe	Average
*Taq*I	482	13	24	0	7	1.85
	1272	13	12	0	5	0.92
	3279	13	29	0	9	2.23
	482/1272	15	53	0	12	3.53
	482/3279	15	53	0	11	3.53
	1272/3279	20	78	0	12	3.90
*Dra*I	482	13	21	0	5	1.61
	1272	13	14	0	6	1.08
	3279	13	20	0	7	1.54
	482/1272	14	54	0	10	4.57
	482/3279	14	59	1	12	4.21
	1272/3279	14	68	0	14	4.86

The level of polymorphism revealed by microsatellite fingerprinting depends upon the probe used. No obvious correlation exists between abundance, fingerprint quality, and sequence characteristics of a particular motif. As expected, digestion with methyl-sensitive enzymes shows that simple sequence motifs are enriched in highly methyl sensitive genomic regions. The high level of intraspecific polymorphism detected by oligonucleotide fingerprinting suggests the use of simple sequence repeat probes as molecular markers for genome mapping. Some probes, such as $(CGGA)_4$ and $(GCGT)_4$, are stable within an accession but polymorphic between accessions. A set of such probes may thus provide an invaluable tool for the identification of accession but be polymorphic between accessions. The 20 probes classified in category 1 (polymorphism probes) revealed between 3.5 and 4.8 between-accession polymorphisms per probe. How many of these polymorphisms are actually useful for linkage analysis will depend on the extent of alleles and clustering, which remains to be determined. However, the number of markers may be considerably increased if parents that show maximum diversity of their fingerprint patterns are selected, and if bands that are polymorphic

within these accessions are also included. Compared to *TaqI*, *DraI* detects more between-accession polymorphisms, but slightly fewer within-accession polymorphisms per probe. It thus seems to be more suitable for genome mapping using SSR probes.

Linkage maps based on DNA fingerprinting have both advantages and limitations when compared to maps derived from other types of markers. Advantages include the high level of detectable polymorphism, the rapid screening of the genome with comparatively few probes, the availability of universally applicable probes without cloning, and the high reproducibility (compared to RAPDs). Limitations arise if extensive clustering of simple repeats occurs in particular genomic regions, or if high mutation rates lead to unexpected fragments in the progeny (Jeffreys et al. 1988a). High levels of instability were indeed encountered with some chickpea fingerprint bands, e.g. one particular band detected by a $(TAG)_5$ probe (Bünger et al., unpublished). Inclusion of parental and F_1 DNA samples in the segregation analysis and the preferred use of accession rather than individual-specific markers (presumably less stable) will help to minimize this problem.

One of the disadvantages that SSRs share with RAPDs concerns the unknown allelic state of a fingerprint band, which means that its occurrence has to be treated as a dominant rather than a co-dominant marker. Finally, to isolate the gene responsible for a trait linked to a fingerprint band, the respective band has to be cloned before it is used as a starting point for chromosome walking.

Once the sequence of the DNA flanking microsatellite is known, suitable PCR primers can be designed to amplify the stretch of DNA incorporating the microsatellite, and its length determined by electrophoresis in acrylamide or agarose gels (Weber and May 1989; Litt and Luty 1989). Such experiments have shown that multiple length variants can be identified at most microsatellite loci, even in a non-polymorphic species such as soybean (Akkaya et al. 1992; Morgante and Olivieri 1993). High polymorphism, somatic stability and co-dominant inheritance make microsatellites a very powerful tool for genetic mapping and genetic diagnostics. One advantage of microsatellite markers is that they can be shared between laboratories by exchanging primer DNA sequences, avoiding the transfer or shipment of probes that other types of markers require. In contrast to the sequence-tagged sites (STS) concept (Olson et al. 1989), sequence-tagged microsatellite sites (STMS; Beckmann and Soller1990) are useful as a common language in genetic mapping. Microsatellite markers are also suited for use in map-based cloning. STMS can also be used in the integration of physical and genetic maps (Beckmann and Soller 1990; Edwards et al. 1991).

We have initiated experiments to clone SSR loci from the chickpea genome. Our strategy included the isolation of size-selected DNA fragments from gel regions harboring the fingerprinting band, ligation into a phagemid vector, transformation of *E. coli*, and identification of positive clones by colony hybridization to the respective oligonucleotide probe. The desired clones were tested for polymorphism by hybridization to genomic DNA of different chickpea accessions. Analysis of the flanking regions of SSR loci may provide an insight into the mechanism of sequence evolution and conservedness of those loci and related-genomes to study the generation mechanism of simple sequence length polymorphisms. The cloned SSR loci can be transformed into RFLP and STMS markers. Following hybridization of PCR analysis they can be used as anchor points for a chickpea genetic map.

Because of the advantages offered by microsatellite markers, they are currently considered the ultimate marker for genome mapping. Microsatellite marker-based high-density genetic maps are being constructed for humans and mice. At present, the disadvantages are high cost and labor.

RAPD Markers

DNA amplification based markers, RAPDs (William et al. 1990), DAF markers, and AP-PCRs (Welsh and McClelland 1990) are based upon the fact that a short oligonucleotide or randomly chosen sequence (primer), when mixed with genomic DNA (template) and a thermostable DNA polymerase (Taq polymerase from *Thermus aquaticus*) and subjected to the temperature cycling conditions of PCR (Innis et al. 1990), will allow the amplification of several DNA fragments. The reaction products can be separated on standard agarose gels and made visible via ethidium bromide staining. Polymorphisms between individuals result from sequence differences in one or both of the primer-binding sites and from small insertions or deletions in the region of the genome that is amplified, or from both. They are visible as the presence or absence of a particular RAPD band. On average, a 10 mer primer will hybridize to a strand of DNA about once every 1 million bases. The small amounts of DNA (5-25 ng) required, the non-radioactive assay performable in several hours, and the simple experimental set-up make RAPD markers attractive for automated breeding applications.

Another shortcoming of RAPD markers, which may hamper their use in different studies, is repeatability and reproducibility. Results may vary drastically if the PCR conditions are altered, and it is therefore mandatory to decide upon a set of optimum conditions and to strictly adhere to them. Many experiments have focused on the effect of parameters that qualitatively or quantitatively alter the amplification products (Munthali et al. 1992;

Weeden et al. 1992). Although template DNA of high purity is crucial for reproducible results, a 10-fold variation in the concentration of the template (3–30 ng per 25 μl reaction) and a 2- to 5-fold variation in the primer and Mg^{+2} concentration is possible without seriously affecting the RAPD pattern. In general, 20 mer primers are more specific than 10 mer primers.

One of the first practical uses of RAPD markers allowed the creation of high density genetic maps and saturation of already existing genetic maps (*Arabidopsis thaliana*: Reiter et al. 1992; tomato: Klein-Lankhorst et al. 1991; pine: Chaparro et al. 1992; sugarcane: Al-Janabi et al. 1993). Many other crop plants are being surveyed for genetic polymorphisms that will lead to genome mapping.

RAPD markers are dominant markers. When identifying heterozygous regions, two closely linked RAPD markers, each amplified from a different parent, may be used as a pair. Amplification of both markers of the pair is diagnostic for a heterozygous genomic region with an uncertainty equal to the recombination distance between two markers. However, the use of RAPD markers in pairs requires twice as many markers as co-dominant markers (RFLPs). Alternatively, RAPD bands may be excised from the separating gel and used as probes to detect co-dominant RFLPs. The utility of this approach, however, depends upon whether or not the RAPD amplification product is homologous to a single copy sequence in the genome, a characteristic specific to the genome being investigated. It is also possible to quantify the intensity of RAPD bands using standard densitometric techniques. An RAPD band derived from a heterozygous region will have half the intensity of a band derived from a homozygous region. However, the reliability of the quantification remains to be determined.

Lack of specificity also inhibits the potential use of RAPDs as genetic markers in comparable mapping and cloning strategies. Comparable maps in related species can be generated with a common set of RAPDs. The lack of specificity of RAPDs is exemplified by the fact that cultivated lettuce and the wild, partially compatible relative *Lactuca saligna*, share RAPD bands. However, a recent survey of 480 10-mer primers for RAPD markers revealed general consistency in primer amplification strength among distantly related genera of flowering plants (*Helianthus* and *Yucca* spp.). Six characteristics of the primer base sequence were analyzed. Of these, total content (G + C) was best at predicting primer amplification strength (ability of amplification in different taxa). Nevertheless, the lack of homology between genomes offers an obvious advantage to RAPDs when constructing maps in polyploid species. Single copy RFLP probes hybridize to multiple sequences in polyploids producing complex banding patterns with difficult to identify alter-

nate alleles. By contrast, most RAPDs will segregate at a simple 3:1 ratio.

Table 2. Disease resistance genes mapped using molecular markers.

Crop	Disease	Marker	Reference
Pea	Bean yellow mosaic virus	Isozyme	Weeden et al. 1984
	Pea enation mosaic virus	Isozyme	Weeden & Providenti 1987
Wheat	Stem breaker foot rot	Isozyme	McMillin et al. 1986
Barley	Rust resistance	Isozyme	Feuerstein et al. 1990
	Powdery mildew	RFLP	Hinze et al. 1991
	Scald or leaf blotch (*Rhynchosporium secalis*)	RAPD	Barua et al. 1993
Maize	Maize dwarf mosaic virus	RFLP	McMullen & Lovie 1989
Tomato	*Fusarium oxysporum*	RFLP	Sarfatti et al. 1989, 1991
	Tobacco mosaic virus	RFLP	Young & Tanksley 1989; Young et al. 1988
	Stemphylium	RFLP	Behare et al. 1991
	Verticillium	RFLP	Juvick al. 1991
	Peseudomonas syringae	RAPD	Martin et al. 1991
Lettuce	Downy mildew	RFLP	Landry et al. 1987; Paran et al. 1991
	Downy mildew	RAPD	Michelmore et al. 1991; Paran et al. 1991; Paran & Michelmore 1993
Rice	Blast	RAPD	Naqvi et al. 1994
	Gall midge	RAPD	Nair et al. 1994

Williams et al. (1993) discuss the various statistical aspects of genetic mapping with RAPD markers. RAPDs as dominant markers are less informative than co-dominant markers (RFLPs). The disadvantage of mapping with dominant markers is that markers linked in the repulsion phase as available in an F_2 population provide little information for estimating genetic distance (Allard 1956). When mapping with dominant markers, it is therefore necessary to work with markers that are only linked in coupling, i.e. markers residing on a single chromatid (such as can be found in a backcross or recombinant inbred population, in haploid or gametophytic tissue, or, alternatively, in an F_2 population where only RAPD markers amplified from a single parent are mapped). Genetic simulations show that dominant markers linked in the coupling phase are as efficient for mapping as co-dominant markers on a per gamete basis.

Our preliminary results with RAPDs to detect inter-accession polymorphism in chickpea have not been very encouraging. Out of the 10 Operon primers tested, only two could detect moderate polymorphisms between accessions ILC1272 and ILC3279 (susceptible and tolerant to ascochyta blight, respectively). Furthermore, the results were not always reproducible. Weeden et

al. (1992) report successful PCR amplification using chickpea genomic DNA. At IARI (New Delhi) polymorphisms at an inter-specific level have been detected using RAPDs (Sharma, personal communication). Considering the labor involved and the low polymorphism detected through RFLPs in chickpea, RAPDs hold great promise. Extensive efforts should therefore be made in this direction.

Targeting Specific Regions of the Genome

Near-isogeneic lines

Several groups have used the RAPD assay as an efficient tool to identify molecular markers that lie within regions of a genome introgressed during the development of near-isogeneic lines (Klien-Lankhorst et al. 1991; Martin et al. 1991; Paran et al. 1991). This approach is particularly useful for the identification of RAPD markers linked to disease resistance. Barua et al. (1993) report RAPD markers linked to a *Rhynchosporium secalis* (Oud.) J.J.Davis resistance locus in barley using near-isogeneic lines and bulked segregant analysis. RAPD markers were 4–6 times more efficient in time and labor. Another advantage of this technology is that a genetic map of the entire genome is not required in order to identify markers linked to a trait of interest.

Instead, specific regions of the genome are focused on. However, there are two disadvantages in using near-isogeneic lines to identify markers linked to a genetic trait. First, it takes several generations of backcrossing to create a near-isogeneic line. Second, several regions of the donor genome that are inadvertently co-introgressed into the near isogeneic line are frequently found (Young and Tanksley 1989). This results in the identification of marker polymorphisms between near-isogeneic lines that are not necessarily linked to the trait that is being studied.

To the best of our knowledge, near-isogeneic lines for disease resistance genes are not available in chickpea. Therefore, there is an urgent need to start breeding programs for the production of near-isogeneic lines in this important grain legume. Availability of such mapping populations will greatly facilitate the tagging of resistance genes.

Bulked segregant analysis

Michelmore et al. (1991) describes the use of RAPD markers to efficiently screen for markers linked to specific regions of the genome. This method, called bulk segregant analysis (BSA), uses two bulked DNA samples gathered from individuals segregating in a single population. Each bulk is com-

posed of individuals that differ for a specific phenotype or genotype. For simple genetic traits, all loci in the genome should appear to be in linkage equilibrium except in the region of the genome linked to the selected locus. Markers linked to this locus should appear polymorphic between the pools for alternate parental alleles. Because many segregating individuals are used to generate the pools, there is only a minimal chance that regions of the genome unlinked to the target locus will also be polymorphic between the pools. Random primers can then be used to amplify loci from each pool and to identify RAPD polymorphisms linked to the trait of interest. Michelmore et al. (1991) successfully used this technique to detect linkage of three RAPD markers to Dm5/8 (a downy mildew locus in lettuce) by screening with 100 RAPD markers. The advantage of this technology is that markers are targeted to a region within the genome and the likelihood of identifying false positive markers is small compared to near-isogeneic line analysis. Selections made from an F_2 population will always be in linkage disequilibrium with respect to selected regions of the genome, and markers can be targeted to any locus where any form of selection can be applied (phenotypic or genotypic). The BSA method is applicable to a variety of simply inherited traits, making construction of near-isogeneic lines or detailed genetic maps unnecessary to identify linked DNA markers.

A pooling strategy based on known genotypes from existing mapping populations has also been followed (Patton et al. 1991). Giovannoni et al. (1991) used this method to target RAPD markers to regions of the tomato genome responsible for fruit ripening and pedicle abscission. Reiter et al. (1992) used it to identify 100 RAPD markers specific to chromosome I of *Arabidopsis thaliana*. As genetic maps approach saturation, pooling by phenotype or genotype will allow researchers to move away from a random approach to map saturation, and focus on specific regions of the genome.

Sequence characterized amplified regions (SCAR)

Despite the speed and general utility of RAPDs, they have several shortcomings: reliability and repeatability, dominant nature, low allelic number, lack of homology among related taxa, and lack of specificity for unique regions of the genome. SCARs, PCR-based genetic markers derived from RAPDs, could eliminate most of these deficiencies and prove applicable to a variety of studies. SCARs identify a specific locus from a defined pair of oligonucleotide primers (Michelmore et al. 1992; Paran and Michelmore 1992). The sequence of these primers is derived from the termini of a band identified as a RAPD marker. Two 24-base oligonucleotides are synthesized corresponding to the ends of the fragment (the 5' ten bases were the same as the original 10 mer-used in the RAPD reaction). These primers, with their

increased specificity, generally amplify a single highly repeatable band, not the 5–10 bands needed for the progenitor 10-base primer. SCARs are similar to sequence-tagged sites (STSs; Olson et al. 1989), but do not involve DNA hybridization for detection and can therefore contain repeated DNA sequences. They are also similar to the PCR amplification of specific tandem repeats with unique flanking primers (Jeffreys et al. 1988b), except that the primers do not flank mini- or microsatellite repeat sequences and therefore generally identify more conserved regions. More than 15 SCARs have been characterized for *Lactuca* spp., most of which are associated with specific disease resistance genes. Ten sets of SCAR primers have also been tested for their ability to amplify homologous sequences in diverse members of the Composite (Kesseli et al. 1992). SCARs have also been used to increase the efficiency of F_2 mapping programs in *Lactuca* spp.

SCARs are simple to generate. However, they do require an investment of time and labor. They can be used in conjunction with RAPDs to tag the reliably of specific regions (e.g. resistance genes), providing multiallelic codominant genetic markers. The increased specificity of SCARs enhances the probability of making comparative genetic maps among related species.

RAPD-DGGE (denaturing gradient gel electrophoresis)

Detection of sufficient DNA polymorphisms among closely related plant lines remains an obstacle to genetic analysis. Dweikat et al. (1993) optimized a denaturing-gradient gel electrophoresis system (Fisher and Lerman 1983) which, when used in combination with RAPD analysis, goes a long way towards overcoming this problem. They have used this approach to estimate pedigree relationships among a spectrum of plant materials in wheat, barley, and oat. In this procedure the usual PCR products amplified using oligoprimers are separated on polyacrylamide gels with a 10–50% denaturant gradient (urea formamide). Denaturing-gradient gel electrophoresis is designed to allow the resolution of sequence differences among fragments of similar or identical size (Fisher and Lerman 1983). The procedure takes advantage of the fact that even single base-pair differences will alter the melting properties (Tm) of a double-standard DNA fragment, thus resulting in altered migration rate. However, other difficulties inherent in the PCR technique that influence reproducibility (Saiki et al. 1988) are not necessarily reduced by modifying the gel system. The RAPD-DGGE system may therefore provide an opportunity not previously available in certain self-pollinated species for the detection of polymorphisms necessary for gene mapping experiments.

Single Strand Conformation Polymorphism (SSCP)

If an RAPD band can be amplified from both parents (no polymorphism) the DNA could be cleaved with restriction enzymes either before or after amplification. This would identify a restriction site polymorphism located between the priming sites. Sequence-based differences in the secondary structure could be detected using this method (Orita et al. 1989).

Multiplex Techniques

Several newly developed techniques, including alumorphs (Sinnett et al. 1990) and amplified fragment length polymorphisms (AFLPs; Zabeau and Voss 1993) permit simultaneous detection of multiple loci distributed throughout the genome. The multiplex ratio of these techniques is high (50–100)—higher than RAPDs (about 10). The advantage of high multiplexing has to be balanced against the disadvantage of the need for a high resolution method, such as a sequencing gel, and the relative complexity of the procedure (for AFLP).

Table 3. Core sequence of some important minisatellite probes used for fingerprinting.

Probe	Repeat Unit	Reference
33.6	5' AGGGCTGGAGG 3'	Jeffreys et. al. 1985
33.15	5 'AGAGGTGGGAGGTGG 3'	Jeffreys et al. 1985
M13	5' GAGGGTGGXGGXTCT 3'	Vassart et al. 1987

Table 4. Comparative characteristics of different molecular markers.

	Isozymes	RFLPs	RAPDs	Microsatellites
Level of Polymorphism	Low	Moderate	Moderate	High
Inheritance	Co-dominant	Co-dominant	Dominant	Dominant
Assay	Fast Starch/polyarcrylamide gel electrophoresis	Slow Agarose gel electrophoresis Southern blotting	Fast DNA amplification Agarose gel electrophoresis	Slow DNA amplification Agarose gel electrophoresis
Detection	Enzyme-specific staining	Radioactive isotopes (also biotin labeling)	Fluorescence (Ethidium bromide staining)	Fluorescence Autoradiography Chemiluminescence
Labor/cost	Less	Moderate	Moderate	High

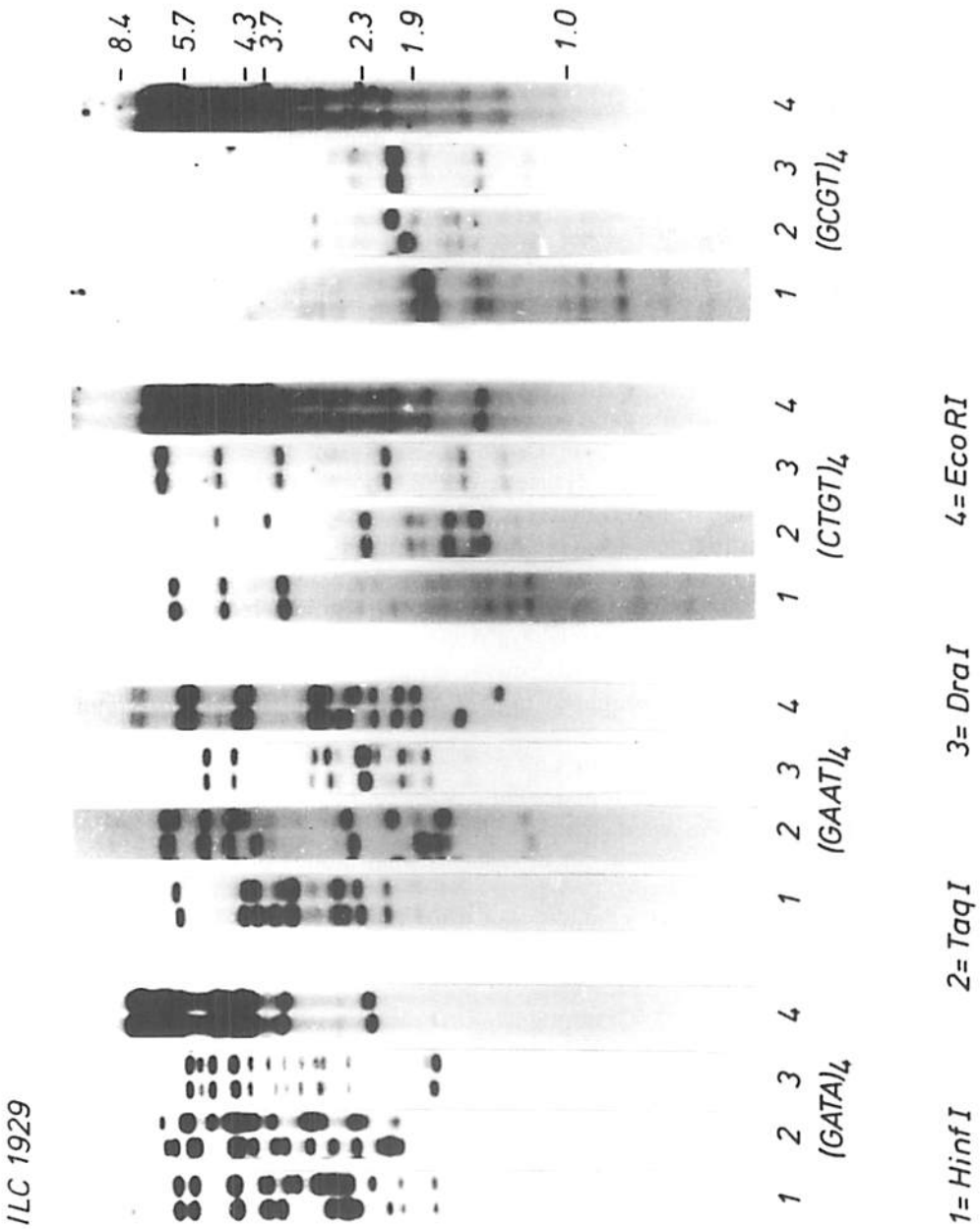

Figure 1. Level of polymorphism within the accessions of chickpea, ILC 1929 as revealed by different probe/enzyme combinations.

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DNA Fingerprinting using Oligonucleotide Probes Specific for Microsatellite DNA Sequences Detect Inter- and Intra-varietal Variability in Chickpea

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Abstract

Oligonucleotides specific for microsatellite sequences, namely $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, and $(GTG)_5$, are used to identify varieties and to estimate intra-cultivar polymorphism in chickpea. Among the four oligonucleotide probes tested, the first three revealed both inter- and intra-cultivar polymorphism, and the probe $(GTG)_5$ did not reveal any polymorphism with the enzymes tested. In general, the maximum intra-cultivar polymorphism was obtained with $(GATA)_4$, followed by $(GACA)_4$ and $(GGAT)_4$. Our study demonstrates that microsatellite-probed DNA fingerprints are very useful for the identification of chickpea cultivars.

Introduction

Oligonucleotides specific for simple sequence repeats or microsatellites are a class of repeats that detect high levels of polymorphism at multiple loci that can serve as a major source of genetic variation (Tautz et al. 1986). This has led to individual identification in humans, animals, and plants (Jeffreys et al. 1985; Rogstad et al. 1988; Ryskov et al. 1988). Complex and highly variable hybridization patterns have been obtained with $(GATA)_4$, $(GACA)_4$, $(CA)_8$, and several other oligonucleotide probes used for DNA fingerprinting (Ali et al. 1986; Schafer et al. 1988; Haberfeld et al. 1991). Recently, oligonucleotide probes have been successfully used for DNA fingerprinting in

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higher plants (Weising et al. 1991; Beyrmann et al. 1992; Vosman et al. 1992). These reports deal mainly with inter- and intra-specific variability in plants. However, systematic research on intra-cultivar or intra-accession variability in plants using oligonucleotide probes is lacking.

Chickpea (*Cicer arietinum* L.) is an important food legume crop in the Indian subcontinent, the Mediterranean region, East Africa, and the Americas. Several high yielding varieties resistant to biotic and abiotic stresses have been developed and released for cultivation in many countries (Singh 1987; ICARDA 1993). In Syria, varieties (Ghab I, Ghab II, and Ghab III), with different levels of resistance to ascochyta blight disease, have been developed and released (ICARDA, 1993).

Reliable identification, regular monitoring of purity, and protection of breeders' rights for varieties released for cultivation are important activities for the seed production industry. Conventionally, rights are established by morphological or physiological traits. These traits are often limited in number and influenced by the environment, making reliable identification and purity testing difficult—sometimes impossible. Use of molecular markers may overcome these difficulties and enable reliable identification and purity testing of crop varieties (Soller and Beckmann 1983). Among the different molecular markers tested in chickpea, isozymes (Kazan and Muehlbauer 1991) and conventional RFLP and RAPD (Udupa et al. 1993) markers are not useful for varietal identification as they detect very little intra-specific polymorphism in chickpea. Recently, Weising et al. (1992) showed that DNA fingerprinting with oligonucleotide probes specific for microsatellites are extremely useful in detecting intra-specific polymorphism in chickpea. In this paper, we report on the use of several such oligonucleotide probes for identification of the cultivars and to assess intra-cultivar variability in chickpea.

Material and Methods

Plant material

Seed of different varieties of chickpea—Ghab I (ILC 482), Ghab II (ILC 3279), and Ghab III (FLIP 82-150C)—was obtained from the Genetic Resources Unit, ICARDA, Aleppo, Syria. Ghab I and Ghab II are landraces, and Ghab III is an improved variety bred through hybridization and selection.

DNA extraction, restriction enzyme digestion and Southern hybridization

DNA was isolated from lyophilized leaves of individual plants using cetyltrimethyl ammonium bromide as described by Weising et al. (1991). The CsCl density gradient centrifugation steps were omitted. Two to three μg of DNA were digested, according to the suppliers' recommendations, with the restriction enzymes *Eco*RI, *Hin*dIII, *Dra*I and *Bam*H1. Electrophoresis was carried out on a 1% agarose gel in a TAE buffer (40mM Tris-acetate, 1 mM EDTA, pH 8.0). Southern transfer and hybridization were performed, and signals were detected colorigenically according to Bierwerth et al. 1992.

Analysis of DNA fingerprint data

Differences in banding patterns were scored on the basis of presence or absence of bands. Similarity indices (Nei and Li 1979) were calculated for all pair comparisons of individual plants of a cultivar. The genetic diversity (H) was calculated according to the following equation (Nei 1987):

$$H = [n/(n-1)] \cdot \left[1 - \sum_{i=1} x_i^2\right]$$

where x is the frequency of i^{th} genotype in the population, and n is the number of individuals examined.

Results and Discussion

We tested four restriction enzymes—*Eco*RI, *Hin*dIII, *Dra*I, and *Bam*H1—and four oligonucleotides—$(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, and $(GTG)_5$—to identify informative probe/enzyme combination for DNA fingerprinting of chickpea cultivars. All these microsatellite sequences were found in the chickpea genome. Nevertheless, informativeness and the fingerprint profiles revealed by different probes and enzyme combinations varied.

Of the four probes used, only three—$(GATA)_4$, $(GACA)_4$, and $(GGAT)_4$—revealed both inter- and intra-cultivar polymorphism (Figs. 1–3). The probe $(GTG)_5$ did not reveal polymorphism with any of the enzymes tested (Fig. 4). Among the polymorphic probes the level of intra-cultivar informativeness, as indicated by the diversity index (Nei 1987), was highest with $(GATA)_4$, followed by $(GACA)_4$ and $(GGAT)_4$. (Table 1)

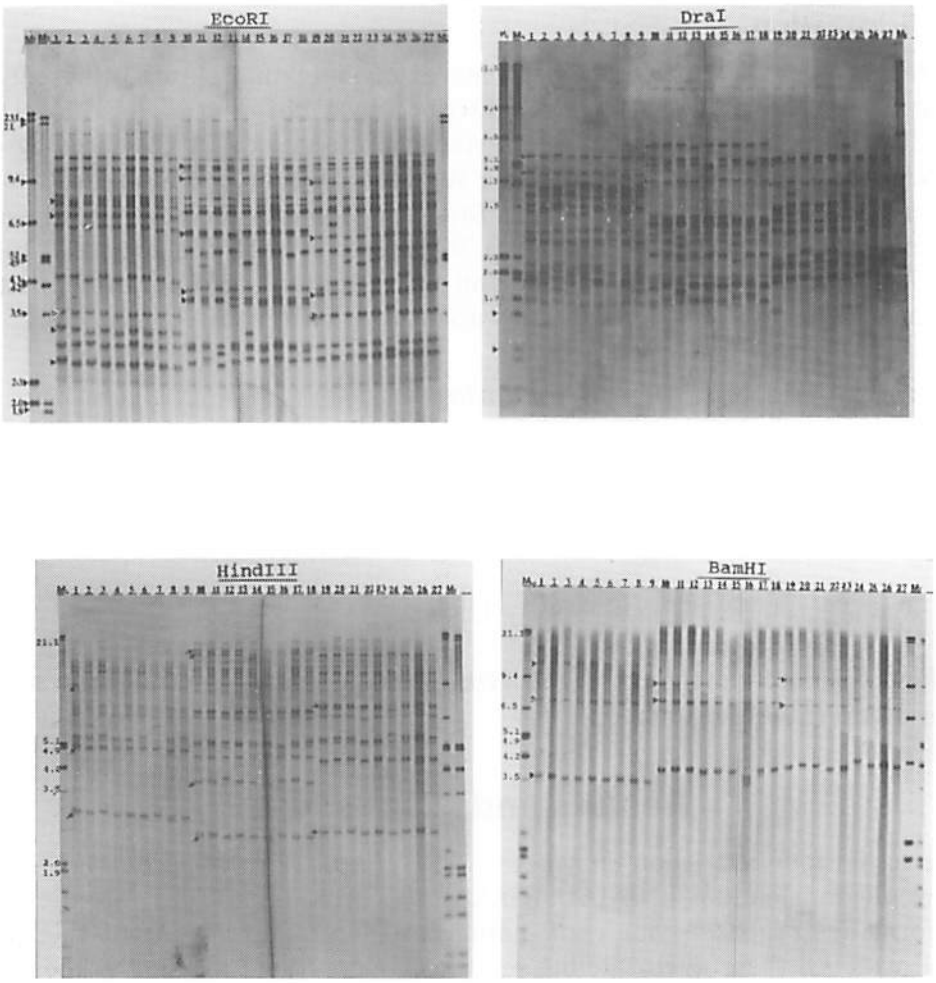

Figure 1. DNA fingerprinting of chickpea cultivars with the oligonucleotide probe $(GATA)_4$ and restriction enzymes, *Eco*RI, *Dra*I, *Hin*dIII, and *Bam*H1. Lanes 1–9 , 10–18, and 19–27 show DNA fingerprinting patterns of individual plants of Ghab I, Ghab II, and Ghab III, respectively. Lane M contains DNA molecular weight markers. Molecular weights are expressed in kilobases. The arrows indicate cultivar-specific bands, which can be used to identify the corresponding cultivar.

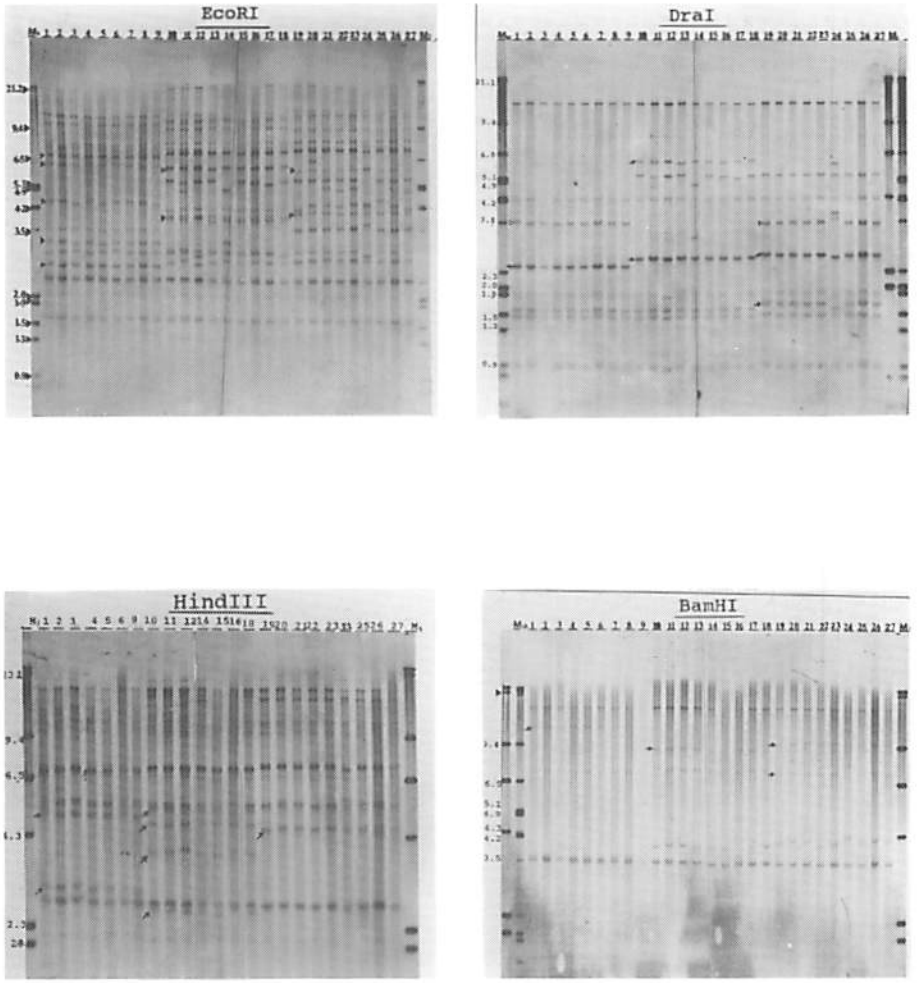

Figure 2. DNA fingerprinting of chickpea cultivars with the oligonucleotide probe $(GACA)_4$ and restriction enzymes, *Eco*RI, *Dra*I, *Hind*III, and *Bam*H1. Lanes 1–9 , 10–18, and 19–27 show DNA fingerprinting patterns of individual plants of Ghab I, Ghab II, and Ghab III, respectively. Lane M contains DNA molecular weight markers. Molecular weights are expressed in kilobases. The arrows indicate cultivar-specific bands, which can be used to identify the corresponding cultivar.

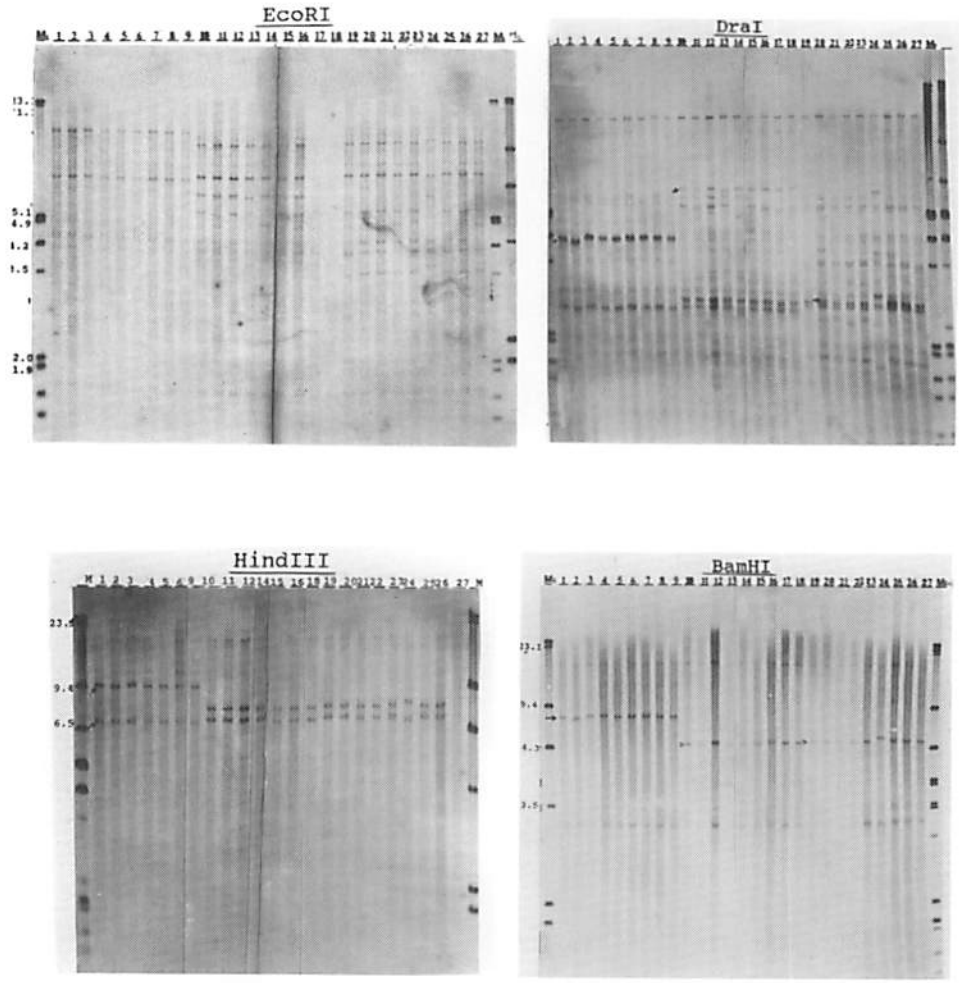

Figure 3. DNA fingerprinting of chickpea cultivars with the oligonucleotide probe $(GGAT)_4$ and restriction enzymes, *Eco*RI, *Dra*I, *Hin*dIII, and *Bam*H1. Lanes 1–9 , 10–18, and 19–27 show DNA fingerprinting patterns of individual plants of Ghab I, Ghab II, and Ghab III, respectively. Lane M contains DNA molecular weight markers. Molecular weights are expressed in kilobases. The arrows indicate cultivar-specific bands, which can be used to identify the corresponding cultivar.

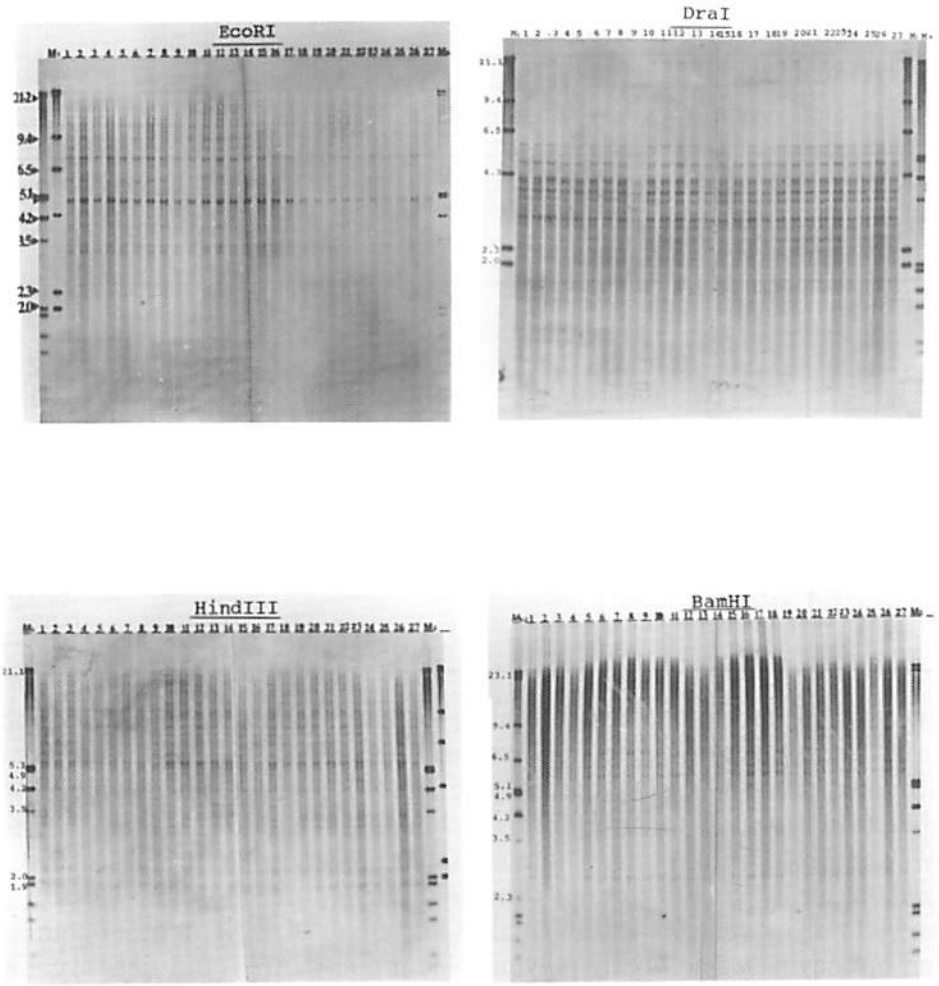

Figure 4. DNA fingerprinting of chickpea cultivars with the oligonucleotide probe $(GTG)_5$ and restriction enzymes, *Eco*RI, *Dra*I, *Hin*dIII, and *Bam*H1. Lanes 1–9, 10–18, and 19–27 show DNA fingerprinting patterns of individual plants of Ghab I, Ghab II, and Ghab III, respectively. Lane M contains DNA molecular weight markers. Molecular weights are expressed in kilobases. Note that this probe did not reveal any polymorphism within or between chickpea cultivars.

Table 1. Genetic diversity within cultivars as revealed by different probe/enzyme combinations.

Probe/Enzyme		Genetic Diversity		
		Cultivar		
Probe	Enzyme	Ghab I	Ghab II	Ghab III
$(GATA)_4$	*Eco*RI	0.97	1.00	1.00
	*Dra*I	1.00	0.97	1.00
	*Hin*dIII	0.97	0.92	0.97
	*Bam*H1	0.00	0.42	0.82
$(GACA)_4$	*Eco*RI	0.83	0.39	0.92
	*Dra*I	0.00	0.89	0.24
	*Hin*dIII	0.52	0.52	0.22
	*Bam*H1	0.83	0.39	0.92
$(GGAT)_4$	*Eco*RI	0.00	0.00	0.42
	*Dra*I	0.24	0.58	0.81
	*Hin*dIII	0.00	0.00	0.00
	*Bam*H1	0.39	0.00	0.24
$(GTG)_5$	*Eco*RI	0.00	0.00	0.00
	*Dra*I	0.00	0.00	0.00
	*Hin*dIII	0.00	0.00	0.00
	*Bam*H1	0.00	0.00	0.00

The level of informativeness revealed by the different polymorphic probes varied greatly according to the enzymes used (Figs. 1-4). With the less informative probe $(GGAT)_4$, the enzyme *Hin*dIII revealed no polymorphism, while *Dra*I revealed polymorphisms within all three cultivars: *Bam*H1 within two and *Eco*RI within one. The moderately polymorphic probe $(GACA)_4$ in combination with *Dra*I did not show any polymorphism within Ghab I, but showed polymorphisms within Ghab II and Ghab III with all other combination of enzymes. The highly polymorphic probe $(GATA)_4$ did not reveal any polymorphism with the enzyme *Bam*H1 for the Ghab I cultivar, but detected very high levels of polymorphism with all other enzymes.

The genetic similarity index (Nei and Li 1979), based on the DNA polymorphism detected by the probe $(GATA)_4$ with the four enzymes, varied greatly. The range was greatest for Ghab III (0.63–0.93), followed by Ghab I (0.69–0.95), and the Ghab II (0.81–0.98). Multiple profiles of hybridizing fragments were obtained for all the probe/enzyme combinations on all three cultivars. The average number of bands hybridized by $(GATA)_4$ was greatest for Ghab II (50.1), followed by Ghab III (44.2), and Ghab I (41.4). (Table 2) The multiple profiles of hybridizing fragments further confirm the results obtained by Weising et al. (1992), that microsatellite sequences are multilocus in nature and are well distributed throughout the chickpea genome.

Table 2. Range of similarity index and average number of hybridizing fragments as detected by the probe (GATA)4 in combination with four enzymes.

	Cultivars		
	Ghab I	Ghab II	Ghab III
Range of Similarity indexes	0.69-0.95	0.81-0.98	0.63-0.93
Average no. of bands hybridized	41.40	50.10	44.20

All the chickpea cultivars used in this study revealed a very high degree of both inter- and intra-cultivar variation between the probe $(GATA)_4$ and the enzymes *Eco*RI, *Dra*I, and *Hin*dIII. However, specific probe/enzyme combinations differed. The probe/enzyme combination $(GATA)_4$/*Bam*H1 revealed a monomorphic pattern only with Ghab I. Therefore this probe/enzyme combination can be used to differentiate Ghab I from Ghab II and Ghab III (Fig. 1). Similarly, the probe/enzyme, $(GGAT)_4$/*Bam*H1 could be used to identify Ghab II (Fig. 3). In addition, cultivar-specific banding patterns revealed by the polymorphic probes could be used for identification of cultivars (see arrows in Figs. 1–3).

The probe $(GATA)_4$, in combination with enzymes *Eco*RI or *Dra*I, could be used to estimate intra-cultivar polymorphism. Since *Eco*RI is the cheapest enzyme among these three, it provides a cost effective way to identify/measure genetic diversity within and among accessions. The high degree of intra-cultivar variability observed in the cultivars shows that further genetic improvement is possible through pure-line selection in these cultivars.

The results presented in this paper show that oligonucleotide-based DNA fingerprinting has a tremendous utility as a powerful tool for reliable identification and genetic purity testing of different chickpea cultivars. No other molecular makers—isozymes (Kazan and Muehlbauer 1991), conventional RFLPs, and RAPDs (Udupa et al. 1993)—tested so far have shown any utility for this purpose. This type of analysis could also assist in assessing genetic diversity in the germplasm collections of chickpea. Genetic diversity study can help find the gaps in the collections to help target collection missions. It can further help target suitable germplasms for their specific adaptation to an environment and for breeding programs.

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Use of Area Under the Disease Progress Curve to Screen Potential Parents for Ascochyta Blight Resistance

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Abstract

Area under the disease progress curve (AUDPC) was used to screen potential parents for resistance to *Ascochyta rabiei* (Pass.) Lab., a very serious pathogen of chickpea (*Cicer arietinum L.*). Of the 46 lines tested under artificial inoculation in the field, four were considered highly tolerant (F88-83C, F84-112C, F84-93C, and F84-80C) another four were tolerant (F83-48C, F83-71C, F84-137C, and F83-98C). The rest of the lines were considered susceptible. AUDPC appears to be a reliable parameter for assessing quantitative resistance to ascochyta blight in chickpea.

Introduction

Chickpea is the second most important grain legume after faba bean. The average yield is about 900 kg/ha. Yield of this crop is very low due to landraces with low potential, late maturity, and susceptibility to disease (Bouslama 1980). Ascochyta blight, caused by *A. rabiei,* is the most important disease of chickpea (Singh and Reddy 1983). It causes considerable losses in sub-humid and semi-arid areas of Tunisia (Kasseber et al. 1976).

Research has been carried out on certain aspects of ascochyta blight and its control by various means. The disease can be controlled to a certain degree by application of fungicides and by cultural practices. But the best strategy to control ascochyta blight is through the use of resistant cultivars. However, resistance is often overcome by new races of the pathogen (Agrios 1978).

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Variability of *A. rabiei* with regard to virulence for chickpea has been reported several times (Halila et al. 1989; Harrabi et al. 1988), and the conclusion drawn that the virulence spectrum of the fungus may be large. In contrast, sources of resistance in the plant are limited.

Techniques for screening germplasm and breeding materials may have limited the development of ascochyta-blight-resistant cultivars. One factor that has hampered the genetic analysis of the *C. arietinum–A. rabiei* interaction and search for resistant lines is the method of scoring. Traditionally, this has been a 1–9 scale more appropriate for field screening of large populations of plants than for genetic studies (Singh et al. 1984). Recently, another scale was developed by Riahi et al. (1990), termed the Linear Infection Index (LII), which is more appropriate for screening potential parents to be used in hybridization program.

The aim of this study was to study disease progress, identify lines with low blight severity, as well as those lines with stable resistance throughout the growth stage of the crop.

Materials and Methods

Plants

Forty six lines of chickpea were evaluated in the field (Table 1). Forty lines were obtained from ICARDA (CIABN A/92) and six cultivars were obtained from INRAT.

Table 1: AUDPC for tested lines of different groups.

Group	AUDPC	Lines
1	250-329	F 88-83C, F84-112C, F 84-93C, F 84-80C
2	330-359	F 83-84C, F 83-71C, F 84-137C, F 83-98C
3	360-374	F 84-83C, F84-87C, F 84-102C, F 89-27C, F 89-62C, ILC 195, F 84-81C
4	375-434	F 85-114C, F 84-78C, F 84-158C, F 84-182C, F 89-38C
5	435-442	ILC 3279, ILC 6090, F 85-148C, F 83-46C, F 84-79C
6	443-449	F 84-92C, F 84-145C, F 90-112C
7	450-509	F 84-79C, F 89-47C, F 84-133C, F 88-1C,F 88-86C, F 88-85C, ILC 191, F 88-82C, F 88-87C, F 89-48C, ILC 482
8	510-749	ILC 72, F 85-99C, F 89-49C, F 85-118C, F 89-110C
9	750-765	ILC 263, Amdoun 1

Screening method

The experiment was conducted at the experimental station of the Institut National Agronomique de Tunisie (INAT) in Morneg during the 1992/1993 season. Sowing was carried out in November. Ten seeds of each entry were planted in single rows. The inter-row and intra-row spacing was 40 and 25 cm, respectively. A susceptible cultivar (Amdoun) was sown every two test rows. The experiment design was a randomized block with two replications. The nursery was inoculated twice during February with a spore suspension standardized to a concentration of 2×10^6 spores/ml, prepared from a fungus culture multiplied and single spored in the laboratory. The isolate was collected at the station the previous year.

Blight score scale

Lines were scored for the disease at one month intervals, beginning with the appearance of the first symptom. We used ICARDA's 1–9 scale (Reddey and Singh 1984). The disease progress curves were analyzed by calculating the AUDPC as follows:

$$AUDPC = D(½(Y1+Yk) + (Y2+Y3+.....+Yk-1))$$

where D = Time interval; Y1 = First disease score; Yk = Last disease score; Y2, Y3 Yk-1 = Intermediate disease scores.

Results were analyzed using multiple comparison based on the Newman-Keuls test with a 5% probability level.

Results

Blight development differed significantly between lines. Compatible to moderately compatible infection responses were observed for all entries. Nine groups of statistical significance were discerned with respect to AUDPC values (Table 1). Out of the 46 lines only eight cultivars were classified as tolerant to *A. rabiei*. Of these, four were highly tolerant (F88-83C, F84-112C, F84-93C, and F84-80C) and four were tolerant (F83-48C, F83-71C, F84-137C and F83-98C). The rest of the lines were classified as susceptible.

Profiles of disease progress curves for all groups were similar, even though their severity was different. The development of ascochyta blight was rapid and severe on the susceptible cultivars (G9; Fig. 1). The disease progress curve for the susceptible group was markedly steeper, as ascochyta blight severity increased from about 6 in March to 9 (plant dead) in April. However, ascochyta blight was lower in tolerant groups (G1 and G2). The epidemic started in the highly tolerant group at the same time as in the susceptible group. The most rapid development occurred after April, due in part to the favorable environmental conditions.

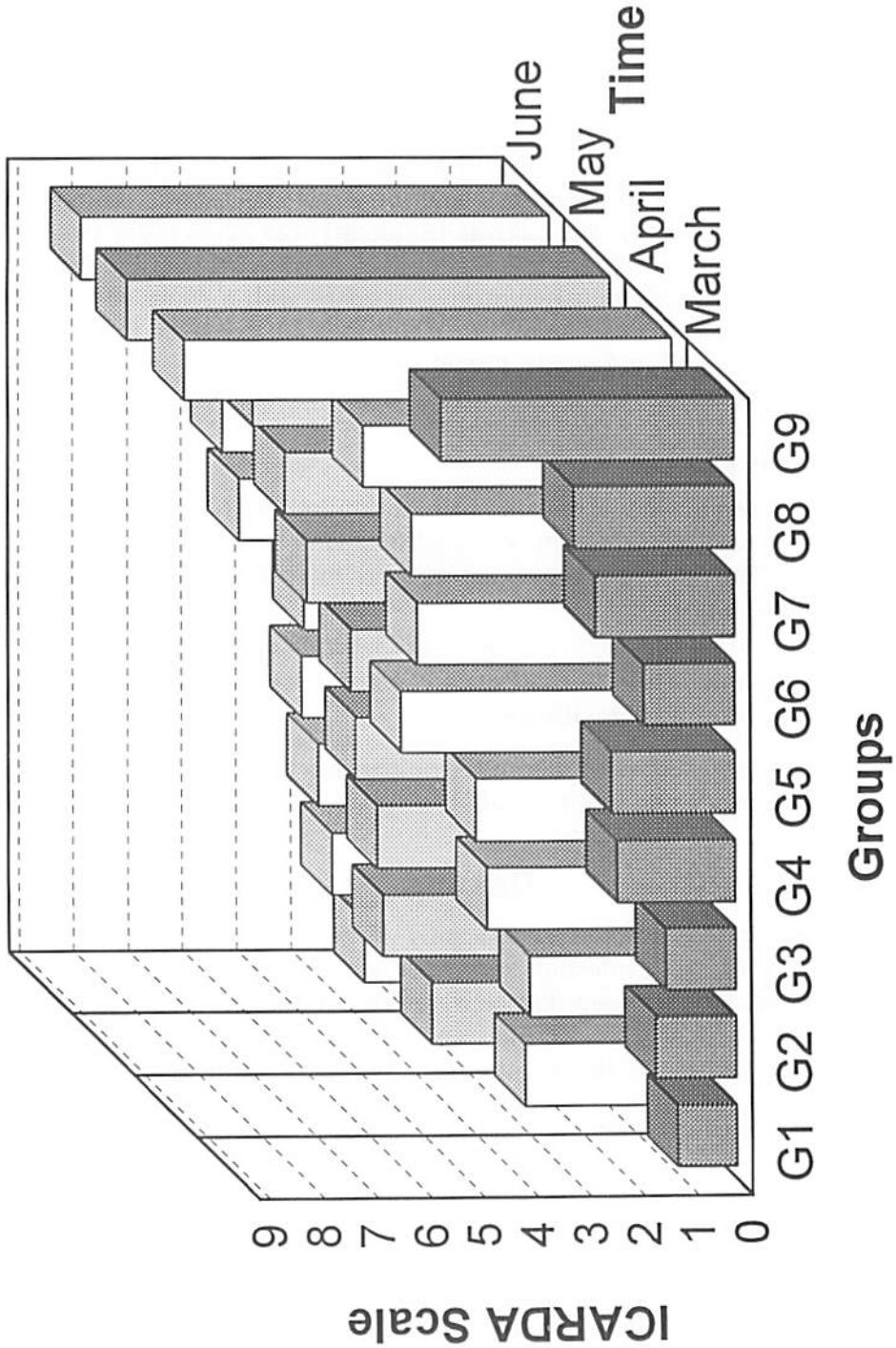

Figure 1. Temporal expression of disease severity on the different groups of chickpea cultivars

At the end of the season, when the crop reached maturity, the amount of disease in the tolerant group was less than half of that in the susceptible lines, and consequently yield loss was lower (Table 2). No difference was observed between the groups (except the susceptible group) regarding percent loss in 100 seed weight. However, the harvest index and the number of pods varied widely among the nine groups. The G1 and G2 groups showed the best resistance to the four parameters of yield components.

Table 2. Yield components of different groups.

Group	AUDPC	HI†	NP‡	Percent loss in 100 g/w	Yield/plant (g)
G1	301.75	35.87^{d}	179.77^{i}	10.00^{a}	29.60^{d}
G2	331.75	35.62^{d}	134.18^{g}	10.50^{a}	27.70^{d}
G3	361.00	34.78^{d}	149.00^{h}	11.14^{a}	26.90^{cd}
G4	393.00	32.60^{cd}	94.12^{c}	11.20^{a}	16.70^{a}
G5	435.00	28.10^{b}	111.99^{e}	11.60^{a}	22.70^{b}
G6	442.00	36.00^{d}	84.70^{b}	11.66^{a}	28.29^{d}
G7	464.00	34.00^{d}	117.89^{f}	12.50^{a}	27.03^{d}
G8	530.80	28.90^{cb}	98.90^{d}	12.60^{a}	23.90^{cb}
G9	757.50	16.00^{a}	25.00^{a}	31.00^{b}	16.30^{a}

†HI = Harvest index

‡NP = Number of pods

Numbers followed by different letters are significantly different according to $LSD_{5\%}$

The correlation between AUDPC and some yield components was high. A negative correlation was observed between the AUDPC and the harvest index (the number of pods and the yield per plant). The highest correlation (r=0.91) was exhibited between AUDPC and percent loss in 100 seed weight (Table 3).

Table 3. Correlation between AUDPC and yield components.

	AUDPC	HI	NP	Percent loss in 100 g/w	Yield/plant
AUDPC	1.00	-0.86	-0.85	0.91	-0.54
HI		1.00	0.77	-0.91	0.75
NP			1.00	-0.79	0.79
Percent loss in 100 g/w				1.00	-0.62
Yield/plant					1.00

Discussion

Two tolerant groups exhibited responses that indicate some degree of resistance to ascochyta blight in the field, yet progress for both groups, as measured by AUDPC, was significantly lower than for the susceptible checks—local Amdoun and ILC 263 (Table 1). Thus, the genotypes in these two

al Amdoun and ILC 263 (Table 1). Thus, the genotypes in these two groups may possess different levels of quantitative resistance in the field. In this study, the term quantitative resistance is a practical term describing a type of resistance that can be assessed by estimating the rate of disease progress by the ascochyta blight pathogen. The rate of disease progress was lower in G2 than in G1. Generally, fully susceptible genotypes show the highest rate of disease progress. However, a high rate of disease progress can occur on genotypes with reduced disease levels when there is a rapid increase of disease from low to moderate over a short time.

The importance of resistant cultivars in control of ascochyta blight has been emphasized from the earliest reports of the disease. Since simple and reliable screening techniques are essential for a successful resistance breeding program (Reddy et al. 1984), AUDPC has been recognized by researchers as one of the most suitable parameters for quantifying a plant disease epidemic, measuring the slow rate of disease development in different cultivars, studying the sporulation of pathogenic fungi in different cultivars, and studying the sporulation of pathogenic fungi in different environments (Pandy et al. 1989).

This study is a preliminary investigation on quantitative resistance, so that large-scale field evaluation of germplasm can be efficiently carried out in breeding programs. In a recent study, genes with additive effects were found to confer quantitative resistance to *A. rabiei* in chickpea. Indeed, Riahi et al. (1994) found that the nature of resistance is quantitative—contrary to the findings of Singh and Reddy (1983) that resistance to *A. rabiei* in chickpea has a qualitative nature.

Investigation into the components contributing to quantitative resistance can also be valuable. For example, if a strong correlation is found between a particular resistance component, such as toxin resistance (Morjane et al. 1992), and quantitative resistance in the field, this component could serve as a trait for selecting this type of resistance in the greenhouse. Correlation between in vitro toxin assessments on protoplasts of chickpea and resistance in the field has been documented in this pathosystem (Dallagi 1992; Morjane et al. 1992). This screening technique should prove attractive to the breeder as it is rapid and inexpensive.

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