DISSERTATION

CHARACTERIZATION OF CHICKPEA (*Cicer arietinum* L.) ACCESSIONS USING MOLECULAR TECHNIQUES.

Submitted by
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WE HEREBY RECOMMENDED THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY FATIHA HAMDAOUI ENTITLED CHARACTERIZATION OF CHICKPEA (*CICER ARIETINUM* L.) ACCESSIONS USING MOLECULAR TECHNIQUES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF CHICKPEA (*Cicer arietinum* L.) ACCESSIONS USING MOLECULAR TECHNIQUES

The productivity of chickpea has not been markedly improved through conventional breeding. The main problem for increasing yield is the susceptibility of the plant to the disease caused by the ascomycete *Ascochyta rabiei*. Because genetic markers may speed up chickpea breeding for resistance to ascochyta blight, isozymes and RAPD techniques have been applied to 56 chickpea germplasm lines which have been screened against ascochyta blight in the field and at the greenhouse in Morocco. Artificial inoculation at three locations, resulted in none of the lines evaluated being immune, seven entries were resistant and the remaining were tolerant or susceptible under Morocco conditions. Two hundred primers for RAPD assay and 15 enzymatic systems were assayed. The fifteen enzymatic systems tested were almost monomorphic and were not able to discriminate among the tested lines. Among the 200 primers tested only 6 primers yielded polymorphism. Forty- one amplification products were produced and among them four were associated with disease resistance to ascochyta blight. RAPD procedure with polyacrylamide gels differentiated among the resistant and susceptible cultivars and produced more polymorphisms than RAPD using agarose gels.

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DEDICATION

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TABLE OF CONTENTS

INTRODUCTION 1

CHAPTER 1

LITERATURE REVIEW 5

PART I- BREEDING CHICKPEA (*Cicer arietinum* L.) FOR ASCOCHYTA BLIGHT RESISTANCE.

INTRODUCTION 5

HISTORY OF BREEDING FOR BLIGHT RESISTANCE 6

Chickpea sources of resistance to ascochyta blight 7

Sources of resistance within cultivated chickpea 7

Sources of resistance to ascochyta blight in wild chickpea species 8

Interspecific hybridization and application of in vitro techniques for blight resistance 8

Inheritance of resistance to ascochyta blight 8

Selection methods for blight resistance 9

Screening for ascochyta blight 9

PART II- IMPACT OF MOLECULAR MARKERS IN PLANT IMPROVEMENT

INTRODUCTION 12

ISOZYME MARKERS 12

Definition 12

Applications 13

Isozymes in some grain legumes 14

DNA MARKERS 14

Description 14

Restriction Fragment Length Polymorphism (RFLP) markers 15

Definition 15

Application 16

Polymerase Chain Reaction (PCR) 17

Definition 17

Random Amplification of Polymorphic DNA (RAPD) 17

Description 17

TABLE OF CONTENTS (CONT.'D)

Application 18

RAPD's in plant disease improvement 19
RAPD's in grain legumes 19

CHAPTER 2

SCREENING CHICKPEA GERMPLASM FOR ASCOCHYTA BLIGHT (ASCOCHYTA RABIEI) IN THE FIELD AND GREENHOUSE UNDER MOROCCO CONDITIONS

INTRODUCTION 21
MATERIALS AND METHODS 23
Field experiments 23
Seed sources 23
Sources of pathogenic isolates 23
Disease scoring 23
Location of the experiments 25
Planting 25
Controlled environment experiment 24
Growing conditions 27
Statistical analysis 27
RESULTS AND DISCUSSION 28
Reaction of the tested germplasm lines to ascochyta blight 28
Field experiments 28
Greenhouse experiment 30
CONCLUSION 33

CHAPTER 3

ENZYMATIC POLYMORPHISM IN CHICKPEA GERMPLASM LINES

INTRODUCTION 35
MATERIALS AND METHODS 37
Plant material 38
Protein extraction 38
Gel formation 38
Vertical electrophoresis 43

TABLE OF CONTENTS (CONT.'D)

Data analysis 43

RESULTS 44
Esterase (EST) 44
Phosphoglucomutase (PGM) 45
Phospho-glucose isomerase (PGI) 45
Aspartate aminotransferase (AAT) 45
Malic enzyme (ME) 45
Leucine aminopeptidase (LAP) 45
6-Phospho-glucose dehydrogenase (6-PGDH) 46
Glucose-phosphate dehydrogenase (GDH) 46
Alcohol dehydrogenase(ADH): 46
β-Amylase (β-AMY), fumarase (FU) and alkaline phosphatase (ALPH) 46
Acid phosphatase (ACPH) 46
Carbonic anhydrase (CA) 46
Propionate-esterase (PR-EST) 46
DISCUSSION 63
CONCLUSION 66

CHAPTER 4

CHARACTERIZATION OF CHICKPEA GERMPLASM LINES USING RAPD TECHNIQUE.

INTRODUCTION 67
MATERIALS AND METHODS 68
DNA extraction 68
Spectrophotometric determination of nucleic acids 69
RAPD primers 71
Template DNA 71
The enzyme Taq 71
DNA ladder 71
RAPD reactions 71
Preparation of agarose gel 73
Agarose gel staining 74
Bulked DNA samples for analysis 74
RAPD with acrylamide gel procedure 74
PCR amplification: 74
Silver staining of gels 76
Data analysis 76

TABLE OF CONTENTS (CONT.'D)

RESULTS 78
Quality and quantity of DNA extracted 78
Level of polymorphism 79
Bulked DNA analysis 81
RAPD procedure using acrylamide gel and silver staining 82
Discrimination of 56 chickpea lines based on RAPD with agarose data using cluster analysis and principal components analysis 92
Discrimination of 12 cultivars by cluster analysis and principal component analysis using RAPD with agarose and RAPD with acrylamide gel data 95
Relationships between RAPD amplification products and the reaction to ascochyta blight and RAPD products and the origins of the lines. 96
DISCUSSION 100
CONCLUSION 103

GENERAL CONCLUSION 104

LITERATURE CITED 106

APPENDIX 1 123

APPENDIX 2 130

APPENDIX 3 146

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important food legume in the crop rotations of West Asia and North Africa. It is an important source of good quality protein in the diets of the people of the region, especially for the poorer sections. Its plant by-products are invaluable sources of nutritious fodder as well. Furthermore, this crop enriches the soil through its symbiotic nitrogen fixation. Thus, the dependency of cereal dominated cropping systems on nitrogen fertilizer is reduced (Saxena, 1984).

In Morocco, chickpea is grown on 85,000 hectares producing 64,000 metric tons per year (Kamal, 1984). Recently, chickpea production has decreased. This reduction in both area and yield (700kg/ha) appears to be due to production problems associated with the susceptibility of chickpea to ascochyta blight and leaf miner. Thus, average yields for chickpea have gone down in the last 10 years (MARA, 1990).

Emphasis on chickpea improvement, therefore, deserves high priority in Morocco. The International Center for Agriculture Research in Dry Areas (ICARDA) in Aleppo, Syria has initiated work with the National Program in Morocco in order to solve the production problems. This research has led to the testing in Morocco of breeding material developed at ICARDA in Syria specifically for Moroccan conditions. Promising genotypes have been identified for multi-location testing.

Winter planting of chickpea in major production areas of Morocco has been tested but the problem of ascochyta blight is one of the major limiting factors for the extension of this winter production system. However, significant gain in production was obtained when sowing local cultivars in winter as compared to spring sowing (Kamal, 1984). Thus, the cultivars developed at ICARDA have a high genetic potential and therefore, might have an advantage for winter production provided genes for resistance to frost and ascochyta blight are found and incorporated. Efforts continue to screen breeding lines for ascochyta blight resistance as well as for developing economically alternative control measures.

Efforts in the development of high yielding genotypes with resistance to ascochyta blight, leaf miner and good seed size (40 g per 100 seeds) are important.

Development of blight resistance is the highest priority. To fill this need, it is important to characterize germplasm collections and screen them for ascochyta blight. In an attempt to restrict further erosion of both the cultivated gene pool and the diversity available within the genus, international collections of chickpea germplasm have been established at ICRISAT(International Crop Research in the Semi Arid Tropic), ICARDA and other countries, among them Morocco. Within these collections, accurate assessment of the level of genetic variation is important in order to minimize duplications, and provide a source of germplasm that is accessible to the chickpea breeder. However, information on taxonomic characteristics as well as genetic diversity of chickpea is poorly documented. Characterization of these genetic materials was traditionally based on visual assessment of morphological traits, which is complicated by the influence of changing environmental conditions from one experiment to another. In order to facilitate the screening of large collections for resistance, molecular methods may be necessary for characterization. There is also a need for use of molecular methods to facilitate the identification of resistance genes rapidly and accurately.

Molecular marker analysis is a rapid and efficient means for analysis of large sample numbers. Therefore, the use of these molecular approaches is a reasonable solution to aid in the characterization of a collection that may serve as the primary source of genetic diversity (Brown, 1989).

Isozyme analysis is one possible procedure for detecting genetic markers. It relies on the detection of polymorphism among enzymes that differ in their electrophoretic mobility (Markert and Moller, 1959). It has the advantage of being rapid and inexpensive as compared to other methods but its use may be limited by lack of polymorphism , i.e., a relatively small number of loci and alleles for analysis (Bernatsky and Tanksley, 1986). Restriction Fragment Length Polymorphism (RFLP) analysis overcomes the problem of limited polymorphism because it works at the DNA level. Unfortunately, thorough characterization of a large collection particularly for agronomic traits, may be impractical and expensive (Anderson and

Fairbanks, 1990). Another type of genetic marker analysis termed RAPD (Randomly Amplified Polymorphic DNA) has a potentially important application in this field and may overcome observed problems using RFLP and isozymes. RAPD procedures are faster than RFLP since there are fewer steps; they are cheaper and often reveal more polymorphisms than isozymes. These molecular techniques offer new opportunities for crop improvement. They can be used for several purposes such as the improvement of breeding programs through the tagging of economically important genes, such as disease resistance genes.

The present study investigates the use of isozymes and RAPDs for characterization of chickpea germplasm lines for use in the improvement of resistance to ascochyta blight. These techniques have been successfully used in many crop species such as lettuce (Waycott and Fort, 1994), mangos (Schnell et al., 1995), black currant (Lanham et al., 1995), rye (Iqbal and Rayburn, 1994), apricots (Gogorcena and Parfitt, 1994) and brassicas (Demeke and Adams, 1994). Availability of a sufficient number of polymorphic markers is a prerequisite for successful identification of those closely linked to the gene of interest. If present, they should speed up chickpea breeding for ascochyta blight.

Biochemical markers have been tested in chickpea for the purpose of avoiding the difficulties encountered with disease symptom screening methods. Some researchers have been investigating the possibility of using isozymes as genetic markers to study the genetic variability of chickpea cultivars. Many enzymes and staining systems have been examined in chickpea. However, little success has been achieved thus far because of the low levels of isozyme diversity obtained and the lack of any correlation between isozyme banding patterns and resistance (Oram et al., 1987; Tuwafe et al., 1988; Ahmad et al., 1992; Kusmenoglou et al., 1992.).

The first objective of this study was to screen 56 chickpea germplasm lines in the field and greenhouse for ascochyta blight resistance thus characterizing the level of resistance for use in correlation with marker analysis.

The second objective was to test an expanded number of enzymatic systems in an attempt to find sufficient polymorphisms to facilitate their use as markers.

A third objective was to use RAPD analysis to characterize chickpea germplasm

accessions. If sufficient polymorphisms are observed, they will be used to identify markers closely linked to the ascochyta blight resistance gene using bulked segregant analysis.

CHAPTER 1

LITERATURE REVIEW

PART I. BREEDING CHICKPEA (*CICER ARIETINUM* L.) FOR ASCOCHYTA BLIGHT RESISTANCE.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) or garbanzo bean is an ancient, well established crop in many countries, and a new rapidly expanding crop in several others, e.g., Australia. The largest producer of chickpea is India, followed by Turkey, and Pakistan (Singh, 1991). Although most of the world's chickpea production and consumption (>70%) is in India, this crop is of importance in many countries in Asia, Africa, Europe and the Americas (Singh, 1987).

Chickpea is produced in 33 countries in the world on approximately 9.5 million hectares, with average yields of 586 to 696 kg /ha. Thus, worldwide about 7 million metric tons per year are produced (Saxena, 1990).

Chickpea is high in protein (ca. 20%) and is used in many dishes either as a whole grain or as flour. Chickpea is also an important source of animal feed in western Asia and northern Africa and is important in maintaining soil fertility by fixing more than 70 kg /ha of soil nitrogen (Saxena, 1984).

Two types of chickpeas are grown. These are "kabuli" and "desi" types. Kabuli type has smooth, generally large, light colored seeds. This type is of particular importance in countries of the Mediterranean region (Singh, 1987). The desi type has yellow to black seeds, generally smaller, with a rougher surface and constitutes about 85% of the total

production. Kabuli seeds as compared to desi type have less seed coat mass, fiber and cellulose, as well as fewer polyphenols, and higher nitrogen, sugar and protein (Saxena, 1984).

Kabuli chickpea is predominantly cultivated in the Americas and Mediterranean basin (West Asia and North Africa, designated as WANA region) and account for 20% of total world production. Desi types are cultivated predominantly in the Indian subcontinent (Saxena, 1984).

Chickpea is an annual diploid species with 2n=16 chromosomes. It is self pollinated, but can outcross up to a rate of 1% (Auckland and Van der Maesen, 1980). It is adapted to grow without rainfall during the growing season (Saxena, 1984). It is grown as a summer crop in the Middle East, around the Mediterranean countries and in the Americas, and as a winter crop in more tropical climates (Nene, 1984; Saxena, 1984).

In the early 1980's, winter sowing was proposed in Morocco as a means of escaping drought and high temperatures which are prevalent for spring sown crops (Saxena, 1980). Winter chickpea production yield potential exceeds 3T/ha when grown under optimal conditions (when ascochyta blight resistant and cold tolerant chickpea lines were winter planted (Nene, 1984)). But, deviations from such conditions may decrease yield substantially. Many factors are responsible for this reduction which might be abiotic, such as drought, salinity and low temperature, or biotic where fungal and viral diseases, nematodes, and pests cause significant damage to the crop (Singh and Reddy, 1989 and 1992).

Ascochyta blight is one of the most devastating diseases of chickpea worldwide. Yield losses from the disease have been reported in 29 countries that account for 97% of the world's production (Nene and Reddy, 1987). The incidence of this disease is not regular. During years that it is prevalent it can completely eradicate the crop (Singh et al., 1988).

HISTORY OF BREEDING FOR BLIGHT RESISTANCE

The extensive blight resistance breeding work jointly undertaken in the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, and the International Crops Research Institute for the semi-arid Tropics (ICRISAT), Pantacheru,

India, has helped in the identification and development of several blight-resistant kabuli varieties. However, none of these varieties were sufficiently resistant in India and Pakistan, due to virulent races of *Ascochyta rabiei* in those areas (Singh et al., 1984).

Some success have been found in the chickpea cultivars developed for winter production systems in the Mediterranean region. This type of production system allows the farmers to take advantage of limited water resources (Singh, 1987). However, ascochyta blight still constitutes a major problem in chickpea especially in the Mediterranean region (Saxena, 1990).

The introgression of genes from related *Cicer* species has been suggested to improve the resistance of the cultigen (Ladizinsky et al., 1988; Singh, 1987). However, only *crosses of Cicer reticulatum* to *C. arietinum* produces fully fertile hybrids. *Cicer echinospermum* is crossable to *C. arietinum*, but the F1 hybrids are partially sterile and the F2 progeny is mostly sterile (Muehlbauer et al., 1987). Biotechnological methods have been initiated to incorporate resistant genes into adapted cultivars as well as to characterize resistance genes but these studies are just beginning and considerable work remains before significant progress is made (Muehlbauer et al., 1989; Singh et al., 1989).

Chickpea sources of resistance to ascochyta blight

Sources of resistance within cultivated chickpea: The international collection of chickpea stored at ICRISAT in India has been screened progressively for wilt resistance, heat tolerance, and protein content (ICRISAT, 1989). In addition, the germplasm has been checked for ascochyta blight resistance and winter hardiness at ICARDA in Aleppo, Syria (ICARDA, 1988).

More than 3500 germplasm accessions have been screened for ascochyta blight in isolation at ICRISAT. Five desi types were found to be resistant to blight at the ICARDA center, and were included in the international chickpea ascochyta blight nursery (Singh, 1987). Evaluation of over 1300 ICARDA breeding lines using six races of *Ascochyta rabiei* revealed that three lines (Flip 84-79C, Flip 85-86C and Flip 90-103C) were highly resistant (ranking 3 in a 1 to 9 scale) (ICARDA, 1991).

Sources of resistance to ascochyta blight in wild chickpea species: Wild species of chickpea possessing resistance to several diseases as well as increased vigor including resistance to several biotypes of ascochyta blight have been identified. A total of 154 pure lines were identified, and were separated and described on the basis of morphological characters. Evaluation of these lines helped in identifying genotypes resistance to ascochyta blight, leaf miner, cyst nematode, seed beetle, and cold (ICARDA, 1988).

Interspecific hybridization and application of in vitro techniques for blight resistance: Hybridization can play an important role through introgression of resistant genes from wild species of *Cicer* into adapted varieties. However, there are crossability barriers to interspecific hybridization and most of the wild species possessing useful characters belong to the tertiary gene pool (Ladizinsky and Adler, 1976; Ladizinsky et al., 1988). The use of tissue culture techniques for embryo rescue and protoplast fusion has been tested for the transfer of genes for blight resistance from wild *Cicer* species to the cultivated species with little success due to regeneration problems (Singh et al., 1989). However, other work on hybridization of chickpea using in vitro techniques is underway at several institutions: ICRISAT, India; ICARDA, Syria; University of Napoly, Italy and the University of Saskatchewan, Canada. Some successful crosses have been reported between *C. arietinum* and *C. reticulatum*. Crosses between *C. arietinum* and *C. echinospermum* resulted in F1 plants but F2 seeds could not be obtained (Singh et al., 1989).

Transfer of desirable genes from the wild progenitor is often accompanied by closely linked genes with deleterious effects (Muehlbauer and Singh, 1987; Muehlbauer et al., 1989 and Tanksley et al., 1989).

Inheritance of resistance to ascochyta blight: Three studies on inheritance of ascochyta blight resistance reported that one dominant gene was responsible for resistance in the materials used (Hafiz and Ashraf, 1953; Vir et al., 1975; Eser, 1976). Recently, Singh and Reddy (1983) reported a single recessive gene conferring resistance in line ILC 191, in addition to a single dominant gene controlling resistance in four other lines (ILC 72, ILC 183, ILC 200 and ICC 4935). Eser (1976) indicated that resistance to ascochyta blight was monogenic and dominant when he crossed a local line 'code No. 8276', that was highly

susceptible to ascochyta blight, with a resistant line 'code No. 70-102'. He found that F2's segregated into a 3 (resistant): 1 (susceptible) ratio.

Selection methods for blight resistance: Pedigree selection is expected to be the most effective method for developing resistance to disease and pests in chickpea. It is currently being used in the development of lines in disease infected plots and in laboratory screening. Bulk advance of resistant plants in early generations is used in some crosses to increase the amount of material handled, while single pod descent is used to maintain variation in advanced populations (Spetcht and Graef, 1989).

Screening for ascochyta blight: Ascochyta blight has been reported in North America, Southern Europe, North and East Africa, West Asia, Southern Russia, and the Indian subcontinent (Nene, 1978). The earliest report of its occurrence is from the " North-west Frontier Province " of India (now in Pakistan) where it was observed in 1911 (Ahmad et al., 1952).

The disease frequently causes heavy losses. All the green parts of the plant are attacked. Dark lesions appear on the stems and leaves first and then on pods (Fig. 2.1 and 2.2, originals). When well developed, the margin of the lesion is dark brown and the center is light brown full of small pycnidia.

Six physiologic races have been identified and characterized in Syria for *Ascochyta rabiei* (Reddy and Kabbabeh, 1985). Generally a mixture of the six races are used in screening for resistance (Muehlbauer et al., 1989). In Morocco 3 to 4 races have been identified preliminarily.

Nene (1978) cites Labrousse in 1933 as the first to artificially inoculate chickpea material for identification of resistance lines. This was accomplished by scattering infected chickpea leaves or branches as well as spraying spore suspensions followed by repeated sprinkler irrigations.

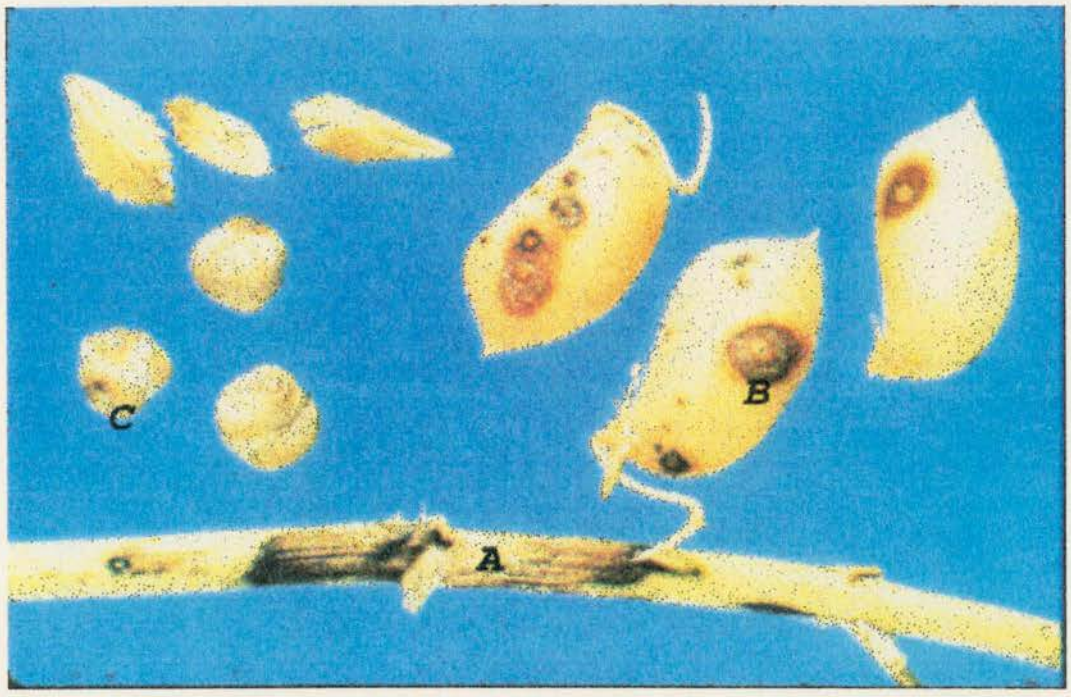


Fig.1.1 Ascochyta blight disease symptoms. A: on stem, B: on pod and C: on seed.

Fig. 1.2. Ascochyta blight disease symptoms on leaves and stem.

PART II. IMPACT OF MOLECULAR MARKERS IN PLANT IMPROVEMENT

INTRODUCTION

Classical characterization of germplasm is usually based on a visible phenotype, for example, plant vigor or general appearance, or on measurable traits such as yield, oil or protein composition (Rafalski et al.,1991). These genetic resource collections are therefore poorly characterized phenotypically (Tanksley et al., 1989).

Many of the complications of a phenotypic characterization can be reduced through direct identification of genotype with a DNA based assay (Beckman and Soller, 1983; Burr et al., 1988). For this reason, DNA markers are being used in the characterization of many plant species, and may play an important role in the future of plant breeding (Tingey and Deltufo, 1992).

Before 1980, isozymes were the most common type of molecular markers used in breeding and genetic research. Some genetic maps using isozymes, in combination with morphological markers were completed by the late 1970s in several crops, including maize (Ott and Scandalios, 1978) and tomato (Tanksley and Rick, 1980). However, rapid development of the use of DNA markers began after 1980 when RFLPs were proposed (Botstein et al., 1980), followed by the establishment of RAPD markers by Williams et al. (1990). Consequently, many studies based on these markers have been carried out in several crop species, such as tomato (Tanksley, 1993; Kleinhofs et al., 1991), maize (Coe et al., 1990), potato (Bonierbale et al., 1988) and *Brassica spp. (*Slocum et al., 1993)

This review is a brief summary on the use of two molecular techniques; isozymes technique traditionally used in the assessment of genetic variability within and between plant populations of many species (Gottlieb, 1981) and DNA markers which have received much attention as a plant breeding tool (Soller and Beckmann, 1983).

ISOZYME MARKERS

Definition: The term isozymes, was first used by Markert and Moller (1959) to describe different molecular forms of enzymes which use the same substrate and share the

same reaction. The shapes and net charges of these isozymic molecules allow them to be separated from one another electrophoretically. Some isozyme variants were called "allozymes" to represent products of different alleles of the same locus (Lewontin and Hubby, 1969).

Some studies indicated that isozymes could be used as genetic markers in plant programs. However, polymorphisms at isozyme loci are often not available in cultivated crops (Gottlieb, 1981). Isozyme polymorphism can be created by interspecific hybridization such as in the case of tomato (Tanksley et al., 1982; Vellejos and Tanksley, 1983) and lentil (Hoffman et al.,1986; Tadmor et al., 1987; Muehlbauer et al., 1989).

Applications: Isozymes have applications in many areas of plant science. Some of the applications of isozyme data according to Moore and Collins (1983) have been: 1) screening variability in plant populations, 2) identifying sexual and somatic hybrids, 3) reducing the number of backcross generations in a backcrossing program, 4) detecting genetic diversity in plant populations and 5) construction of linkage maps for plant chromosomes (Tanksley and Rick, 1980).

Isozymes have also been used successfully in the identification of many plant species (Torres, 1978; Ellstrand and Lee, 1987; DeWald et al., 1988; Pascal et al., 1993), and in the characterization of germplasm collections in several crop species such as soybean (Larsen and Benson, 1970; Kiang and Gorman, 1983), barley and oats (Almgard and Norman, 1971) *Annona cherimola* (Ellstrand and Lee, 1987), Spanish chemoya (Pascal et al., 1993), white beans (Weeden, 1984), avocado (Torres et al., 1978), salvia (Hashemi et al.,1993), potato (Pierce and Breubaker, 1973) and pineapple (DeWald et al., 1988). It also has been used in clonal identification (Bringhurst et al., 1981) and for taxonomic purposes and plant evolution studies (Stebbins, 1989; Anderson and Fairbanks, 1990).

Another area where allozyme polymorphisms have practical significance is their use in locating and tagging economically important genes, such as disease resistance. Nematode resistance in tomato was the first reported isozyme-tagged trait in crops (Rick et al., 1974). The Resistance gene was found to be associated with an acid phosphatase locus. This isozyme was used successfully to transfer resistance to other tomato plants without reduction

in quality. Other examples include the linkage between Pgi-1 and Adh-2 and genes for cold tolerance in apple (Vellejos and Tanksley, 1983) and a close association between the gene for straw breaker foot rot resistance and an endopeptidase allele in wheat *(Triticum aestivum* L.) (McMillin, 1983).

Isozyme markers have also been successfully used in many crops to identify quantitative trait loci. Using polymorphism at 12 isozymes in an interspecific backcross population of tomato, Tanksley et al., (1982) detected and mapped more than 20 QTLs (Quantitative Trait Loci) affecting variation of four quantitative traits. In maize, Kahler and Wehrhahn (1986) studied an F2 population and identified associations between isozymes and eleven quantitative traits. In another study of an F2 population of maize, Edwards et al., (1987) detected QTL affecting variation in 82 quantitative traits.

Isozymes in some grain legumes: Some examples of food legumes for which isozymes have been applied include the selection for resistance to race 1 of F*usarium oxysporum* f. Sp. *pisi* in pea using an esterase (Hunt and Barnes, 1982), selection for resistance to pea enation mosaic virus using alcohol dehydrogenase (Weeden and Provvidenti, 1987) and the resistance to bean yellow virus using the gene encoding phosphoglucomutase (Weeden, 1984). QTLs-isozymes associations were detected in lentil, Tahir (1990) investigated associations among 14 isozyme markers and seven quantitative traits using recombinant inbred line populations. He detected six genomic regions which appear to contain QTLs.

DNA MARKERS

Description: Several DNA based techniques have some advantages over protein analysis. These techniques directly reflect the relatedness or phylogeny of the populations studied. Very small amount of DNA isolated from only a few cells can be analyzed. Due to the high stability of DNA, even mummified or fossilized material can be used (Pääbo, 1989). These DNA markers reflect heritable differences in homologous DNA sequences between individuals. These differences result from base-pair changes, rearrangements (e.g.,translocation and inversions), insertions or deletions at the homologous DNA region.

These markers are stable and lack pleiotropic effects on other traits. These properties make them extremely useful genetic markers as compared to morphological or even protein markers (Havey and Muehlbauer, 1989).

Different molecular techniques have been developed. They are based either on polymerase chain reaction (PCR) or southern hybridization techniques. The PCR technique permits the detection of variation in the amplified DNA regions as in the case of Random Amplification of Polymorphic DNA (RAPD) (Williams et al., 1990). While the southern hybridization technique detects variations in the lengths of restriction fragments (Beckman and Soller, 1983). The most practical and broadly used techniques in this area are RFLP, PCR and RAPD.

Restriction Fragment Length Polymorphism (RFLP) markers

Definition: RFLP refers to inherited differences in sites for restriction enzymes (for example, caused by base-pair changes in the target site, that result in differences in length of fragments produced by cleavage with the relevant restriction enzymes). The DNA sequence variation was referred to as restriction fragment length polymorphism (RFLP) (Botstein et al., 1980). Differences in RFLP's between two genotypes can be detected by digesting DNA from both genotypes with the same restriction enzyme and electrophoretically separating the fragments in agarose gels. The digest is then subjected to "Southern blotting", a technique that involves transferring digested DNA onto a nylon membrane, immobilizing the fragments and making them accessible to radioactively labeled DNA probes (Botstein et al., 1980). Fragments complementary to each cloned sequence will bind to that radio labeled clone. The membrane is exposed to x-ray film to visualize the bands. Only hybridized fragments would be visible. Probes can be selected based on their ability to reveal clear DNA polymorphisms and to detect one or a few polymorphic loci (Gebhardt and Salamini, 1992).

RFLP's reveal a high degree of polymorphism in some cultivated crops (maize, or brassica) whereas in other species (peanut, beans, chickpea and melon) very little polymorphism was observed (Havey and Muehlbauer, 1989).

Applications: While RFLPs were first described for adenoviruses (Grodzicker et al., 1974), they soon became useful for human genetic studies (Jeffreys, 1979). The extention of the use of RFLP technique to plant genetics and breeding was also soon recognized (Beckman and Soller, 1983). Since that time, many studies using RFLP polymorphisms were undertaken for genetic mapping in several plant species including maize (Helentjaris, 1987), tomato (Bernatzky and Tanksley, 1986) lettuce (Landry et al., 1987), potato (Bonierbale et al., 1988) rice (McCouch et al., 1988) and soybean (Muehlbauer et al., 1991).

RFLP's were also used to locate some genes controlling economically important traits. RFLP markers have been found to be linked to genes controlling resistance to many diseases, such as downy mildew (*Bremia lactuca)* of lettuce (Landry et al., 1987) and leaf blast (*Magnaphorthe grisea*) of rice (Yu et al., 1991).

They have also provided identification of several Quantitative Trait Loci (QTL's) for several agronomic, quality and stress traits in many crop species. In tomato, for example, six QTL's affecting variation in fruit size, four QTL's affecting variation in soluble solids and five QTL's controlling variation in fruit pH have been identified (Paterson et al., 1988) and mapped (Paterson et al., 1991). Six QTL's controlling variation to heat tolerance in maize have also been identified using RFLP markers (Ottaviano et al., 1991).

Other uses for RFLP analysis include the measurement of genetic distances, relationships among species and genotype identification (Gebhardt and Salamini, 1992).

The major advantage of RFLPs over morphological and RAPD markers is that they can be used to distinguish homozygote from heterozygote individuals in segregating populations. In addition, RFLP markers lack phenotypic effects and epistatic interactions. These attributes make this technique useful for genetic studies and mapping purposes (Beckman and Soller, 1986). However, its use is limited because of restriction endonucleases and the Southern blotting and radioactive labeling. These make RFLP analysis time consuming and expensive, especially where limited funds and means exist (Anderson and Fairbanks, 1990; Rafalski et al., 1991).

Polymerase Chain Reaction (PCR)

Definition: The polymerase chain reaction (PCR) is another DNA-based assay (Saiki et al., 1988; Ochman et al., 1988 and Oliver, 1990). It is a molecular method which uses two oligonucleotide primers of varying length that specifically hybridize to opposite strands. Using a repetitive series of cycles involving DNA denaturation, primer annealing and extension of the annealed primers by DNA polymerase, amplification of the target DNA region occurs. The number of products approximately doubles after each cycle. DNA regions of interest can therefore be amplified many million-fold in this way (Erlich et al., 1991)

Using short oligonucleotide primers of known sequences and thermostable DNA polymerase, PCR allows the amplification of specific regions of DNA (Saiki et al.,1988). Very small samples of DNA can be amplified and the segments can be compared for polymorphism either directly or after digestion with four base-pair restriction enzymes on stained agarose gels without recourse to southern hybridization (Saiki et al., 1988).

Random Amplification of Polymorphic DNA (RAPD)

Description: The Random Amplified Polymorphic DNA (RAPD) developed by DuPont scientists (Williams et al., 1990) is a recent technique for detecting DNA polymorphism. This method requires only small amounts of DNA and no prior knowledge of the genome in question is necessary. It is based on random amplification of DNA fragment, via PCR, using a single short primer (e.g., 9 or 10 bases) of arbitrary sequence and a lower annealing temperature than the average PCR reaction.

A single species of primer binds to the genomic DNA at two different sites on opposite strands of DNA template. The Taq polymerase starts at each 3'DNA site complementary to the 5'primer site and copies and extends the target sequence of that strand until either the 5' end of the DNA strand is encountered or the 2,000 base pair limit of PCR is reached (Williams et al., 1990). This whole sequence is repeated 20-50 times (40 is often used). During the following cycles, the double stranded molecules of both original DNA segments and the copies are separated with heat. They are then cooled to allow the primer to anneal to complementary sequences, and then heated to allow the taq polymerase to

replicate the target sequence (Kesseli et al., 1992). Through this process, the target DNA sequences are amplified a million or more times (Rafalski et al., 1991; Williams et al., 1990). Most often, it is not possible to determine whether an amplified segment is from a locus that is heterozygous or homozygous. The amplified products which RAPDs provide are dominant markers and therefore are either present or absent (Williams et al., 1990). RAPD markers can be separated on standard 2% agarose gels by means of electrophoresis. They are visualized with ethidium bromide staining and illuminated by ultraviolet light.

Compared to RFLP, the RAPD assay avoids many of the technical limitations of RFLP analysis, which is laborious and costly. This large amounts of genomic DNA and cloned probes that may be specific to an organism and radioactive isotopes or PCR assay which depend on DNA sequence knowledge of the organism or gene under study are not required (Innis et al., 1990; Krawets, 1989). RAPD technology offers then several advantages such as using random primers, using low amounts of DNA and being simple and relatively cheap (Martin et al., 1991). Moreover, the technique does not require radioactive isotopes. As in the case of many techniques, RAPD assay has some disadvantages such as lack of reliability and repeatability among different laboratories and lack of homology among related species (Kesseli et al., 1992).

Applications: The use of RAPD markers in crop improvement has contributed to many studies. For example, they have became a basic tool for genetic studies of related organisms (Hadrys et al., 1992). Another application of RAPD's is their use in "tagging" pest-resistance genes and identifying genotypes carrying specific resistance genes (Paran et al., 1991; Ohmori et al., 1995; Borovkova et al., 1995).

RAPD markers have also been used for creating of genetic maps. By using this assay, Reiter et al., (1992) were able to place over 250 new genetic markers on a recombinant inbred population of *Arabidopsis thaliana*. Chapparo et al. (1992) were able to establish a 191-marker RAPD map of loblolly pine. Other genetic maps in a variety of organisms such as *Arabidopsis thaliana* (Reiter et al., 1992) and wheat (He et al., 1992; Hu and Quiros,1992) have been established using the RAPD technique.

RAPD's have been successfully used in the characterization of genetic resources in

RAPD's have been successfully used in the characterization of genetic resources in many plant species, such as lettuce (*Lactuca sativa* L.) (Waycott and Fort, 1994), strawberry (Gidoni et al., 1994) and citrus cultivars (Denz et al., 1995).

RAPD's have been used to demonstrate that the genetic resources of black currant is narrower than would be expected by the analysis of parentage (Lanham et al., 1995). In olive (*Olea europapea* L.), Fabbri et al. (1995) screened seventeen cultivars by RAPD assay and found a high level of polymorphism in the germplasm with two main groups. In mango, Schnell et al. (1995) examined twenty- five accessions for RAPD markers for the identification of mango cultivars. The uses of RAPD analysis for *Mangifera* germplasm classification and clonal identification were possible. In potato and sunflower, somatic hybrids and inbred lines have been characterized using RAPD and isozyme markers (Rasmussen and Rasmussen, 1995 ; Teulat et al., 1995).

RAPDs in plant disease improvement: RAPD's may be used in plant disease improvement to identify markers linked to genes for resistance. It has been successful in many crops such as lettuce in which RAPD markers linked to downy mildew resistance genes were identified (Paran et al., 1991). In tomato, Martin et al. (1991) established linkage between RAPD markers and resistance genes to Pseudomonas. Recently, Ohmori et al. (1995) identified RAPD markers linked to the gene conferring resistance to tomato mosaic virus. In barley, Borovkova et al. (1995) identified RAPD and RFLP markers linked to the stem rust resistance gene RPG4 in a doubled haploid population of barley using bulked segregant analysis. And in apples, the identification of a marker linked to the Vf gene for scab resistance was the first RAPD marker identified for scab resistance (Yang and Kruger, 1994).

RAPDs in grain legumes: RAPD markers have permitted the generation of linkage maps and identification of germplasm accessions in most of the grain legume crops. Genetic diversity of European and Mediterranean faba bean germplasm has been established using RAPDs. These markers were successful for the classification of germplasm and identification of different groups in faba bean *(Vicia faba)* (Link et al., 1995). In lima bean (*Phaseolus lunatus* L.), Nienhuis et al. (1995) established genetic relationships among cultivars and land races by means of RAPD markers. For lentil (*Lens culinaris* ssp), RAPD

markers have also been used to estimate intra and interspecific variations in the genus (Aboelwafa, 1995).

CHAPTER 2

SCREENING CHICKPEA GERMPLASM FOR ASCOCHYTA BLIGHT (*ASCOCHYTA RABIEI*) IN THE FIELD AND GREENHOUSE UNDER MOROCCO CONDITIONS

INTRODUCTION

Ascochyta blight is the most devastating disease of chickpea and is caused by the highly pathogenic fungus *Ascochyta rabiei* (Pass). This fungus may survive over two years in naturally infected tissue at 10-35°C provided the relative humidity is between 0-3% (Nene and Reddy, 1987). However, infected seeds are the main source of primary infections (Kaiser, 1973). The fungus has been isolated from infected seed which had been stored for more than 117 weeks at Safiabad (Iran) under summer temperature exceeding 45°C (Kaiser, 1972). Secondary spread of the fungus occurs through spores produced in pycnidia. Under prolonged wet and windy conditions with temperatures around 20°C, the fungus spreads rapidly causing mass mortality and epidemics (Nene and Reddy, 1987).

Many cultural techniques have been used to control this disease. However most of them are costly and/or impractical. Practices include burning of plant debris, deep plowing, crop rotation and use of healthy seeds (Saxena, 1984). In addition to these methods, heat therapy has been successful: the exposure of infected seeds to temperatures of 55, 60, and 65°C for 6 to 22 hours has completely eradicated the fungus from the seed. However, germination of the seeds was adversely affected as more than 50% of the seeds failed to germinate (ICARDA, 1988).

Ascochyta blight can also be controlled by frequent spray applications of fungicides. However, this method is quite expensive and control depends on weather conditions and optimal time of application. Chemical control measures suggested are (1) seed treatment with

Benomyl, Thiram or Primaricin (2) foliar sprays with Bordeaux mixture , Zineb or Captan (Kaiser, 1973). Some of these chemical have been effective in research plots, but since frequent applications were needed, these results can not be economically applied to large production areas.

Development of resistant cultivars to ascochyta blight is therefore the best and the most economical way to control this disease. Thus considerable efforts have been devoted to the identification of stable sources of resistance and their subsequent transfer into a good yielding cultivars.

Screening tests to identify sources of resistance to ascochyta blight is a prerequisite for the improvement of resistance to ascochyta blight. However, classical screening using field and greenhouse for the evaluation of the material is time consuming and costly. In this report 56 germplasm lines of chickpea were screened in two field locations. As occurrence and severity of ascochyta blight is known to be highly influenced by environmental factors (Reddy and Kabbabeh, 1985) which may complicate field screening, a controlled environment experiment was also conducted under greenhouse conditions for rapid screening of the chickpea lines and to compare them to the multilocational testing.

Variability of the fungus is very high (6 genetic races found in Syria (Singh, 1987) and 3 to 4 in Morocco (Lamnouni, person. Com.)), thus 56 germplasm lines originating from diverse locations were selected for screening against *Ascochyta rabiei* in the field at two locations in Morocco.

MATERIALS AND METHODS

Field experiments

Seed sources: Fifty six chickpea (*Cicer arietinum L.*) lines, comprising 6 desi (ICC) and 10 kabuli (ILC) and 33 kabuli breeding (FLIP) lines, 3 local Moroccan cultivars (Local, PCH 46, PCH 37) and 4 other Kabuli and Desi (RH, PI and S) accessions plus 1 susceptible check to ascochyta blight (ILC263) were evaluated in this study. These lines were provided by the ICARDA food legumes program. The origin and parents of this material is listed in Table 2.1.

Sources of pathogenic isolates: Ascochyta plant debris were collected from infested chickpea plants and used as native inoculum in the fields. These chickpea debris were collected from different locations in the semi-arid regions (Chaouia , Abda and Merchouch) of Morocco. American isolates were supplied by Dr. Kaiser, plant pathologist, Department of Plant Pathology, Washington State University as well. Other isolates from America were collected during a survey conducted by myself, Dr. Muehlbauer, and Dr. Kaiser in the Palouse region of Eastern Washington during July, 1994. However, these latter isolates failed to cause disease in lines tested herein, even in the greenhouse. Thus, only the isolates collected in April, 1993 from the semi-arid regions of Morocco, were used for the screening trials.

Disease Scoring: Usually ascochyta disease symptoms are scored twice, first at the early stage of chickpea development and then at the podding stage. A scale from 1 to 5 where 1 = no disease and 5 = dead plants is used. The percentage of damage to the plant, visibility of symptoms at the first scoring, size and deepness of lesions on stem or branches, and the number of broken branches are evaluated in scoring the material as resistant or susceptible. This scale (1 to 5) has recently been extended to 1- 9 with five defined categories of severity where 1 = no visible lesions on any plants (highly resistant), 3 = lesions are visible on less than 10 % of the plants, no stem girdling (resistant), 5 = lesions are visible on up to 25 % of the plants, stem girdling on less than 10 % of the plants but little damage (tolerant), 7 = lesions on most plants resulting in the death of a few plants (susceptible) and 9 = all plants are dead (Singh, 1987).

Table 2.1. Parents and origin of chickpea cultivars tested

Cultivar	Origin	Parent
Flip 92-72C	ICARDA/ICRISAT	ILC571 X FLIP85-122C
FLIP 84-87C	ICARDA/ICRISAT	ILC72 X ILC215
FLIP 92-189C	ICARDA/ICRISAT	ILC100 X FLIP82-150C
FLIP 90-112C	ICARDA/ICRISAT	ILC3856 X ILC4296
FLIP 88-83C	ICARDA/ICRISAT	F82-100C X ILC200
FLIP 90-76C	ICARDA/ICRISAT	(ILC171 X F82-127)XILC171)
FLIP 91 -8C	ICARDA/ICRISAT	F84-48C X ILC4293
FLIP 88-85C	ICARDA/ICRISAT	ILC 629 X F82-144C
FLIP 89-78C	ICARDA/ICRISAT	F82-87 X F 85-46C
FLIP 92-78C	ICARDA/ICRISAT	ILC6055 X F85-122C
FLIP 84-156C	ICARDA/ICRISAT	ILC200XILC482XF84-48C
FLIP 83-48C	ICARDA/ICRISAT	ILC72 X ILC215
FLIP 83-77C	ICARDA/ICRISAT	ILC72 X ILC215
FLIP 92-152C	ICARDA/ICRISAT	F85-122C X F85-137C
FLIP 84-81C	ICARDA/ICRISAT	ILC72 X ILC215
FLIP 92-112C	ICARDA/ICRISAT	ILC 1934 X F85-122C
FLIP 91-23C	ICARDA/ICRISAT	ILC482 X 78C
FLIP 84-87C	ICARDA/ICRISAT	ILC72 X ILC215
FLIP 92-139C	ICARDA/ICRISAT	F84-43C X F85-122C
FLIP 91-62C	ICARDA/ICARDA	ILC3777 X F84-92C
FLIP 92-181C	ICARDA/ICRISAT	F82-59CXF84-145CXF82-59C
FLIP 83-92C	ICARDA/ICRISAT	ILC73 X ILC897
FLIP 92-70C	ICARDA/ICRISAT	ILC571 X F85-122C
FLIP 90-56C	ICARDA/ICRISAT	S85088 X ILC3856
FLIP 91-14C	ICARDA/ICRISAT	F85-122 X F 85-112C
FLIP 84-109C	ICARDA/ICRISAT	ILC1920 X ILC187
FLIP 92-187C	ICARDA/ICRISAT	F84-176 X F84-155C
FLIP 92-64C	ICARDA/ICRISAT	LC6055 X F85-122C
FLIP 92-34C	ICARDA/ICRISAT	ILC3520 X F84-92C
FLIP 85-54C	ICARDA/ICRISAT	F82-65C X F82-69C
FLIP 92-18C	ICARDA/ICRISAT	FLIP85-122C X FLIP85-137C
FLIP 92-132C	ICARDA/ICRISAT	ILC571 X F 85-122C
FLIP 9-18	ICARDA/ICRISAT	unknown
ILC 3279	FORMER USSR	Selection from STEPNOJ-1
ILC 195	FORMER USSR	Selection from Vysokoroshyj-30
ILC 482	TURKEY	ACC.No. 26780-68
ILC 5924	BULGARIA	6709
ILC 183*	ICARDA/ICRISAT	Unknown
ILC 72*	ICARDA/ICRISAT	Unknown
ILC 19I*	ICARDA/ICRISAT	Unknown
ILC3397	ICARDA/ICRISAT	Unknown
ILC 200	FORMER U.S.S.R	Selection from Stepnoj-1
ILC 1929	SYRIA	Syrian local

Table 2.1. Parents and origin of chickpea cultivars tested (Cont.'d)

Cultivar	Origin	Parent
ILC 263(Check)	SYRIA	Unknown
LOCAL	MOROCCO	Unknown
PCH 46	MOROCCO	ILC 3820
PCH 37	MOROCCO	ILC 3815
ICC 4935*	INDIA	BG-212 (P-100X106)
ICC 322	INDIA	P-242-1
ICC 5003	INDIA	850-3/27
ICC 6098	INDIA	JG - 74
ICC 8933	INDIA	K - 315
ICC 4918	INDIA	Annigeri
PI456883	INDIA	Unknown
RH 79177	ICARDA/ICRISAT	Unknown
S 8502762	ICARDA/ICRISAT	Unknown
S 85085	ICARDA/ICRISAT	ILC295 X ILC 202

* Lines bearing *Ascochyta rabiei* resistance, recessive or dominant gene

Location of the experiments: Three ascochyta blight experiments were conducted. Two were at the research experiment stations (Jemaa de Shaim and Sidi El Aydi) in Morocco. Jemaa de Shaim is a station located in a semi-arid area with 200 to 350 mm of rainfall per year. The Sidi El Aydi station is located in the Settat region in the zone of high production of food legumes and is characterized by a moderate climate with the same range of rainfall. The third experiment was set at the greenhouse at the Settat center in Morocco.

Planting: Chickpea germplasm lines (Table 2.1) were tested under Moroccan conditions and in the greenhouse. Planting was done in November 20, 1995. Seeds were divided into two replicates, planted in the ascochyta blight screening nurseries using a randomized complete block design. The experiments were planted in the above described stations. Each experiment was planted with a single row planter. Rows were spaced 60 cm apart and were 2 meters long. A susceptible check was planted after every two entries and also around the experiment to facilitate spread of the disease. Disease development was encouraged through irrigation, necessary only once at the Sidi El Aydi station. Maximum and minimum temperatures and total precipitation were monitored throughout the growing season

at the two stations. The experiments were managed in accordance with the local recommendations for production with respect to land preparation, fertilizer, pest, disease, weed control, etc. Fifteen day-old plants were inoculated with ascochyta blight in the field according to the growth stage of the plant (Table 2.2). Infected plant debris collected from previous years were scattered on the field to provide disease inoculum. Weekly observations were made to record the presence of any insect or disease that may interfere with ascochyta blight.

Controlled environment experiment

At the greenhouse, seeds were sowed in plastic trays (35x25x8cm) in sterilized sand. There were 4 seedlings in each tray and two replicates. Isolates used for inoculation were collected from different regions of Morocco where the disease is prevalent and according to the genetic study of the fungus undertaken in Morocco (Lamnouni, 1994). These isolates from these regions have had different levels of severity when tested on chickpea differential lines. These isolates were mixed together and used in preparing spore suspensions. The cultures were cultured and maintained on potato-dextrose agar at 5°C. The spores were produced on chickpea seeds, prepared by autoclaving 100g of chickpea seeds in 50ml of water for 30min in a flask. The seeds were inoculated from a 7-day-old culture of *Ascochyta rabiei* and incubated for 10 days at 20°C. The spore suspension was made by soaking infected seeds in sterile distilled water for 30min, stirring with a glass rod and passing the suspension through double-layered muslin cloth. The spore suspension was also made from infected debris in the same manner. The suspension was adjusted to the required spore concentration using a hematocytometer. Seedlings were inoculated by spraying spore suspensions ($2x10^6$ spores per milliliter) of a mixture of isolates of *A. rabiei* onto seedlings and plants were covered with plastic to preserve moisture. Air temperature was maintained at 20°C(±1°C) in the greenhouse. Relative humidity was maintained between 65 and 70%. Disease scores were recorded at two stages (Table 2.2) and plants were scored on a scale of 1-9 as described by Singh et al., (1988), where 1=no symptoms and 9=plants killed.

Table 2.2 Inoculation and rating dates of ascochyta blight on 15 days old chickpea lines at three locations during 1995-96.

Experiment	Localization	Inoculation	Rating
1	Sidi El Aydi	Jan. 7	Mar.4 and Apr. 25
2	Jemaa de Shaim	Jan. 10	Mar. 14 and Apr. 28
3	Greenhouse	Jan. 15	Feb. 23 and Mar. 28

Growing conditions

In both field locations (Sidi El Aydi and Jemaa de Shaim) the inoculations (straw debris) were applied during cool and humid weather conditions followed by a long wet period with mild temperatures. At Sidi El Aydi, daily temperatures ranged from 17 to 37°C and relative humidity was high at night (60 to 80%). At Jemaa de Shaim, the days were hot when not raining but most of the time was humid with cool and humid nights. Daily temperatures ranged from 32 to 42°C.

Statistical analysis

Scores of the lines evaluated at 3 locations and 2 different stages of development were compared to the check using the analysis of variance (ANOVA, SAS software). A randomly complete block design (RCBD) with 2 replications and a check repeated every two entries was used for each experiment. Scores of the 56 lines of each replication and screening date and those of the susceptible check were compared.

Ratings in the greenhouse were compared to the field results using the coefficients of correlation calculated using SAS procedure. The coefficients of correlation (Pearson correlation coefficients) is a measure of association denoted by $\varrho_{Y,X}$.

RESULTS AND DISCUSSION

The field experiments were carried out during 1994-96. The results of 1995-96 are only presented as weather in Morocco during 1994-95 was exceptionally dry. Temperatures were also high and this as well as the dryness resulted in a weak disease infestation.

Evaluation of disease infestation was done twice, at the early stage of plant development and at the podding stage on the check and the two replication entries. Visual damage scores were taken by a consensus of two persons at the greenhouse and a team of 6 persons according to the 1-9 scale adopted by ICARDA scientists for this disease in the field.

Ascochyta blight was not as evident at the early stage of plant growth but increased at the podding stage in all three experiments (Appendix 1: Fig. 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6). This was no doubt due to the hot weather (35 to 45°C) which enhanced natural infection of ascochyta blight at all locations.

Reaction of the tested germplasm lines to ascochyta blight

Field experiments: In the field, symptoms developed 5-6 days after inoculation with death of the susceptible check (ILC263) within 10 days of inoculation. No genotype tested was immune. These results agree with that of Iqbal et al. (1994) who made the same observation when screening 467 lines at Islamabad in Pakistan. These results suggest that the fungus is very aggressive throughout the regions where the host is grown and supports the hypothesis of high variability of the fungus (Reddy and Kabbabeh, 1985). This obviously complicates an ascochyta blight breeding program. Though there was considerable variation in the reaction of the lines across locations, seven lines (F92-72C, ILC 200, ILC 72, ILC 5924, FP92-187C, F92-139C and F92-78C) were graded as resistant (score 3 to 4) at two to three sites and presented a difference which was highly significant as compared to the susceptible check. These lines may not be released since they have small seeds which would not be acceptable by farmers in Morocco. However, they can be used as a source of resistance in the chickpea breeding program. Another 7 lines differed significantly from the check at both rating times (early stage and podding stage) and were graded as tolerant (ILC 183, ICC4918, RH79177, F84-81C, F90-56C, F92-112C and F92-152C). Yet another 7 lines

were graded as moderately susceptible and presented scores significantly different from the check in at least one site during the first stage (ICC 8933, ICC 4918, F83-92C, F85-54C, F89-78C, F91-14C and F92-18C). The 35 remaining lines were susceptible at the 3 locations. Some moderatly susceptible lines were designated with the susceptible ones instead of being moderately susceptible since they were completely killed in previous years at the same locations (F9234, S85027, S85085 , F92132, ILC1929, F84156, F84109, ICC322, ICC5003, F8885, PCH46, ILC482, F92181, F9123, F92133 ect.).

The desi lines (named ICC) were moderately susceptible to highly susceptible, while the kabuli lines mostly showed resistance or tolerance (Table 2.3). This suggests that the kabuli germplasm has better resistance to ascochyta blight than desi germplasm. This result agrees with that of Reddy et al. (1992) who screened 151 kabuli lines and 40 desi lines to *Ascochyta rabiei* in 48 disease-endemic locations in 20 countries. They found that 18 out of 191 lines were resistant with just one desi line showing resistance.

The Moroccan cultivars (local , PCH 34 and PCH 46) were highly susceptible to ascochyta blight. This agree with previous results over several years when comparing winter and spring sowing. The local cultivars were more highly susceptible in winter than in spring. This problem is associated with the conditions in winter which are more condusive for ascochyta blight disease development, than those of the spring. Rainfall is usually rare and the weather is very dry in the semi-arid regions in spring.

Resistant lines (ILC183, ILC 72, ILC 191, ILC 200 and ICC4935) screened previously in Syria (Singh and Reddy, 1983) were scored under Moroccan conditions as resistant for only three lines (ILC 183, ILC 72 and ILC 200) while the other two (ILC191, ICC4935) were moderately susceptible. This is likely associated with the variability of the fungus in the semi-arid regions and the crop rotation system which is mainly cereal-chickpea especially at Jemaa de Shaim. This kind of rotation, increases the inoculum in the soil every year with the absence of chemical control. Disease scores were high in both fields but higher at Jemaa de Shaim than Sidi El Aydi station (significative difference between scores were observed at these two locations). This might be due to a naturally high level of ascochyta infestation in that region, an area known to have severe epidemics. This natural level of

inoculum may have enhanced artificial inoculation, thereby increasing the severity of the disease as indicated in Fig. 2.5 to 2.10 in Appendix 1. Differences are highly significant between lines graded as resistant or tolerant and the susceptible check (Table 2.3). No significant differences were shown between the susceptible check (scored 9 in the three experiments) and lines graded as susceptible to ascochyta blight, especially at the podding stage.

Greenhouse experiment: In the greenhouse, disease symptoms appeared on the plants within a week of inoculation. For most of the lines the reaction of the plants to ascochyta blight under constant conditions was lower than in the field (Appendix 1: Fig. 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6). This might be explained by a high level of natural infestation that occurred in the field which enhanced the inoculum and also more the natural conditions (mild to high temperatures, humidity and wind). A positive correlation was observed between results of the controlled-environment experiment and screening in the field at Jemaa de Shaim and Sidi El Aydi. Coefficients of correlation were significantly higher between the greenhouse scores and Sidi El Aydi experiment scores as compared to those of Jemaa de Shaim and greenhouse (ρ=0. 80 and 0.73, versus 0.52 and 0.48 for screening at the early and podding stages respectively). This association is due probably to the low infestation of ascochyta at Sidi El Aydi as compared to Jemaa de Shaim and may also be due to the difference of temperatures and humidity recorded in both field experiments located in different microclimates (200 miles a part). This result (positive correlation) agrees with that of Haware et al., (1995) who conducted their experiments in the greenhouse and at Hisar in India where *A. rabiei* is endemic and found a similar result (positive correlation between the scores taken in the field and in the greenhouse).

Table 2.3 Average disease scores of the 56 chickpea lines evaluated at three locations (ANOVA procedure/SAS software) .

	Cultivar	Scores at the early stage			Scores at the late stage		
	#	JSH	SDL	GH	JSH	SLD	GH
1	F9272C	3**	2**	3**	6	4.5 *	4.5*
2	F8487C	5	4**	4**	7.5	5	6
3	F92189C	3**	2.5**	2**	6	5	3.5**
4	F90112C	3**	4.5*	3**	7	7	7
5	F8883C	5	5.5	3**	9	8	7
6	ICC6098	4**	4.5*	4**	8	8	6
7	LOCAL	6	6	4.5*	9	7	8
8	ILC200	3**	2**	1**	4.5*	5	3**
9	F9076C	3**	3**	3**	7.5	5	4.5*
10	F918C	6	4**	3*	8	5.5	6
11	ILC 72	3**	1.5**	1**	6.5	4**	3.5**
12	F8348C	3**	4**	4**	8	6	6
13	F8377C	5	4**	3.5**	8	6	7
14	F92152C	3**	3**	1**	7.5	4.5*	4.5*
15	F8481C	3**	2**	2**	8	4.5*	4**
16	F92112C	3**	3**	2.5**	7	5.5	5
17	ILC 191	6	4**	3.5**	7	7	6.5
18	F9123C	5	4.5*	4**	7.5	7	9
19	F92133C	5	4.5*	3**	8	6.5	6
20	F8487C	3**	3 **	4**	6	4**	7
21	F92139C	3**	3**	1**	6	4.5*	4.5*
22	F9162C	6	3**	3**	9	7	6
23	F92181C	5	4**	3**	7.5	7	7
24	F8392C	3**	3**	3**	7.5	7	7
25	ILC195	6	5	5	8.5	7.5	7
26	ILC482	6	5.5	4.5*	8	8	8
27	ILC5924	3**	3**	1**	6	4**	3.5**
28	ILC183	5	3**	3**	8	5.5	4.5*
29	PI456883	6	5	5	8.5	9	8
30	PCH46	7	6	4**	9	7	8
31	PCH37	7	5.5	4.5*	9	9	7
32	ICC4935	5	2**	2**	7.5	6	5
33	ICC322	4**	4**	3**	8	6.5	6
34	ICC5003	4**	3**	4**	7	7	8
35	F8885C	3**	4**	4**	7	6	6
36	F9056C	5	5.5	5	9	8	9
37	F8978C	4**	4.5*	3.5**	7	5.5	6
38	F9278C	3**	2**	2**	4.5*	4.5*	4**
39	F84156C	3**	4.5*	4.5*	6.5	5.5	7

Table 2.3 Average disease scores of the 56 chickpea lines evaluated at three locations (ANOVA procedure/SAS software) (cont'd).

	Cultivar #	Scores at the early stage JSH	SDL	GH	Scores at the late stage JSH	SLD	GH
40	ILC1929	5	4**	4**	8.5	5.5	5.5
41	F9270C	5	5	5	9	6	7
42	F9056C	4**	3**	3*	9	5.5	5.5
43	F9114C	4**	4**	4**	8	7	8
44	F84109C	5	5	4.5*	8	6.5	7
45	ILC3279	5	4.5*	3.5**	8.5	6	6
46	F92132C	4**	4**	3.5**	8	8	6.5
47	RH79177	3**	3**	3**	5.5	5	5
48	S85027	6	4.5*	4**	9	7	7
49	S85085	6	5	4.5*	9	8	7.5
50	F92187C	3**	2**	2**	6.5	4.5*	3.5**
51	F9264C	5	5	5	7	7	8
52	F9234C	7	3.5*	3.5*	8.5	6	6
53	F8554C	5	5.5	4.5*	7	8	7
54	F9218C	5	4**	4**	9	5.5	6.5
55	ICC8933	6	3**	3**	8	4.5*	6
56	ICC4918	6	3**	3**	6	5	5
57	ILC263	9	9	9	9	9	9

* Significant difference as compared to the susceptible check at $\alpha = 0.05$

** Significant difference at $\alpha = 0.01$.

Table 2.4. Pearson correlation coefficients between greenhouse disease scores and those of the field at two different stages of plant development.

	Jemaa de Shaim		Sidi El Aydi	
	Early stage	late stage	Early stage	Late stage
Greenhouse scores at the early stage	0.53	0.50	0.77*	0.65*
Greenhouse scores at podding stage	0.52	0.48	0.80*	0.73*

*Significant difference between the coefficient of correlation at Sidi El Aydi as compared to those of Jemaa de Shaim at 5%.

CONCLUSION

None of the 56 chickpea germplasm lines were evaluated as immune from screening for reaction to ascochyta blight disease carried out during 1995-96 in the fields and in the greenhouse with artificial inoculations. Seven entries were resistant, and 7, 7 and 35 lines were moderately resistant (tolerant), moderately susceptible and susceptible, respectively.

Artificial inoculation in the fields was effective as it has the probable advantage of releasing inoculum whenever conditions are favorable (Nasrellah, 1991). The effectiveness of the artificial inoculation method seems to be enhanced by weather conditions (mild

temperatures, good humidity) that prevailed during inoculation and development of the disease. Spring cultivars (PCH 37, PCH 46 and local), sown in the winter (November) under Morocco conditions were highly susceptible to ascochyta blight. Desi chickpea type lines were also more susceptible than kabuli type lines but frequences were quite low.

Greenhouse screening scores were positively correlated with those of the field experiments and seemed accurate for chickpea evaluation. Multilocational testing remains important to identify stable resistance to ascochyta blight.

The selected lines (F92-72C, ILC 200, ILC 72, ILC 5924, F92-139C, F92-78C and F92-187C) graded as resistant, also showed desirable attributes for direct exploitation as valuable sources of resistance to *Ascochyta rabiei* in hybridization programs of chickpea.

CHAPTER 3

ENZYMATIC POLYMORPHISM IN CHICKPEA GERMPLASM LINES

INTRODUCTION

Electrophoresis is a traditional technique for the separation of mixtures of ionic compounds. The development of starch as a stable support for electrophoresis (Smithies, 1955) increased the usefulness of the technique. Other solid support systems, such as polyacrylamide gels and agarose, have also been introduced. At the present time many isozyme studies can be carried out utilizing relatively inexpensive equipment and materials. Both starch and polyacrylamide gels, by adding appropriate reactants and staining solutions, can reveal isozymes (Hunter and Markert, 1957).

Starch gels are used in a horizontal apparatus which can be kept cold on ice trays (Shaw and Prasad, 1970). Lack of clarity and the relatively poor resolution of starch frequently complicates the differentiation among isozymes (Suurs et al., 1989).

Polyacrylamide gels are very transparent and give excellent resolution. These gels may be prepared with various concentrations resulting in different pore sizes. The disadvantages of using polyacrylamide is the fact that it is neurotoxic and expensive compared to starch.

Bands can be visualized by staining of the gel. A homozygote at a particular locus has only one band for the enzyme. If the phenotype is heterozygous, two bands will be seen for monomeric enzymes, one for each allele in the chromosome compliment. Three bands will be seen for a dimeric enzyme, one band for each of the homodimeric enzymes and an intermediate band for the heterodimeric enzyme. Similarly, for a tetrameric enzyme, there will be five bands present.

In chickpea, isozyme analyses have been done using starch gel or acrylamide. Six to

27 different enzymatic systems have been examined by Oram et al.(1987), Tuwafe et al.(1988), Gaur and Slinkard (1990) as well as Kusmenoglou et al. (1992). These analyses revealed low variability within the accessions tested. Therefore, in this study, we used acrylamide as well as starch gels in an attempt to observe the greatest polymorphism possible in the chickpea accessions used for screening tests.

Isozyme markers linked to resistance to ascochyta blight offers great potential to aiding breeding programs. These markers could replace the costly and laborious traditional methods of identifying resistance with a biochemical technique with one that might potentially be done at the seedling stage.

Isozyme markers as a traditional tool are still being used along with the relatively recent molecular markers such as RFLPs and RAPDs (Havey and Muehlbauer, 1989; Anderson and Fairbanks, 1990; Paterson et al., 1991; Baruffi et al., 1995; Rasmussen and Rasmussen, 1995; Cisnero and Quiros, 1995; Lin and Ritland, 1996; Reamonbuttner et al., 1996 and Gustine et al., 1996). The identification of markers that might be closely linked to resistance genes of ascochyta blight is of major interest.

The main objective of using the isozyme assay was to expand the number of enzymatic systems used previously (Tuwafe et al.,1988 and Kusmenoglou et al.,1992) in hope of observing sufficient polymorphism and thus identifying markers linked to ascochyta resistance genes.

The enzyme systems and their abbreviations used in this study are listed in Table 3.1

Table 3.1 Abbreviations of the 15 enzyme systems assayed

Enzyme	Abbreviation
Aspartate amino transferase	AAT
Esterase	EST
Propionate esterase	PR-EST
Glucose dehydrogenase	GDH
6-Glucose phosphate dehydrogenase	6-PGD
Carbonate dehydrogenase	CA
Alkaline phosphatase	ALPH
Leucine amino peptidase	LAP
Malic enzyme	ME
Fumarase	Fu
Phospho-glucose-isomerase	PGI
Phospho-glucose-mutase	PGM
β-amylase	β-AMY
Acid phosphatase	ACPH
Alcohol dehydrogenase	ADH

MATERIALS AND METHODS

The procedures used for acrylamide in this study were those developed in the Department of Horticulture and Land Scape Architecture at Colorado State University (Stephens, 1995). The procedures followed for the starch gel were those used at the agronomy laboratory of the IAV(Institut agronomique et Veterinaire, Morocco). The steps followed for electrophoresis and staining are outlined herein.

Two gel electrophoresis systems were used. The starch system was used as a reference since it was used in chickpea in previous studies (Oram et al., 1987 and

Kusmenoglou et al., 1992) and acrylamide was used in an attempt to improve the resolution of the enzymatic systems. Fifteen enzymatic systems were tested as noted in Table 3.1. These systems were analyzed with acrylamide and starch gels. Acid phosphatase, alkaline phosphatase, carbonic anhydrase and esterase propionate were tested only in starch gel. With acrylamide gels no band appeared for these systems. 6-Phospho-glucose dehydrogenase was revealed by both techniques. Malic enzyme, esterase, phosphoglucose isomerase, phosphoglucose mutase, amylase, glucose dehydrogenase, fumarase, asparate aminotransferase, leucine aminopeptidase and alcohol dehydrogenase were revealed easily in polyacrylamide gels. A minimum of 10 to 12 gels were evaluated for each enzymatic system to screen the 56 lines with 10 to 12 lines per gel and 2 repetitions with more gels in case of very faint or no bands at all. The phenotypes which exhibited differences were assayed two or three times to confirm their consistency.

Plant material

Fifty-six chickpea accessions plus a check susceptible to ascochyta blight, provided by ICARDA and originating from various countries (Table 2.1) were tested. These lines were maintained in the greenhouse at 27±3°C daytime and 20±3°C night time under natural day length. Samples of leaf tissue were taken from two weeks old plants and immediately placed in plastic bags on ice. These samples were weighed and subjected to an extraction technique using 0.5g leaf tissue from each sample.

Protein extraction

Each sample of 0.5 g of tissue was ground on ice using either a Polytron homogenizer for 20-30 seconds or a mortar and pestle for two minutes using 1.5ml of extraction buffer (Table 3.2) per sample. The homogenate was then centrifuged in a Baxter scientific Biofuge 17 R refrigerated centrifuge at 11,068 g for 30 minutes. The supernatants were divided into 24µl aliquot to which 2µl of bromophenol blue tracking dye was added. These were stored at -70°C until further use.

Gel formation

Starch gel: Horizontal slab starch gels were prepared using the system described by Scandalios (1969). The gel buffer consisted of 9 parts Buffer A (0.05M tris and 0.007M

citric acid) and one part Buffer B (0.038M lithium hydroxide and 0.19M boric acid). The gel buffer (200ml) was boiled and rapidly mixed with 38g starch (Connaught Starch Hydrolyzed) previously suspended in 75ml gel buffer (buffer #6, Table 3.2). The hot viscous starch suspension was evacuated and poured into the gel form (16x18x0.8cm), covered with a glass plate, and cooled to 20°C.

The paper wicks (Whatman paper #3) were inserted into a vertical cut in the gel 5cm from the cathodal end across the 18cm width. Ten to twelve samples could be assayed simultaneously. The electrode reservoirs were filled with 150ml buffer B, and thin sponges soaked in Buffer B were used as bridges to the gel. The gel was covered with plastic in a cold room (4 ±1°C) for the electrophoretic run. After a 10 minutes run, 300 volts at 65mA were applied for 10 minutes. The wicks were then removed and electrophoresis continued at 300 volts until the dye had moved 8cm past the origin (4hr). The gel was then cut horizontally into 3 slices. Each slice was stained for a separate enzyme.

Each gel slice was assayed by placing it into a large glass tray with a buffered solution containing the appropriate substrates.

Acrylamide gel: Polyacrylamide separating gels 0.8 mm thick were formed using an Idea Scientific system. This apparatus allowed the preparation of up to 15 gels at a time. Each 10X10 cm plate requires approximately 6 ml of solution to fill to the desired height. The stock solutions listed in Table 3.2 were made and stored in the refrigerator. A fresh preparation of 28 mg ammonium persulfate in 29 ml of distilled water was made each time the gel was prepared.

The gel plates were set up as follows. A spacer was placed vertically on each side of a glass plate(10x10cm). A second plate was placed over that. A few drops of water were placed on the back of this last plate to seal the glass plate to another one and prevent gel leakage. These plates formed the first gel sandwich and these procedures were repeated until the desired number of gels were prepared. A plastic plate and a large cork were placed on top of the sandwiches and held together with a rubber band. The apparatus was then sealed using silly putty placed around the inside edge of the pouring frame to prevent gel leakage. Separating gels of 6.3%-11.6% were then prepared by mixing the solutions listed in Table 3.3,

and immediately poured between the glass plates. Bubbles were removed by gently tapping on the apparatus. The top surfaces of the gels were then covered with butanol saturated with dH_20 and left to polymerize for a minimum of 3 hours. Following polymerization, the gel was washed a minimum of 20 times with dH_20, blotting between each wash to remove all traces of the butanol. The prepared gels were then stored in the refrigerator until further use.

In preparation for electrophoresis, a gel sandwich was slipped into the upper electrophoresis reservoir. Clips were used to attach the gel to each side of the reservoir. The gap around the sandwich was sealed with 1.5% agarose.

A 4.3% stacking gel was then prepared by mixing solutions in Table 3.4. This solution was then added to the top of the separating gel with a Pasteur pipette and the comb inserted. After the stacking gel was polymerized for a minimum of 1 hour, the appropriate electrode buffer (Table 3.2) was then added in both the anode and cathode compartments of the electrophoresis cell. The comb was then carefully removed. Each well was rinsed by using a Pasteur pipette to force buffer into the wells before the extracted protein samples were loaded. Twenty µl samples were then loaded into the wells of the polyacrylamide gel using a micropipette.

Table 3.2. Buffer compositions

Buffer	Components
1. Phosphate solution A (0.2M)	Sodium phosphate monobasic ($NaH_2PO_4.H_2O$) 27.8 g/L).
2. Phosphate solution B (0.2M)	Sodium phosphate dibasic ($Na_2HPO_4 . 7H_2O$) 53.6 g/L.)
3. 1M Phosphate buffer pH 6.0	12 ml Na_2HPO_4 + 88 ml NaH_2PO_4
4*. Tris-citrate buffer pH 8.65	Tris 0.007M+citric acid 0.004M pH 8.65.
5*. Tris-borate EDTA pH 8.6	Tris 0.9M+boric acid 0.5M+EDTA 0.019M.
6. Sodium borate pH 9.6	Boric acid 0.02M + 0.1N NaOH 0.1N.
7. Lithium borate	1.6g $LiOH.H_2O$ + 11.6 g boric acid (anhydrous free base). Dissolve and adjust pH to 8.3, and make to 1 l with distilled H_2O.
8. Tris-glycine	6.5g Trizma base + 28.8 glycine. Dissolve and adjust pH to 8.3. Make to 1 l with distilled H_2O. Dilute 1:10 for use as electrode buffer.
9. Buffer A	0.05M tris and 0.0076M citric acid.
10. Buffer B	0.038 M lithium hydroxide and 0.19M boric acid.
11. Tris-acetic acid	0.5M tris adjust pH to 5 with acetic acid.
12. Extraction buffer (1)	0.1M tris HCl + 0.1M KCl + 0.005M EDTA 0.04M. 2-mercaptoethanol + 0.1M sucrose. Adjust pH to 7.5 with HCL.
13. Extraction buffer (2)	0.2M Tris HCl pH 8.5 + 1Msucrose + 10% PVP 40. Add dithiothreitol (DTT) 8 mg/ml before use.

4*: buffer for FU and ACPH. 5*: buffer for gel and electrodes for EST-PR and CA (100:900 of stock solution: H_2O for the anode (-) and 200:800 for the cathode (+) and for gel 50/950 ml stock solution /H_2O. PVP-40 : (Polyvinylpyrrolidone, average molecular weight 40,000).

Table 3.3. Separating gel ingredients

Solution number	Solution Ingredients	Volume for 12 gels (6.3%)
1	36.6 g Tris + 48 ml 1N HCL. Make to 100 ml with distilled water.	10 ml
2	30 g acrylamide + 0.74 g bis-acrylamide, Make up to 100ml with distitilled water.	17 ml
3	28 mg Ammonium persulfate solution per 20 ml distilled water (Solution should be prepared fresh).	40 ml
	TEMED*	0.02 ml
	Distilled water	16 ml

*TEMED:N N N'N'-Tetramethylenediamine

Table 3.4. Components of stacking gel

Solution Number	Ingredients	Volumes for 4.3% solution
4	5.7 g Tris + 80 ml TEMED, add enough H3PO4 to bring pH to 6.9. Made to 100 ml with distilled water.	0.5 ml
5	10 g acrylamide + 2.5 g bis-acrylamide. Made to 100ml with distilled water.	1.5 ml
6	4 mg riboflavin. Made to 100 ml distilled water (stable for up to 2 weeks)	0.5 ml
7	40 g sucrose made to 100 ml with distilled water	2.0 ml

Vertical electrophoresis

Vertical electrophoresis was carried out in an Idea Scientific Apparatus in a cold room at 3±1°C. A voltage of 150 V and a current of 40 ma was used to stack all samples to the bottom of each well. After 10 minutes the voltage was increased to 400 V and 50 mA for about 60-100 minutes. When the dye had migrated within about 0.5 cm of the bottom of the gel, electrophoresis was stopped. The gel sandwich was then removed from the apparatus and the glass plates were separated. The stacking gel was removed and the upper corner of the gel was notched. The gel was then put into the appropriate stain (Table 3.5. Appendix 2) and incubated until bands appeared. When the bands appeared the solution was discarded and the gel rinsed 3 times in tap water. The gel was fixed for 30 minutes in a solution of 20 % ethanol, 10% glycerol before drying.

After staining, the gels were photographed with a Nikkon 35 mm camera with Black and White or color film with a blue or yellow filter respectively.

The gel was dried on the bench in a drying apparatus, between two pieces of ultraclear cellophane (Idea Scientific # 1080) at room temperature.

Data analysis

The isozyme products (bands) revealed by each enzymatic system were scored as a 1 when the band is present and 0 when it is absent. Cluster analysis was performed using Statistica program. Matrix based on the percent of match was transformed into a dendrogram using Unweighted Pair Group Method (UPGMA).

RESULTS

The two extraction buffers (1) and (2) (Table 3.2) and two extraction techniques (a mortar and pestle for 2 minutes or a kinetamatic polytron homogenizer [Brinkmann Instruments] for 20 to 30 seconds) were compared for chickpea identification. Both extraction buffers and techniques gave comparable results. However, the polytron extraction technique seemed to be the most practical and fastest. This technique requires less time than with mortar and pestle and minimized oxidation and denaturation of some enzymes which may lead to blurring of the bands (Stephens, 1995).

The ME, GDH ADH, and FU enzymatic systems yielded single bands and were not polymorphic (Appendix 2: Fig. 3.5; 3.8; 3.9 and 3.10). These systems require further experimentation to optimize the extraction buffer and or technique in order to improve the resolution.

Other enzymatic systems (PGM, PGI, AAT,LAP, ALPH, and AMY(Appendix 2: Fig. 3.2, 3.3, 3.4, 3.6 and 3.11) were found to give consistent results using both starch and polyacrylamide gels. Bands were clear and easy to evaluate. They, however, exhibited little polymorphism among the lines tested. The final group of enzymatic systems showed promising and consistent results (EST, 6-PGD, ACPH, CA, and PR-EST (Appendix 2: Fig. 3.1, 3.11 and 3.12). Despite some problems in intensity of the bands, they yielded reproducible bands. These systems were the most polymorphic and were evaluated for potential markers for chickpea identification. Fresh protein samples (within 24 hours) were necessary in case of esterase and propionate esterase. The frozen samples gave no bands at all or only faint ones. The observed bands were faint which made the reading difficult as there were many loci (5 and 4 loci for EST and PR-EST systems respectively, App. 2: Fig 3.1 and 3.12,).

Esterase (EST): In chickpea the banding patterns showed five zones of activities (Fig. 3.1). The enzyme activity as demonstrated by banding patterns was faint and therefore difficult to record accurately (Appendix 2: Fig. 3.1). Improvement in the esterase system may make it useful for cultivar separation in chickpea. It is suggested that a more advanced stage of leaves be tested. An evaluation of the banding patterns indicated that two loci exhibited

some variation with 5 different phenotypes observed (9% within the 56 tested lines) (Fig. 3.1).

Phosphoglucomutase (PGM): The phosphoglucomutase phenotypes were monomorphic in all accessions. The anodal form could be clearly distinguished on the zymogram, and yielded one phenotype with one band only (Fig. 3.2). The cathodal form (PGM-2) was also monomorphic but less clear than the anodal one (Appendix 2: Fig. 3.2). This enzyme demonstrated no polymorphism, and thus has little use in the characterization of chickpea lines.

Phospho-glucose isomerase (PGI): Two loci of PGI (PGI-1 and PGI-2) were present in the leaf extract of chickpea PGI-1 yielded faint bands which were difficult to read and was monomorphic in all lines studied (Appendix 2: Fig. 3.3). PGI-2 displayed strong bands but was also monomorphic (Fig. 3.3).

Aspartate aminotransferase (AAT): The distribution of the isozyme bands of this enzyme suggested that there were four loci in chickpea (Fig. 3.4). The anodal loci had one band per individual and all phenotypes were faint while the cathodal loci had clear bands and seemed to be monomeric (Appendix 2: Fig. 3.4). One might assume that this locus was not as active in leaf tissue as in seeds, as reported by Kusmenoglou et al. (1992).

Malic enzyme (ME): Four zones of malic enzyme activity were observed on the anodal portion of the gel (Fig. 3.5). These four loci exhibited only one banding pattern for all accessions tested (Appendix 2: Fig. 3.5). The monomorphic characteristic of ME was thus, not helpful in the discrimination of cultivars.

Leucine aminopeptidase (LAP): Two loci were identified for leucine aminopeptidase (Fig. 3.6). This result contrasted with that of Gaur and Slinkard (1990) who found only one locus in chickpea using different lines than those tested herein. Two LAP loci have also been identified in avocado (Torres et al., 1978), cotton (Suiter, 1988), alfalfa (Quiros, 1983) and soybean (Kiang and Gorman, 1983). Only one banding pattern was observed for all entries for both loci. The activity of LAP-1 was not as clear as LAP-2 (Appendix 2: Fig. 3.6)

6-Phospho-glucose dehydrogenase (6-PGD): After staining the gel, two main areas of banding were found (Appendix 2: Fig.3.7). These two zones of activities were close to the anodal part of the gel. PGD-1 had an Rf value of 0.32 while PGD-2 had an Rf value of 0.35 (Fig. 3.9). Both 6-PGD-1 and 6-PGD -2 loci were invariant and seemed to be monomorphic. This result agrees with work by Kusmenoglou et al. (1992) who also found 2 loci for 6-PGD and both were monomorphic.

Glucose-phosphate dehydrogenase (GDH) : There was only one locus with an Rf value of 0.38 for GDH (Fig.3.8). The chickpea lines exhibited no polymorphism (Appendix 2: Fig. 3.8).

Alcohol dehydrogenase(ADH): ADH phenotypes were mono-banded in all cultivars tested (Fig. 3.9). ADH activity as revealed by the staining used was faint but reproducible. Two zone of alcohol dehydrogenase activity were observed on the cathodal portion of the Tris citrate/lithium borate gel and was easy to score. The anodal zone was very faint and difficult to score (didn't appear on Fig. 3.9, Appendix 2). Electrophoretic phenotypes were the same for all germplasm lines assayed.

Fumarase (FU) , alkaline phosphatase (ALPH) and β-Amylase (β-AMY): One zone of activity was seen for fumarase, alkaline phosphatase and β-amylase, after electrophoresis (Fig. 3.10, 3.11 and 3.12). β-amylase couldn't be photographed because the background of the gel was black and the bands appeared transparent. One banded phenotype for each entry was observed. The fumarase, amylase and alkaline phosphatase systems were monomorphic which failed to facilitate identification of chickpea lines (Appendix 2: Fig. 3.10 and Fig. 3.11).

Acid phosphatase (ACPH): The activity of this system was low but the result were consistent (Appendix 2: Fig. 3.11). This system displayed 3 variants (Fig. 3.13), within the 56 tested lines under conditions outlined herein. However, the resolution of this system was very low. Further investigation using seeds instead of leaves and more accessions might ascertain the potential variability and the resolution of this system in the future.

Carbonic anhydrase (CA): Most of the accessions tested exhibited a single band except for some lines which gave no bands for CA. However, this may be a problem of

staining or denaturation while thawing and freezing samples. One locus was identified for CA (Appendix 2: Fig. 3.12). Two consistent alleles were present for the lines studied (Fig. 3.14). This system might be used in association with other enzyme systems for chickpea identification if further test using diverse chickpea lines were assayed.

Propionate-esterase (PR-EST): The propionate esterases were multi banded and presented 4 loci (Fig.3.15). This multi banded system offered the possibility of use as a marker for chickpea. Four consistent variants were observed with some faint bands (Appendix 2: Fig. 3.12). Despite repeating this system many times clearer results were not obtained. Further investigation to improve the system is not warrented

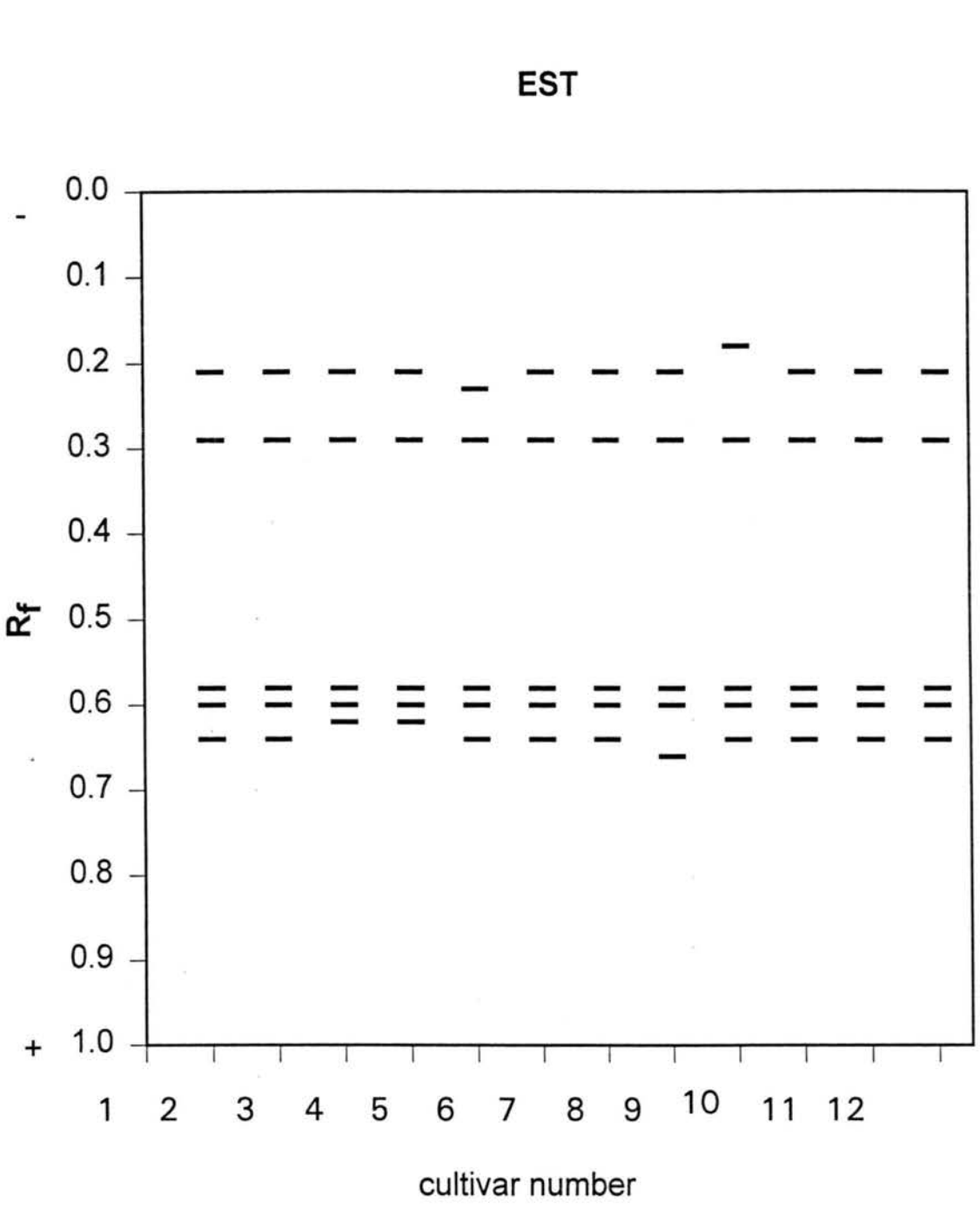


Fig. 3.1 Electrophoretic patterns of esterase isozymes of different germplasm lines of chickpea. Phenotypes from left to right: (1) F92-72C, (2) PCH 46, (3) ILC 72, (4) ILC 200, (5) F92-189C, (6) F92-112C, (7) F 92-139C, (8) ICC 4935, (9) RH79177, (10) S8579177, (11) ICC 4918.

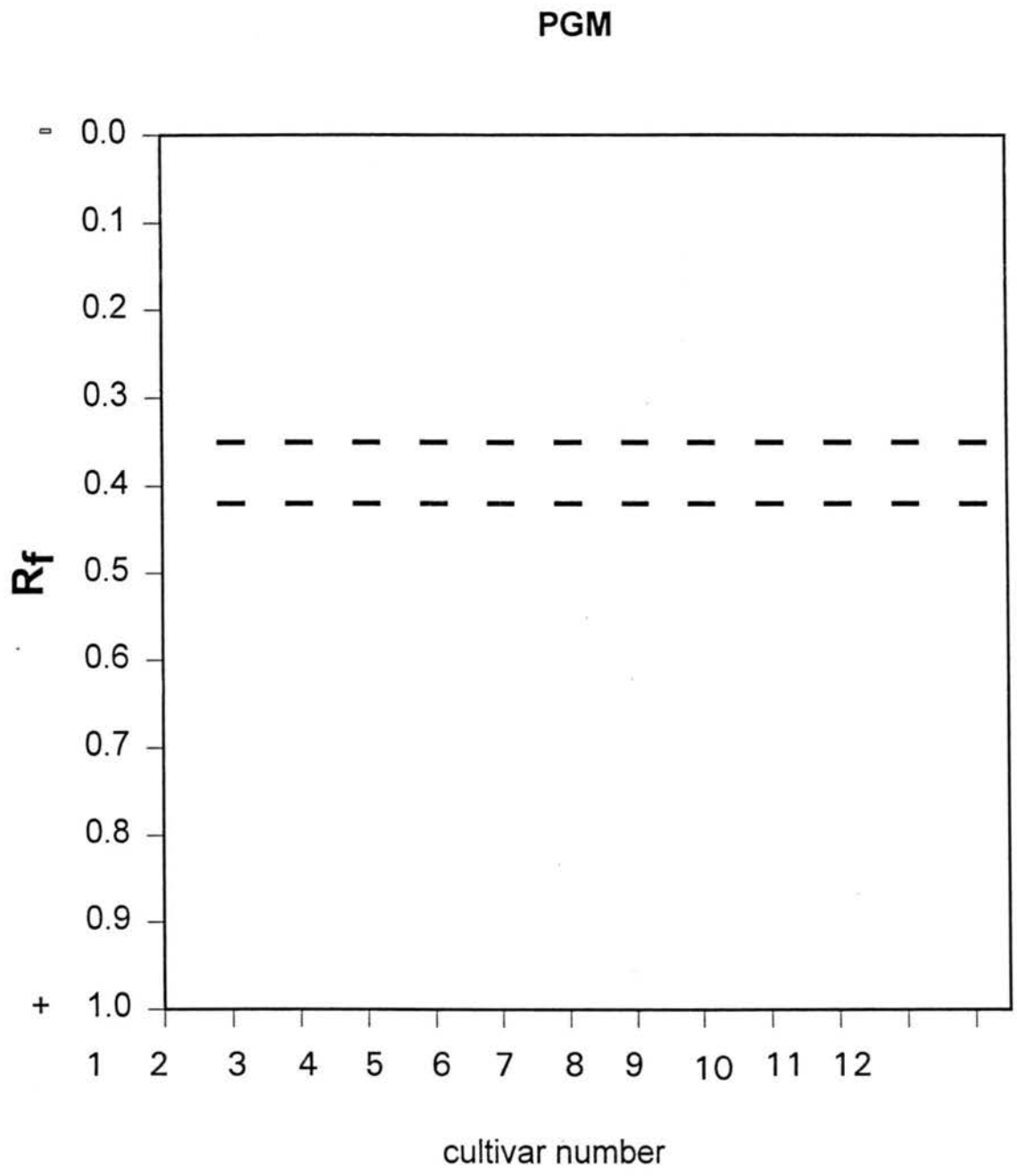


Fig. 3.2 Diagramatic patterns of phosphoglucose mutase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ILC 191, (4) F91-23C, (5) F83-92C, (6) F92-78C, (7) F84-152C, (8) F90-56C, (9) F91-14C, (10) F92-132, (11)F85-54C, (12)ICC8933.

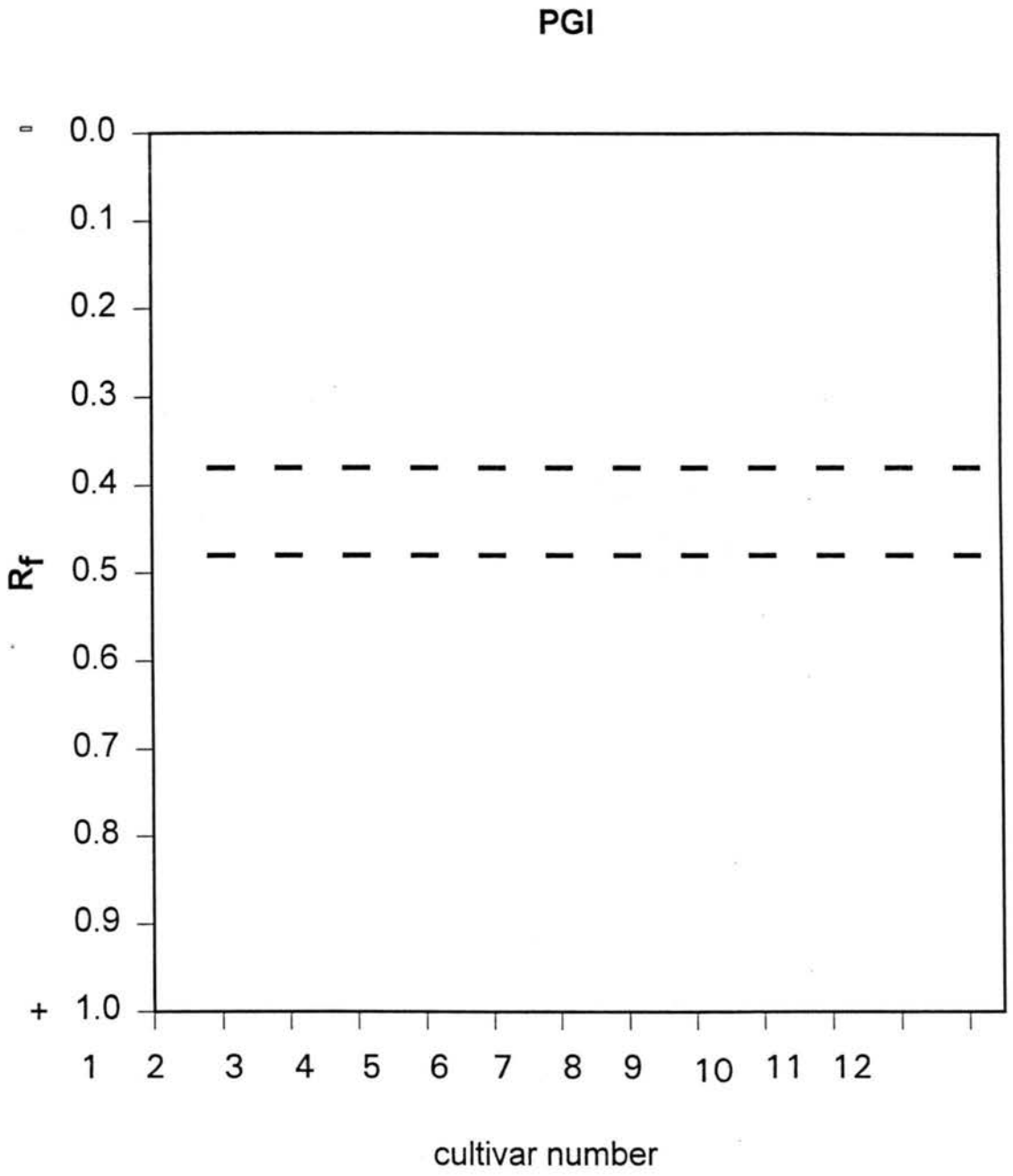


Fig. 3.3 Diagramatic patterns of phosphoglucose isomerase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) F83-77C, (3) ILC 200, (4) ILC 482, (5) ILC 195, (6) ICC 5003, (7) ICC322, (8) ILC 1929, (9) ILC183, (10) PCH37, (11)LOCAL, (12)F83-92.

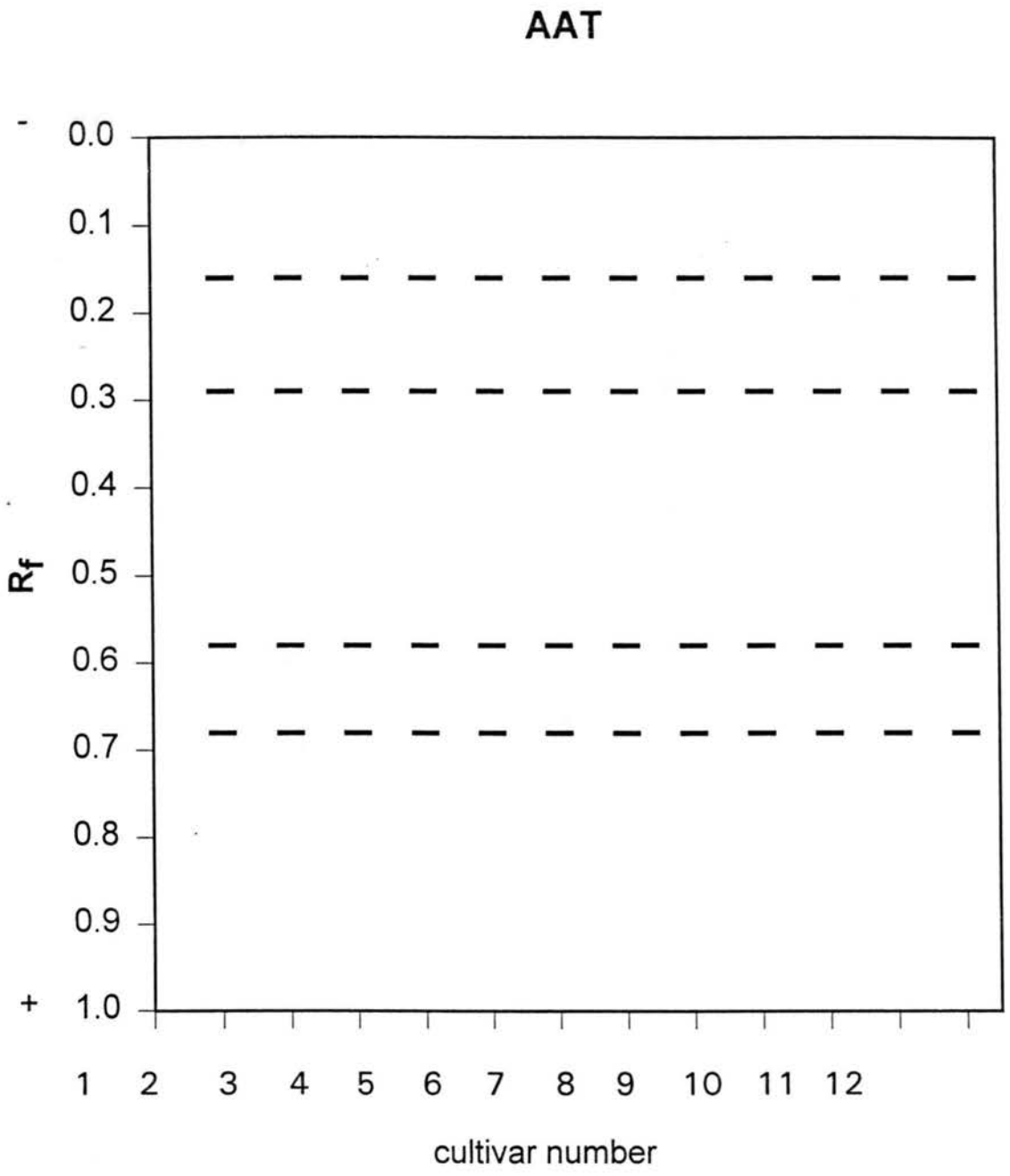


Fig. 3.4 Electrophoretic patterns of aspartate aminotransferase isozymes of different germplasm lines of chickpea. Phenotypes from left to right: (1) F88-83C, (2) F92-112C, (3) F91-8c, (4) ILC 195, (6) ILC 200, (7) ICC 6098, (8) ICC 322, (9) PCH 34, (10) PCH 37, (11) RH79177, (12) S8579177.

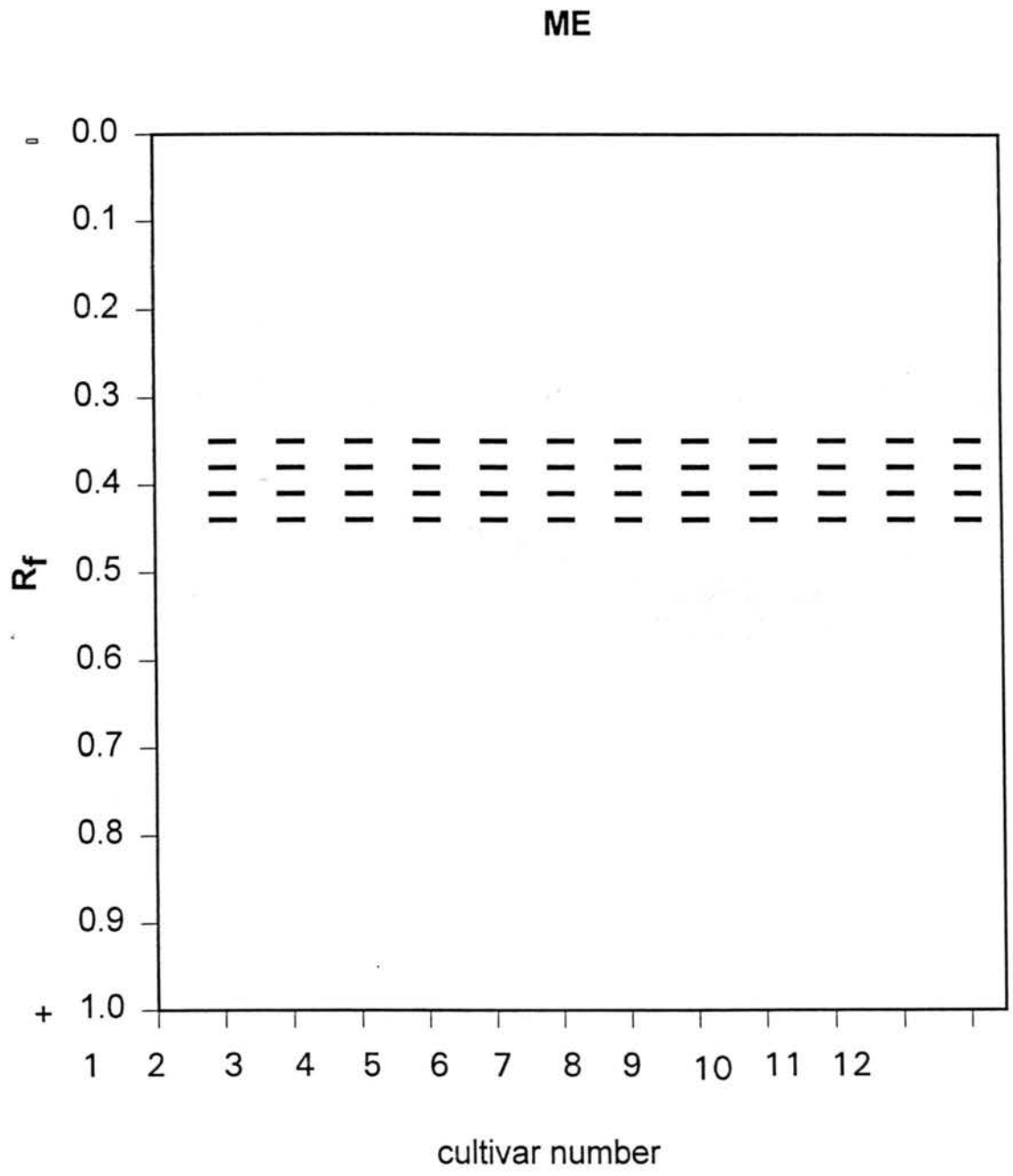


Fig. 3.5 Electrophoretic patterns of malic enzyme isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ILC 191, (4) F91-23C, (5) F83-92C, (6) F92-78C, (7) F84-152C, (8) F90-56C, (9) F91-14C, (10) F92-132, (11)F85-54C, (12)ICC8933.

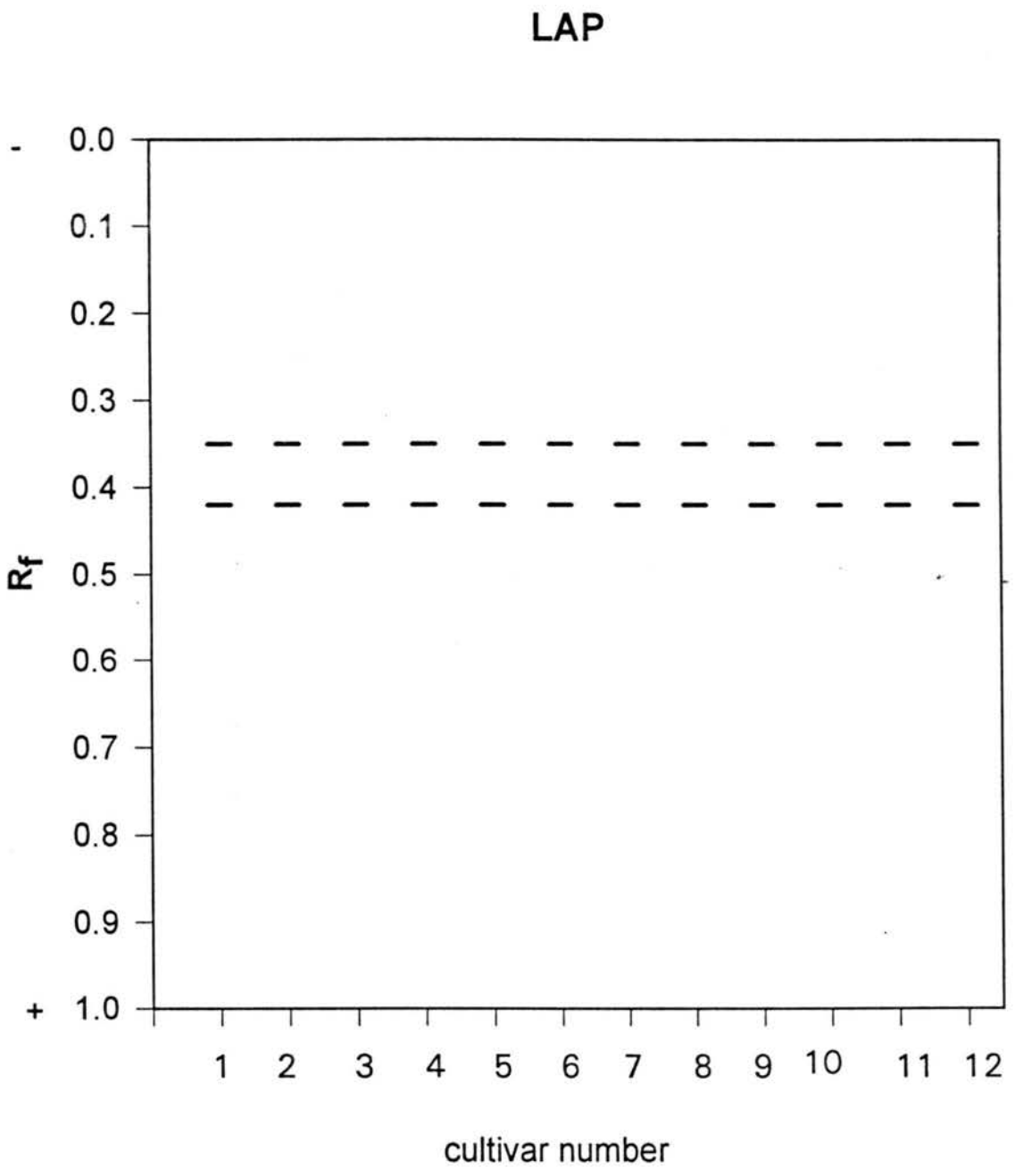


Fig. 3.6 Electrophoretic patterns of leucine aminopeptidase isozymes of different germplasm lines of chickpea. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ILC 191, (4) F91-23C , (6)F92-78C , (7) F84-152C, (8) F90-56C, (9) F91-14C, (10) F92-132C, (11)F85-54C, (12) ILC263.

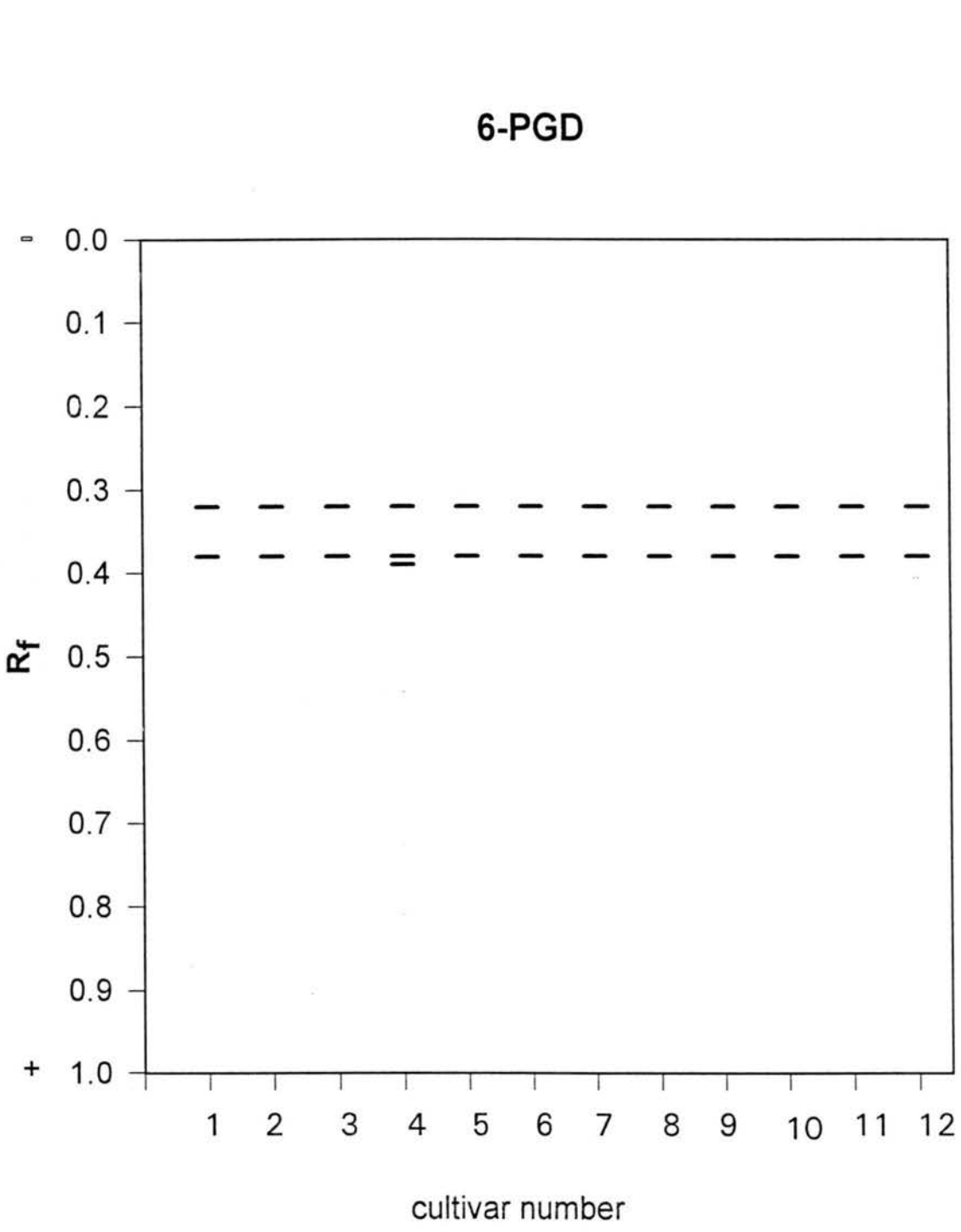


Fig. 3.7 Diagramatic patterns of 6-phosphoglucose dehydrogenas isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) ILC 200, (3) ICC 322, (4) ILC 3279, (5) PCH37, (6) RH79177, (7) F92-187C (8)F92-139,(9) ILC1929, (10) PCH34, (11)ILC72, (12)F83-92.

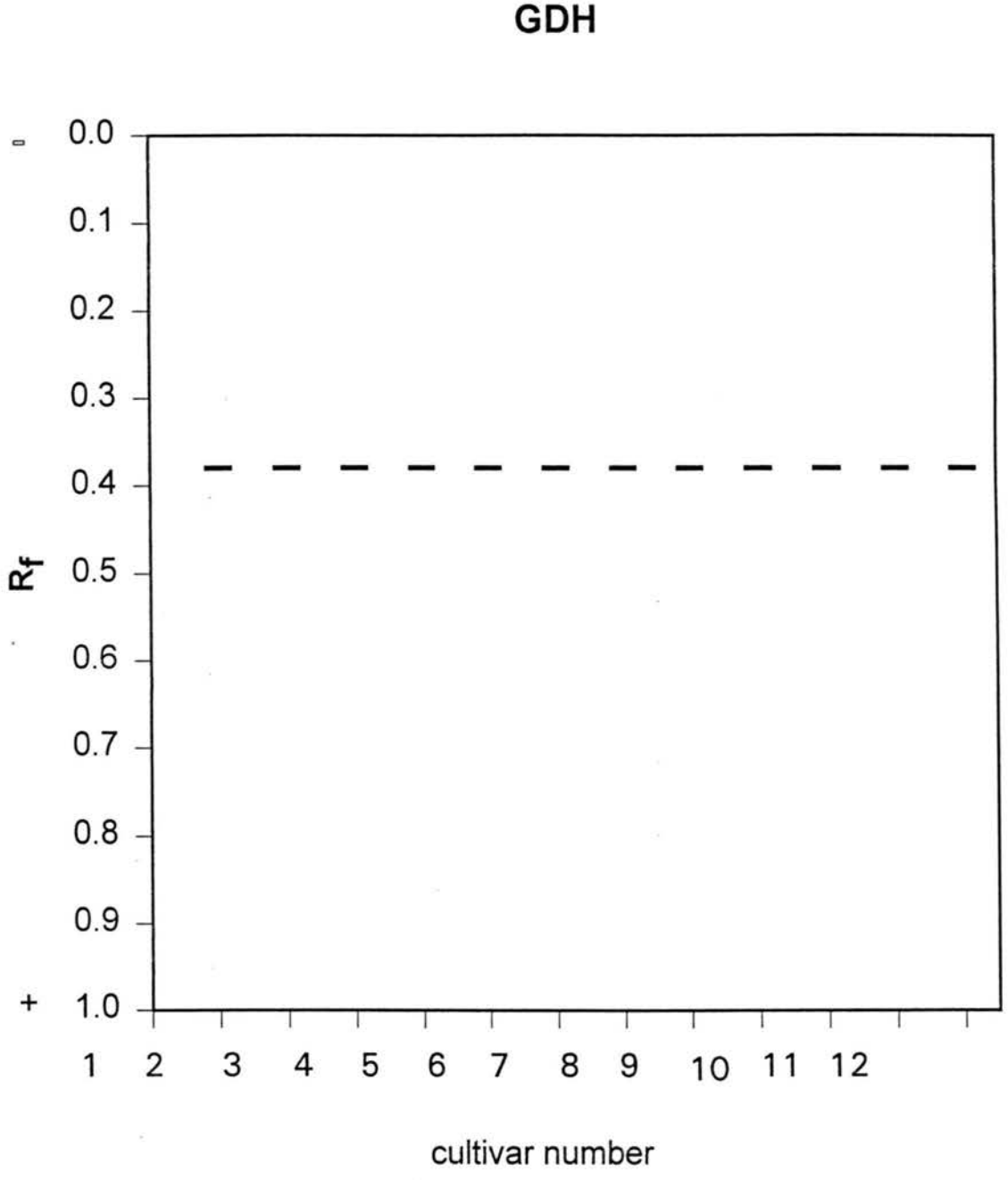


Fig. 3.8 Diagramatic patterns of glucose dehydrogenase emzyme iozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) F83-77C, (3) ILC 200, (4) ILC 482, (5) ILC 195, (6) ICC 5003, (7) ICC322, (8) ILC 1929, (9) ILC183, (10) PCH37, (11)LOCAL, (12)F83-92.

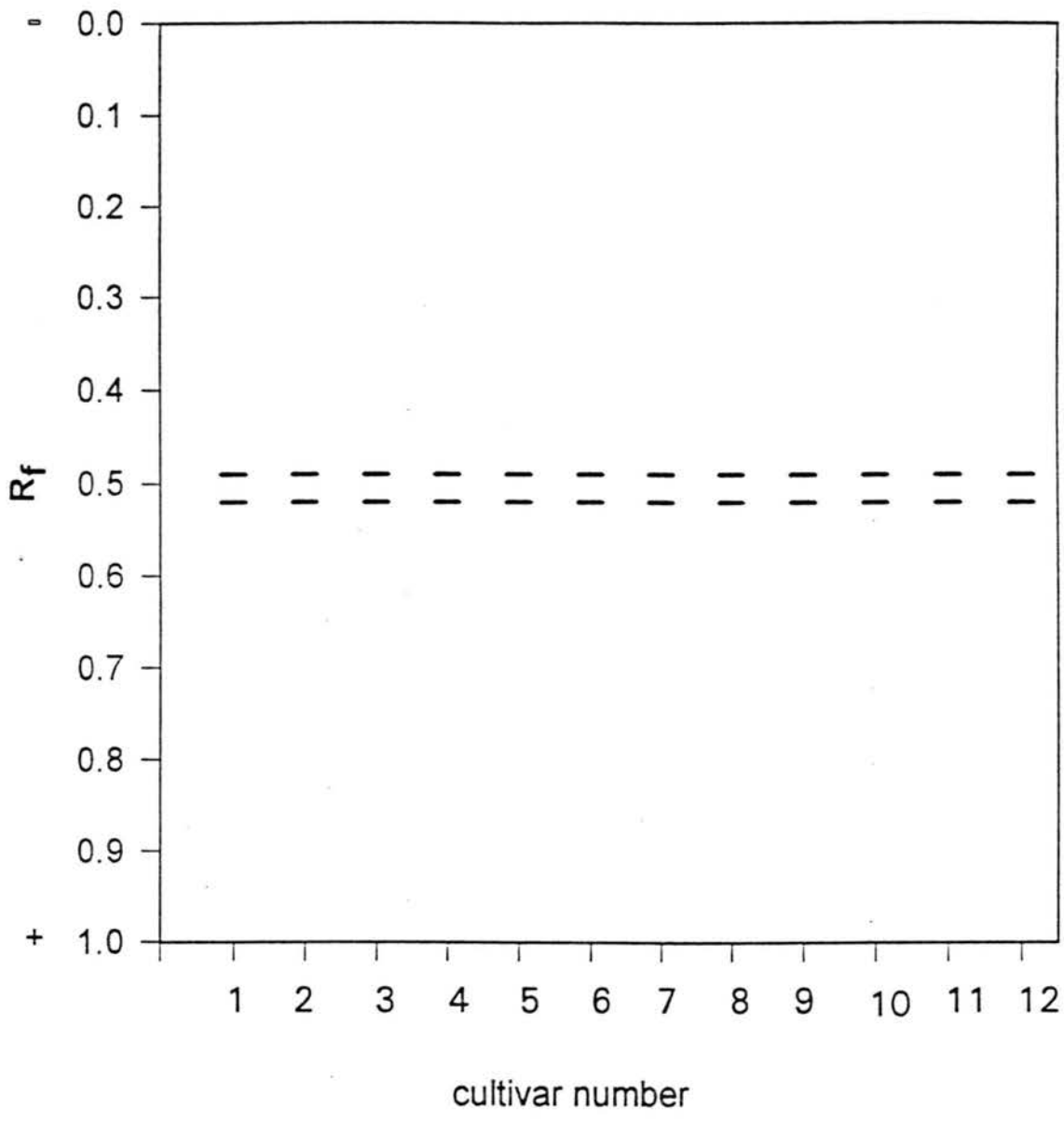


Fig. 3.9 Diagramatic patterns of alcohol dehydrogenase emzyme isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) F83-77C, (3) ILC 200, (4) ILC 482, (5) ILC 195, (6)ICC 5003, (7) ICC322, (8) ILC 1929, (9) ILC183, (10) PCH37, (11)LOCAL, (12)F83-92.

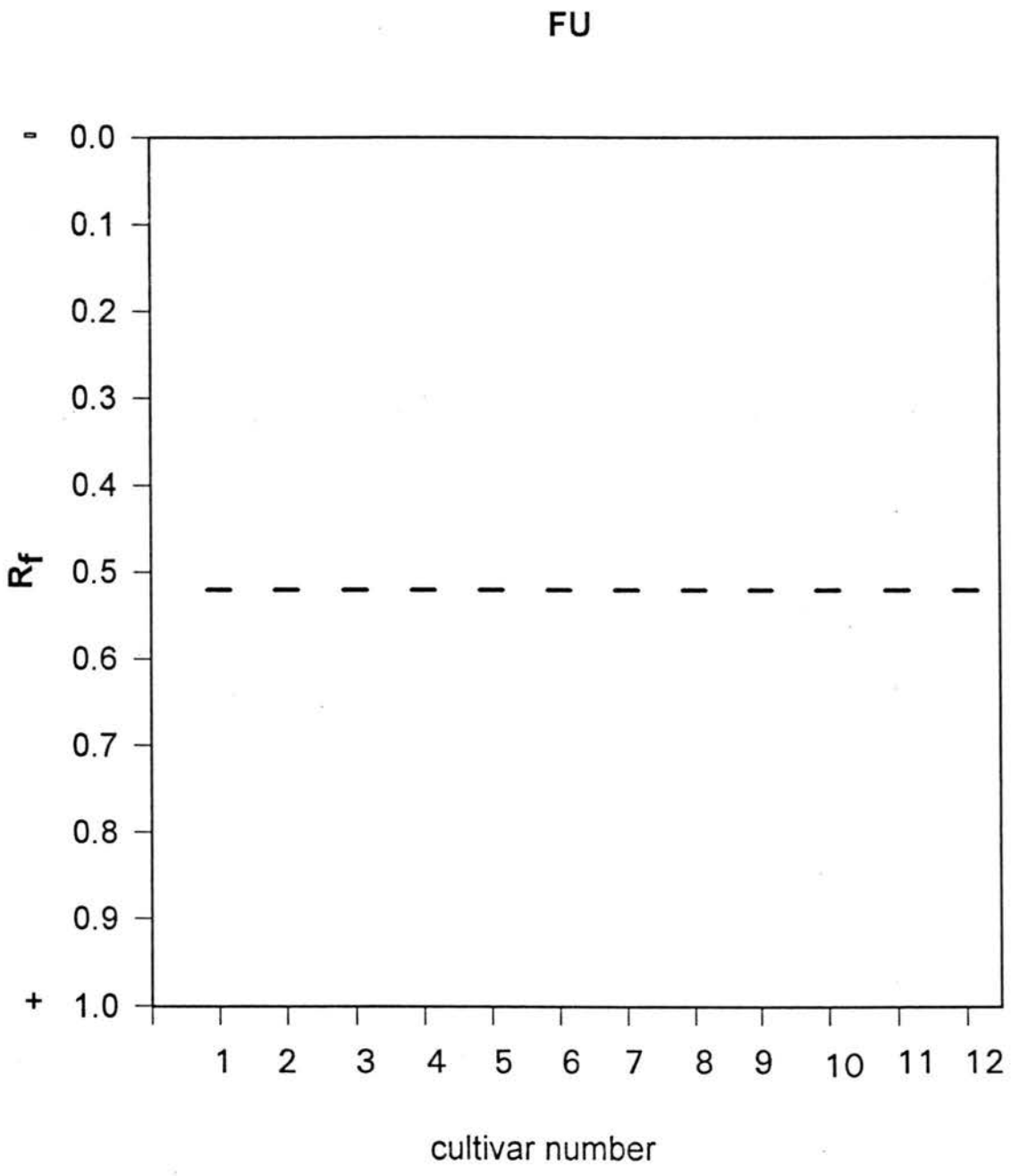


Fig. 3.10 Diagramatic patterns of fumarase emzyme iozymes of 12 chickpea lines (1) F92-72C, (2) F83-77C, (3) ILC 200, (4) ILC 482, (5) ILC 195, (6) ICC 5003, (7) ICC322, (8) ILC 1929, (9) ILC183, (10) PCH37, (11)LOCAL, (12)F83-92.

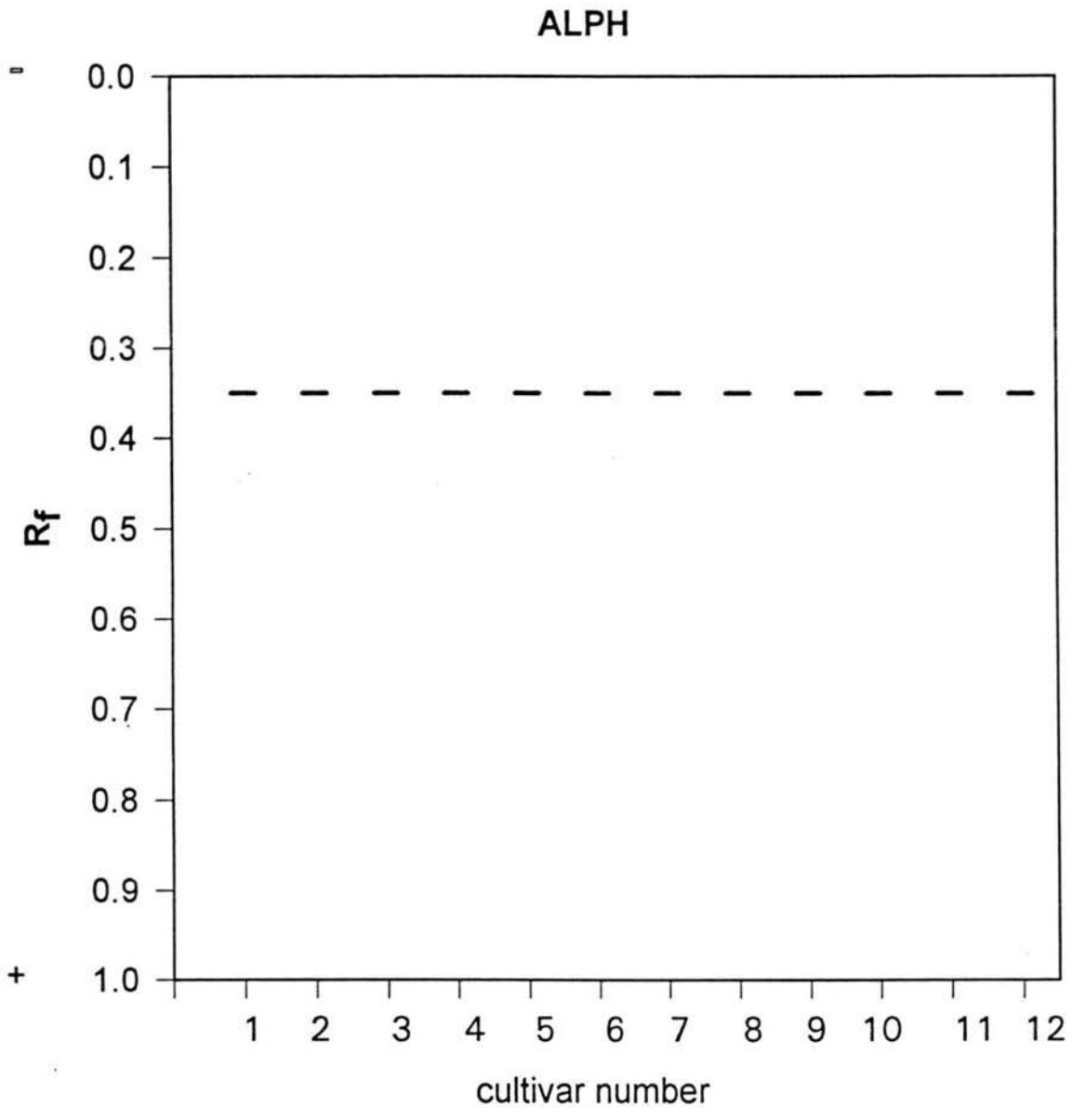


Fig. 3.11 Diagramatic patterns of alkaline phosphatase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) ILC 200, (3) ICC 322 (4) ILC 3279, (5) PCH 37, (6) RH 79177, (7) F92-187C, (8) F92-139C, (9) ILC1929, (10) PCH34, (11)ILC72, (12) ILC 191.

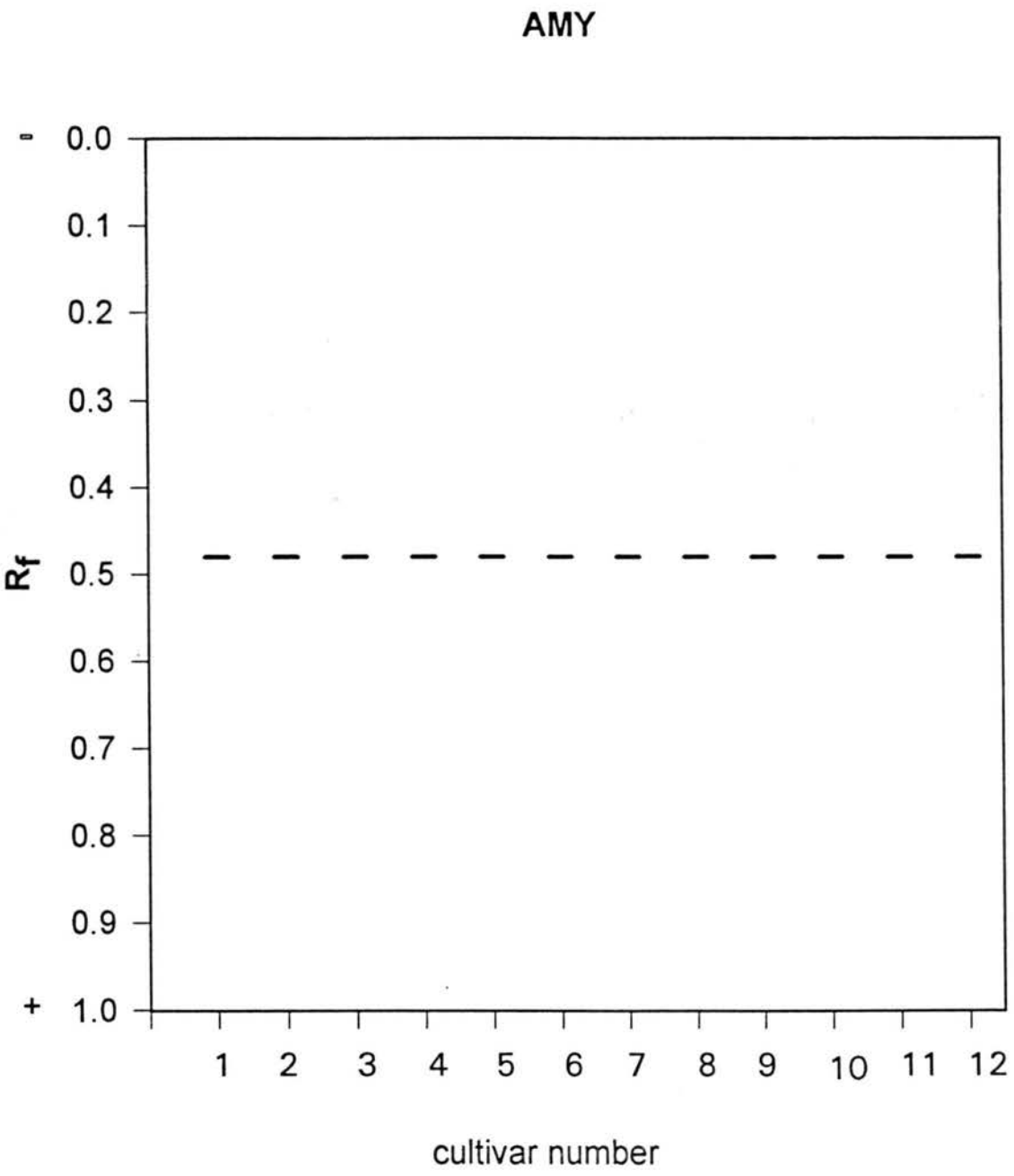


Fig. 3.12 Diagramatic patterns of amylase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) F83-77C, (3) ILC 200, (4) ILC 482, (5) ILC 195, (6) ICC 5003,(7) ICC322, (8) ILC 1929, (9) ILC183, (10) PCH37, (11)LOCAL,(12)F83-92.

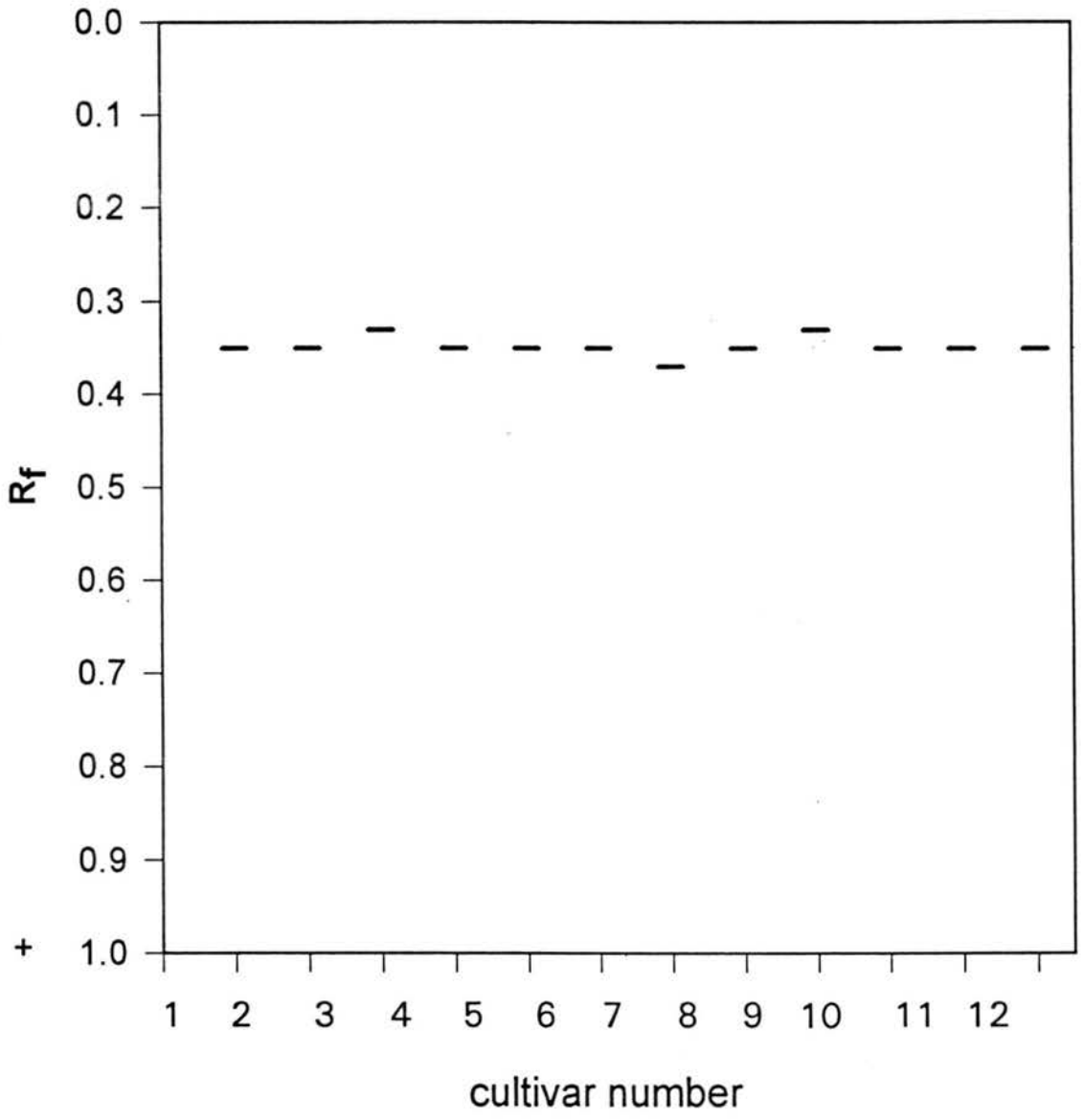


Fig. 3.13 Diagramatic patterns of acid phosphatase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F92-72C, (2) ILC 200, (3) ICC 322 (4) ILC 3279, (5) PCH 37, (6) RH 79177, (7) F92-187C, (8) F92-139C, (9) ILC1929, (10) PCH34, (11) ILC72 (12) F92-72C

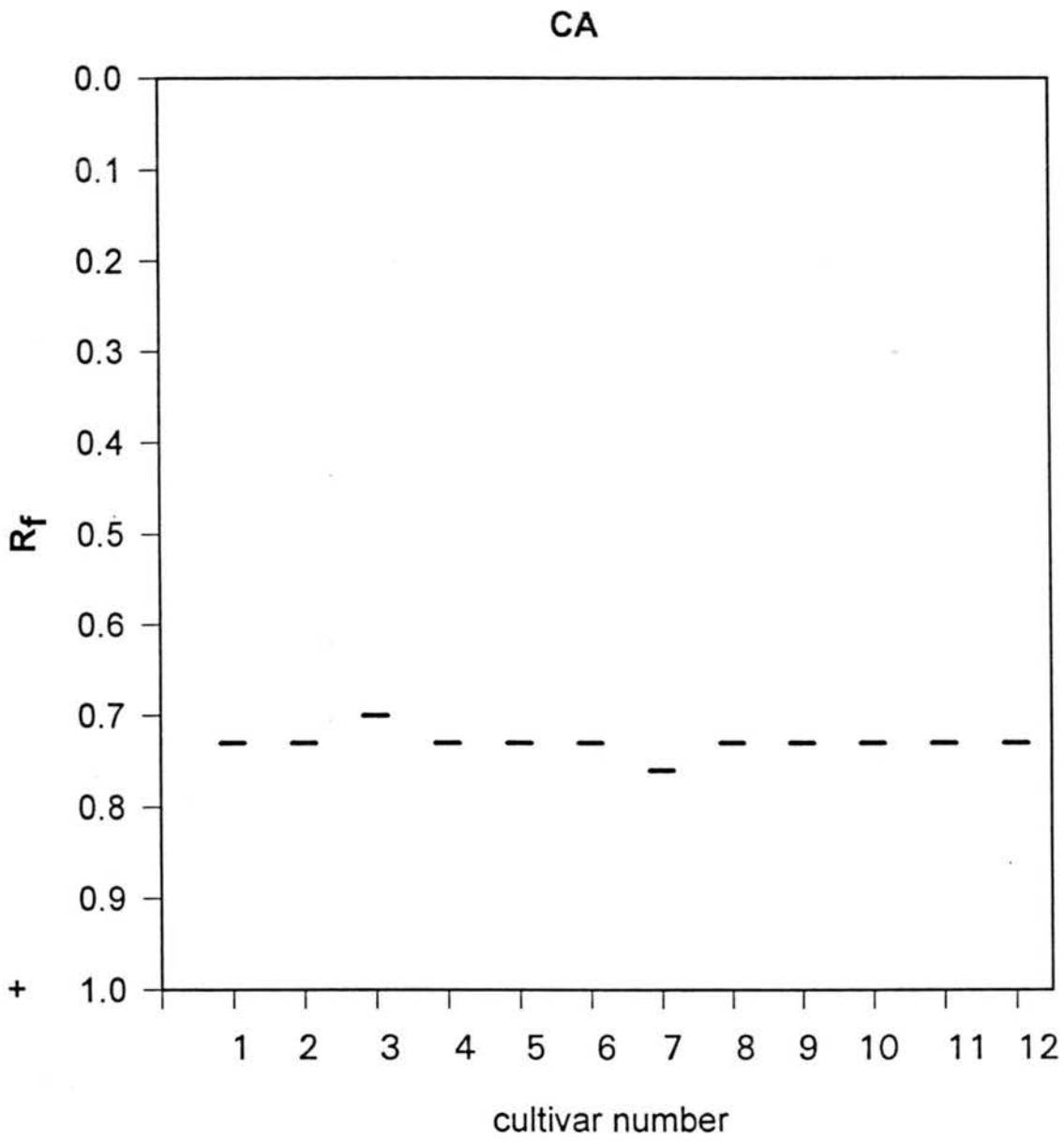


Fig. 3.14 Diagramatic patterns of carbonic anhydrase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ICC 322 (4) ILC 3279, (5) PCH 37, (6) RH 79177, (7) F92-187C, (8) F92-139C, (9) ILC1929, (10) F91-14C, (11)ILC72 and (12) F 85-54C.

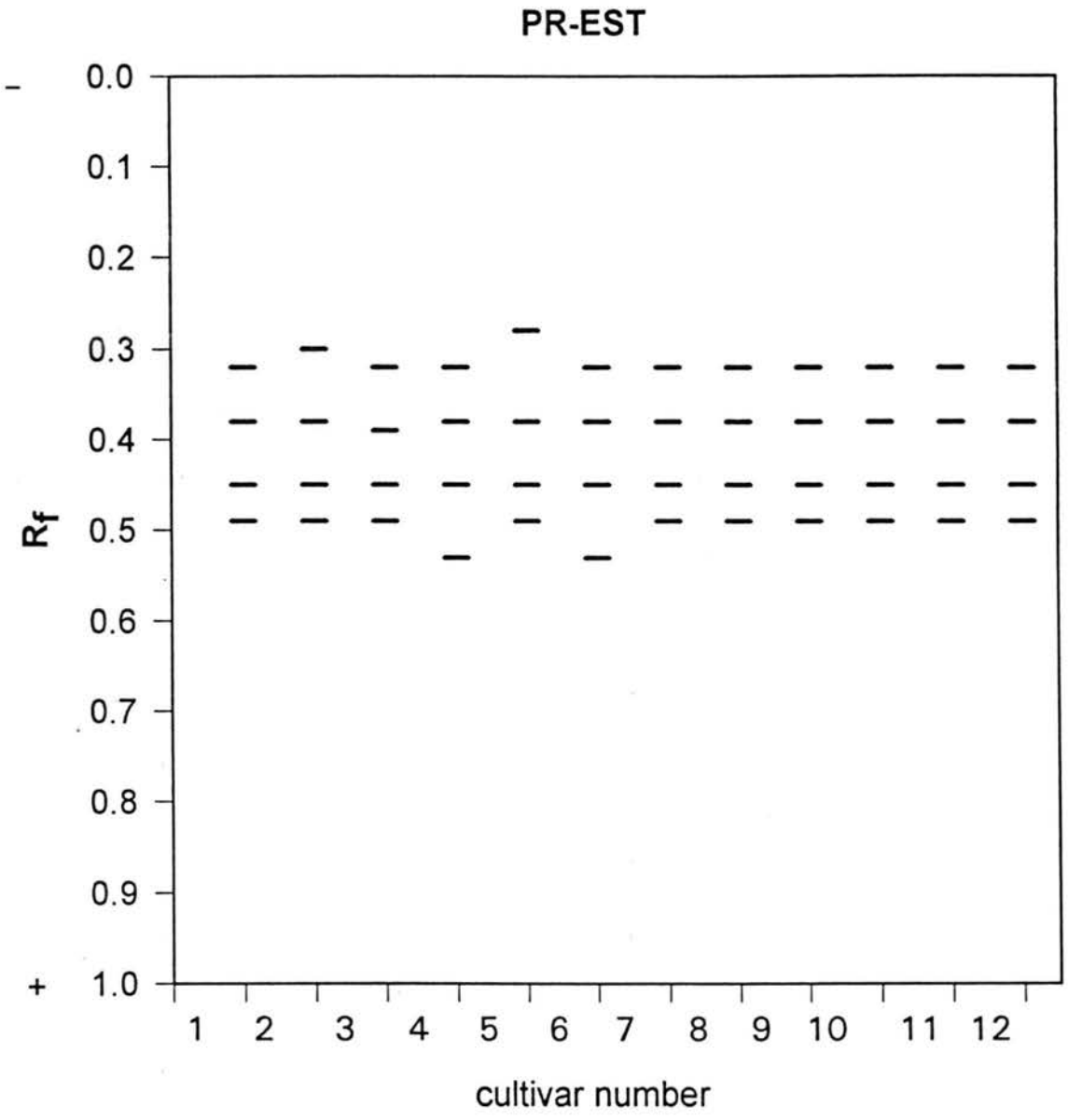


Fig. 3.15 Diagramatic patterns of propionate esterase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ICC 322 (4) ILC 3279, (5) PCH 37, (6) RH 79177, (7) F92-187C, (8) F92-139C, (9) ILC1929, (10) F91-14C, (11)ILC72 and (12) F 85-54C.

DISCUSSION

Very low polymorphism was observed within the 56 chickpea lines. This low variability encountered was not helpful in the differentiation of the resistant and susceptible lines.

Most enzyme systems yielded strong bands with low variation among lines. Some were found to have faint bands but still low polymorphism (malic enzyme, alcohol dehydrogenase, fumarase, gluconate dehydrogenase, aspartate amino-transferase). Thus, these systems presented little information in the identification of chickpea accessions. Since only leaves at one growth stage were used, the faint bands observed in some systems may be in part due to the stage of growth observed in the case of leucine amino peptidase and peroxidase in alstroemeria (Stephens, 1995). For both enzymes in Alstroemeria, clear bands were obtained in January and February but at other times only indistinct or no bands could be identified. This would suggest that there may be a strong influence in terms of time of year or growth stage effect on the expression of these enzymes. The weakness of the activity of these systems might also be related to the extraction technique or the components that affect migration such as voltage or gel concentration. Improved results may be obtained if other organs such as seeds were tested.

Some enzyme systems (PR-EST, and EST) yielded sometimes clear to indistinct bands which complicated gel reading and exhibited 4 to 5 loci for EST and PR-EST respectively in chickpea. A large number of esterase loci have been reported for other plant species. For potato, Desborough (1983) identified 5 loci for esterase and suggested that potato isozymes may be tetramers. For peppers, McLeod et al. (1983) observed 4 loci for esterase and demonstrated that the three anodal esterases were monomeric proteins, while the cathodal locus codes for a dimeric protein. And in tomato, seven loci have been identified for esterase which were a mixture of monomers and dimers with three loci confirmed as coding for dimeric isozymes and three for monomeric (Bernatsky and Tanksley, 1986).

The problem of low resolution of esterases may be due to the growth stage that effect the expression of these enzymes, as suggested in the case of alstromeria (Stephens, 1995). Since the objective of our study was to expand the number of enzyme systems used in

previous studies and most of the systems tested herein or in the litteraure yielded readable bands using 15 day-old plants we used the same stage for all the systems. The expression of these enzymes may have also been affected by the plant organ used for protein extraction. It has been demonstrated that many isozymes are tissue specific. Weeden (1984) observed that of 10 loci useful for distinguishing white seeded bean cultivars, only malic enzyme, rubisco, adenylate kinase, esterase and acid phosphatase exhibited differences in activity or presence of bands when different tissues were used. In another example, aconitase 1 and aconitase 3 were found to be present in *Annona* flowers only and not in leaf extracts (Ellstrand and Lee, 1987).

Esterase isozymes desplayed some variation (9%) within the 56 lines. This observable variation in chickpea esterases amongst lines was also reported by Kusmenoglou et al. (1992) as well as by Tuwafe et al. (1988).

Most of the enzyme systems tested were monomorphic and did not aid in the identification of markers linked to ascochyta blight gene of resistance.

According to these results, chickpea (*Cicer arietinum*) showed very low polymorphism among the 56 lines tested even though some of them originated from different geographic regions. This is surprising in view of the diverse environments of the tested lines, i.e. lines from Asia, Europe and Mediterranean countries. Some of these countries have grown chickpea for at least 7,000 years (Van der Meson, 1972). Furthermore, the presence of abundant genetic variation for other qualitative and quantitative traits (personnel observation, Muehlbauer and Singh, 1987) are evident. Other studies of chickpea revealed the same low level of polymorphism, Oram et al. (1987) studied chickpea isozyme variability using 27 loci in 20 cultivated chickpea accessions representing 11 countries of origin and concluded that chickpea was relatively poor in genetic variation as revealed by isozymes. Tuwafe et al. (1988) also surveyed isozyme variability as well using six enzyme systems (acid phosphatase, esterase, malate dehydrogenase, alcohol dehydrogenase, 6-phosphogluconate dehydrogenase and peroxidase) in 1,392 accessions of cultivated chickpea from 25 countries, and found polymorphism for only 4 loci. Gaur and Slinkar (1990) studied the genetics of 20 enzyme systems in chickpea and did not find any genetic variation for isozymes in *C.*

arietinum (AAT, 6-PGD, PGM, PGI, ADH and AMY were monomorphic) and consequently utilized interspecific hybrids of *C. arietinum* with *C. reticulatum* and *C. echinospermum* to study their genetics and linkage. Kusmenoglou et al. (1992) also reported low variability in the chickpea lines they tested. This again suggests a narrow genetic variability in the cultivated chickpea and indicates the limited variability present at isozyme loci. Therefore, the results obtained here are in general agreement with those obtained in the literature (Oram et al., 1987; Tuwafe et al., 1988; Gaur and Slinkar; 1990 and Kusmenoglou et al., 1992).

Recently, Tayar and Waines (1996) and Labdi et al. (1996) examined genetic variability among annual species of *Cicer* using isozymes and found a high level of polymorphism in 8 of 11 wild Cicer species. However, the cultigen (*Cicer arietinum*) showed only two loci (ADH and EST) which were polymorphic among 14 enzyme system used (Labdi et al., 1996). In contrast with this result, Ahmad et al. (1992) studied isozyme polymorphism of the genus *Cicer* of 8 wild species of Cicer with 25 accessions representing chickpea *Cicer arietinum* with 16 enzymatic systems and found no polymorphism at all in those accessions originating from six different geographic regions. The most striking feature of those data (Ahmad et al., 1992) was that the proportion of polymorphic loci of the *Cicer arietinum* was lower than the values obtained for the wild *Cicer* species (0.128 vs 0,09 and non existent respectively for the 8 wild *Cicer* species, the 56 lines tested herein and the 25 accessions tested by Ahmad et al., 1992). This difference between cultivated chickpea and wild progenitors may be interpreted as founder effect during chickpea domestication (Ladizinsky, 1985). Also, the low level of heterozygote observed in this study (1 plant (ILC 3279) out of 56 in the case of 6-PGD (Appendix 2: Fig. 3.11), was expected since *Cicer arietinum* is predominately self pollinated (Van der Mesen, 1972). Furthermore, out crossing has been estimated to be less than 1% (Singh, 1987).

Since one of the objectives of this study was to identify a marker linked to ascochyta blight resistance, sufficient polymorphism is a prerequisite to achieve this goal. Since only a low level of polymorphism was identified herein, no isozyme marker linked to ascochyta blight resistance genes could be observed. One approach to increase isozyme polymorphism might be through introgression of isozyme variants from *C. reticulatum* into *C. arietinum* .

This species exhibited some polymorphisms and intercrosses with *Cicer arietinum* to produce normal fertile progenies (Gaur and Slinkar, 1990).

Until more polymorphic isozyme loci are identified in cultivated chickpea, the application of isozyme markers in identification of resistance genes appears limited. The low genetic polymorphism as revealed by isozymes in this study and the previous ones appears to be due to the limited level of polymorphism detected by the isozyme technique as is true in other crop species (Gottlieb, 1981). Therefore, it may be not suitable for chickpea characterization.

CONCLUSION

Screening techniques are highly influenced by environmental conditions and inoculum density. If isozyme markers linked to ascochyta blight resistance were found, breeding populations could be screened in the absence of the fungus. Unfortunately, isozyme polymorphism in cultivated chickpea are limited. Four enzymatic systems (6-PGD, ACPH, EST and Est-PR) were polymorphic with about 9% polymorphic loci among the 34 loci revealed for the 15 enzymatic systems tested. In many cases of plant identification, isozyme bands or systems can be used just to facilitate the identification of cultivars and it is extremely rare that one band can identify a given variety. As with morphological characteristics, one must use at least two and possibly several criteria for positive identification. Wade (1976) reported that even though two grape cultivars were quite distinct morphologically, he could not separate them with four polymorphic enzymatic systems studied. Therefore, the enzymatic systems described may be of limited use in variety identification by acrylamide or starch gel electrophoresis.

This limited use due to the low polymorphism observed in chickpea lines tested may be explained by the close relatedness of germplasm lines tested since many of them are breeding lines and some of them have one common parent. However, the low variability encountered in some recent genetic studies on chickpea (*Cicer arietinum*) and other annual species of *Cicer* (Tayar and Waines, 1996 and Labdi et al., 1996) suggests that chickpea has a low genetic variability.

CHAPTER 4

CHARACTERIZATION OF CHICKPEA GERMPLASM LINES USING RAPD TECHNIQUE

INTRODUCTION

The Randomly Amplified Polymorphic DNA (RAPD) technique is a genetic assay that has been recently developed (Welsh and McClelland, 1990; Williams et al., 1990) and is based on the amplification of DNA with arbitrary primers in a polymerase chain reaction.

The method detects abundant polymorphism in most organisms which results from either insertion/deletion in the amplified regions or base changes that alter primer binding (Williams et al., 1990). This technique provides usually dominant markers because polymorphism is detected as presence or absence of the bands (Kesseli et al., 1992). Most often, it is not possible to determine whether an amplified segment is from a locus that is heterozygous or homozygous (Rafalski et al., 1991).

Recently, RAPD markers have been used in the characterization of germplasm of many species (Williams et al., 1990; Hu and Quiros, 1992; Demeke and Adams, 1994 and Brovkova et al., 1995) and in the identification of markers linked to disease resistance (Michelmore et al., 1991).

In chickpea (*Cicer arietinum* L.) there are several hundred accessions that lack a precise description. This research and that of other authors (Tuwafe et al., 1988; Gaur and Slinkard, 1990 and Kusmenoglou et al., 1992) have shown isozymes were inadequate for chickpea characterization. Therefore, the need to use a molecular approach based on DNA analysis to solve this problem is justified.

This research reports results of RAPDs as a technique for differentiating chickpea germplasm lines with the intent of identifying markers associated with ascochyta blight. RAPD with acrylamide gel was also tested on some relevant accessions

MATERIALS AND METHODS

The RAPD assay we employed used 200 decamer primers to screen the 56 chickpea lines. The reproducibility of RAPD markers was examined by re-amplifying and rescoring the lines for the polymorphic primers. Only those primers giving repeatable banding patterns were considered used for analyses.

DNA extraction

DNA extraction procedure # 1: A total of 56 chickpea cultivars (Table 2.1) obtained from ICARDA, Syria which were screened for ascochyta blight at the INRA center of semi-arid crops in Morocco were examined. These plants were grown in the greenhouse at Colorado State University under natural day length with temperature of 27 ± 3°C day time and 20±3 °C night time. Upper young leaf samples were collected from two weeks old plants, labeled and placed into plastic bags and kept in ice until grinding. DNA was extracted following the protocol of CTAB procedure reported by Murray and Thompson (1980). Two grams of fresh tissue were ground to fine powder in the mortar and pestle using liquid nitrogen. The powder was then added to 10 ml of preheated (60°C) 2% CTAB buffer (Table 4.1) with occasional mixing and incubated in a water bath (60°C) for 30 minutes. Ten ml of chloroform/isoamyl alcohol (24:1) were added to the solution and mixed gently for 10 minutes. The solution was centrifuged at 4000rpm (1900g) for 15 minutes. The aqueous phase was transferred with a sterile Pasteur pipette into another cortex tube to which 75% volume of cold isopropyl alcohol (7.5 to 8ml) were added to the supernatant. This was mixed gently to precipitate the nucleic acids and the solution was incubated in a freezer for 30 minutes. DNA was then hooked or the solution was centrifuged at 4000rpm for 5 minutes to pellet DNA. DNA was washed in 10ml of 70% ethanol and allowed to dry for 30min in a vacuum or if the DNA was a pellet, the solution (70% ethanol and DNA) was again centrifuged at 4000 rpm for 5 minutes and the supernatant was decanted followed by drying the pellet for 10 minutes in a vacuum with storage in the freezer (-20C) until further

use. Samples of DNA were dissolved in 500ml of TE buffer (Table 4.1) and stored at -20°C. The DNA was then quantified using the spectrophotometer. Dilutions (1:30) were made for reading on the spectrophotometer (Beckman DU 640).

DNA extraction procedure # 2: This second procedure developed by Scott Reid (personal communication) has been used in the Department of Horticulture for buffalo grass and alstromeria and was adapted to chickpea to see if improvement in purity of the DNA was possible. The first steps cited above, from the CTAB extraction of DNA to its dissolution in tris-EDTA buffer were the same for both procedures. This second procedure required 2µl of 10mg/ml RNAse stock to create 50µg/ml with incubation for 60 minutes at 37°C to clean the sample of RNA. Two µl of 10mg/ml proteinase (final concentration of 50µg/ml) were added to the solution followed by incubation for 20 minutes at 37°C. A 1/10 volume of 3M sodium acetate buffer (Table 4.1) was added to the solution and mixed gently. Two volumes of 95% ethanol were added to precipitate DNA and the solution was mixed by inversion. To allow DNA to precipitate, the solution was stored at -20°C for 30 minutes. The samples were then centrifuged at 10,000 rpm for 15 minutes at 4°C to pellet the DNA. DNA pellets were then washed with 70% ethanol and allowed to air dry. Samples were dissolved in 500ml of TE buffer (Table 4.1) and stored at -20°C.

The DNA was then quantified using the spectrophotometer. Dilutions (1:30) were made for reading on the spectrophotometer (Beckman DU 640).

Spectrophotometric determination of nucleic acids

For quantifying the amount of DNA present in each sample, readings were taken at wavelengths of 260 nm which allowed the calculation of nucleic acid concentration in the sample. An OD of 1 corresponds to approximately 50µg/ml for doubled stranded DNA, or 40µg/ml for single stranded DNA and RNA or 20µg/ml for single stranded oligonucleotides. The ratio between the reading at 260 nm and 280 nm (OD260/OD280) provides an estimate of the purity of the nucleic acid relative to protein only. Pure preparations of DNA have OD260/OD280 values between 1.86 and 1.91. If there is contamination with protein or phenols, the OD260/OD280 will be significantly different from the values given above, and accurate quantification of the amount of nucleic acids will not be possible (Sambrook et al.,1989).

Table 4. 1. Buffers used for the extraction and storage of DNA

Buffer	1L	0.5L
1. CTAB Extraction Buffer.		
2% CTAB	20g	10g
Tris HCL 100mM pH 8.0	100ml of 1M tris HCL	50ml of 1M tris HCL
1.4M NaCl	81.816g	40.908g
20mM EDTA pH 8.0	40ml of 0.5M EDTA	20ml of 0.5M EDTA
Optional: 1%PVP-40	10g	5g
add 2-mercaptoethanol at 0.1-1.0% just pior to use		
2. 0.5M EDTA pH8.0		
Na_2EDTA	186.1g	93.05
H_20	800ml	400ml
Adjust pH to 8.0 with NaOH pellets(about 20g pellets/L). Adjust volume and autoclave		
3. Tris EDTA (TE)		
10mM Tris pH 7.6 (or 8.0)	10ml of 1M tris pH 8.0	5ml of 1M tris pH 8.0
1mM EDTA pH 8.0	2ml of 0.5M EDTA pH 8	1ml of 0.5M EDTA
H_2O	988ml	494ml
4.Tris low EDTA (TLE)		
10mM Tris pH 7.6	10ml of 1M tris pH 7.6	5ml of 1m tris pH 7.6
0.1mM EDTA pH 8.0	0.2ml of 0.5M EDTA pH 8.0	0.1ml of 0.5M EDTA
5. 3M Sodium Acetate pH 5.2 and 6.8		
$NaOAc.3H_20$	408.1g	204.5
H_2O	800 ml	400ml
Adjust pH with acetic acid, then adjust volume to 1L or to 0.5L		
6. 1M Tris pH 8.0	500ml	250ml
Tris base	60.55g	30.275g
H_2O	400ml	200ml
HCL(concentrated)	21ml	10.5ml
7. 1M tris pH 7.6		
Tris base	60.55g	30.275g
H_2O	400ml	200ml
HCL (concentrated)	30ml	15ml

RAPD primers

The ten base long primers used (sets 4 and 5) in this study were obtained from The University of British Colombia, protein unit service. Primers and their nucleotide sequences are listed in Appendix 3, Table 4.2.

The dried primers (10 µg) were reconstituted in 200 µl TLE (Tris Low EDTA- 10 mM Tris, 0.1 mM EDTA) (Table 4.1).

Template DNA

Template DNAs were diluted to 12.5 ng /µl to provide a final concentration of 1.0 ng

DNA / µl reaction mix or 25ng/ reaction mix. DNA's were in normal TE buffer (Table 4.1) and working dilutions of DNA were made with sterilized water.

The enzyme Taq

The enzyme Taq DNA Polymerase was obtained from *Thermos aquaticus* strain YT(1). Each PCR-reaction contained 0.25 units of this enzyme (2μl / sample).

DNA ladder

The DNA ladder, a 1KB molecular marker (Life Technology) was used. The concentration of DNA ladder used was 250 µg/ 250 µl. Ten ml of a solution of 1µl DNA ladder, 19.5µl filtred sterile water and 19.5µl gel loading buffer (10ml H_2O +3ml glycerol + 0.25g bromophenol blue) were used in the gel.

RAPD reactions

RAPD reactions used dATP, dCTP, dGTP, and dTTP, as well as a purchased 10XPCR buffer solution containing 1.5 mM magnesium chloride (Perkin- Elmer), 25mM $MgCl_2$, 10 mer primer, 25 ng genomic DNA, and 0.25 units of Taq DNA polymerase (Perkin Elmer) per reaction. The volumes were performed as indicated in Table 4.3. A master mix was made including all components needed for 31 samples except the template DNA, and Taq DNA polymerase which were added at the end. The solution was mixed gently then spun down in a microcentrifuge for 10 min at 1000 rpm to homogenize the solution. An aliquot of the master mix (21µl) was transferred to 0.5ml PCR tubes and the appropriate template DNA (2 µl) was added to each tube. These mixes were overlaid with 50 µl mineral oil (3 to 5 drops with Pasteur pipette), gently mixed, centrifuged briefly, and placed in Perkin

Elmer/Cetus DNA Thermal Cycler 480. Tubes were heated to 95°C for 5 minutes and 2µl of Taq were added to each tube (0.25 units) and tubes were centrifuged before replacing in the thermal cycler. The amplification was carried out according to the following program: 7 minutes at 94°C and 45 cycles of 94°C 1min; 36°C 1min; 72°C for 2 min and 5 min at 72°C then storage at 4°C. In the first stage of each cycle, the complementary strands of the DNA molecules were separated by heating the mixture to 94°C for one minute. The temperature was then lowered rapidly to 36°C and held for two minutes whereupon the 10-mer synthetic DNA primer strands bind to complementary sequences on the genomic DNA. Finally, the temperature was increased to 72°C for two minutes for the elongation stage.

The temperature and time values given herein have been found to be optimal for eucalyptus and buffalo grass in the Department of Horticulture laboratory (Colorado State University), and also optimal for some grain legumes such as peas and lentil (Agronomy laboratory, Washington State University). These values were also optimal for chickpea when tested for the first time at the Department of Agronomy laboratory at Washington State University.

Table. 4.3 Reaction mix components

Addition order	Components per reaction mix	Volume for master mix (µl)	Final concentration
1	Sterile deionized water (13.5µl)	418.5	
2	Buffer (2.5µl)	77.5	1x
3	DNTP's		
	A (0.25µl)	7.5	200µM
	T (0.25µl)	7.5	200µM
	C (0.25µl)	7.5	200µM
	G (0.25µl)	7.5	200µM
4	$Mgcl_2$(2µl)	62	5mM
5	Primer (2µl)	62	0.2µM

Preparation of agarose gel

The ends of a perplex gel mold were sealed with tape. Seventy eight ml of 1xTris-acetate-EDTA (TAE) buffer (Table 4.1) were added to 1.248g of agarose (Gibco electrophoresis grade agarose) for a 1.6% gel. The solution was then heated in the microwave for 10 minutes and cooled to 60°C. The gel was poured into the tray and air bubbles were removed by using a Pasteur pipette. A comb was then inserted into the gel. When the gel was completely set, the comb and tape were removed and the gel with the tray were placed into the electrophoresis tank. One liter of 1xTAE buffer was poured into the electrophoresis tank until covering the gel to a depth of 3 to 5 mm.

The PCR reactions were mixed with 6 µl of Sigma Gel Loading Buffer (10x), which contains 0.05% (W/V) bromophenol blue, 40% (W/V) sucrose, 0.1M EDTA (pH 8.0), and 0.5% (W/V) sodium lauryl sulfate. Reactions were then centrifuged and a 15 µl aliquot was loaded into the previously prepared agarose gel. DNA samples were loaded into gel wells slowly to prevent spill over to adjacent wells. The first lane was loaded with a 10 µl DNA ladder for sizing DNA fragments. The lid of the electrophoresis tank was then closed and the

electrical leads were connected to the power system, a 5000V microprocessor (Buchler) The cathode end (black) was at the point of DNA sample application. The power supply was programmed for a 50 V (constant) for 3 hr 30 mn.

Agarose gel staining

The gel was removed from the electrophoresis tray and stained by immersing it into a solution of ethidium bromide (15 µl ethidium bromide in 300 ml distilled water) for 30 minutes. The gel was then destained in distilled water for 10 minutes. After that, the gel was viewed on a UV transilluminator and photographed. Each band that was produced was assigned a band number and the fragment size was visually estimated and data recorded for statistical analysis.

Bulked DNA samples for analysis

Two bulks of DNA were made from extracted DNA of different lines of chickpea tested with one pool of resistant DNA lines (F9272C, F92139C, F9278C, ILC 200, ILC 5924 and ILC 72) and one pool of susceptible DNA lines (F8487C, ILC 482, PCH 37, ILC3279, S85027 and F9218C). The same DNA volume and concentration were used to mix the DNA of each line used in the bulks.

RAPD with acrylamide gel procedure

RAPD with acrylamide gel procedure followed herein was that of Antolin et al. (1996).

Plant material analyzed: Twelve chickpea lines were tested . Six resistant lines (F92-72C, F92-139C, F92-78C, ILC 200, ILC 5924 and ILC 72) and six susceptible (F84-87C, ILC 482, PCH 37, ILC3279, S85027 and F92-18C).

DNA isolation: DNA was isolated according to procedure #2 as described in section four and was resuspended in 200µl TE (10Mm Tris-Hcl, 1mM EDTA, pH 8.0).

PCR amplification: Primer sequences used are listed in Table 4.2. PCR reactions were done following the protocol described in section four.

Gel electrophoresis: Large gels (38 X 50cm) were poured and run with the Bio-Rad Sequigen system. Shark tooth combs were used to form wells for loading. Two glass plates, the solid plate and the notched one were used to pour a gel. The outer glass (solid) plate was

treated with bind silane (2-methacryloxy-propyl-trimethoxysilane, Sigma Chemical Co.) to adhere the gel to the plate for staining. The adhesive glass plate was thoroughly scrubbed with an abrasive plastic pad and grease cutting dish washing soap. It was then rinsed with tap water followed by another distilled water rinse and dried with paper tissues. The plate was sprayed with 95% ethanol (EtOH) and cleaned with tissue paper. Bind silane (5µl) was mixed in 1ml of 95% EtOH and 0.5% glacial acetic acid (v/v). This was poured onto tissue paper and wiped across the surface of the adhesive glass plate. After 5min the plate was again sprayed with 95% EtOH and cleaned with tissue paper. This last step was repeated three times. The latter washes remove excess bind silane that could dissolve in the gel and transfer to the opposite plate. While treating the adhesive plate, it was important not to handle the opposite, untreated glass plate, with the same gloves because the bind silane could be easily transferred. Gels were extremely difficult to remove intact if the non-adhesive plate became contaminated with bind silane. The opposite plate was washed in the same manner as described above for the adhesive plate (wash with a soft sponge and grease cutting soap, rinsed in dH_20 followed by three times with 70% EtOH and then treated twice with 1 ml Sigmacote (Sigma Chemical Co.) with tissue paper prior to each use.

In order to make 100ml of gel solution, 16.7ml of 30% acrylamide was mixed with 20 ml of 5x TBE (54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA (pH 8.0)to make 1 liter), 58.3 ml of distilled water and 5ml of glycerol. The acrylamide solution consisted of 29.4g acrylamide and 0.6g N,N'-methylenebisacrylamide dissolved in 100 ml dH_2O. Immediately prior to pouring the gel, the gel solution was filtered through two layers of filter paper (Whatman #1). An ammonium persulfate (100µl of a 25%(w/v) solution per 100 ml gel solution) and TEMED (100µl per 100ml gel solution) were then added and the gel was poured. Following polymerization the wells were washed out with 1XTBE. To each 25µl PCR reaction, 7.5 µl loading buffer(10 mM NAOH, 95%formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) was added. The tube was tapped to mix the contents followed by a spun down. From this solution, 5-6 µl was loaded directly onto the SSCP gel.

Electrophoresis conditions: Electrophoresis was performed at room temperature (20-26°C) for 18 hours with a constant voltage of 350V and amperage of 14mA and 5W. The gel apparatus was set up with 1xTBE in the lower buffer chamber and 1.5xTBE in upper

buffer chamber. Each chamber used 1L of solution.

Silver staining of gels: Following electrophoresis, the glass plate with the adhered gel was removed and placed in 10% acetic acid in a large plastic tray and placed on a rotary shaker for 20min. The gel was stored in a fixative solution(2% GAA [glacial acetic acid] in 2L dH_20) before proceeding to the next step. The fixative was saved for later use in terminating the development reaction. The gel was rinsed three times in dH20 with 2 min of agitation per wash. Fresh dH_2O was used in each wash. The gel was transferred to a stain containing 0.15% (w/v) silver nitrate and 0.15% (w/v) of a 37% formaldehyde in dH_2O and agitated for 30 min. The gel was removed from the staining solution, drained and rinsed for 10s in dH_2O. The gel was then placed in developing solution and agitated. The developer was a 3% sodium carbonate solution which was chilled to approximately 10°C and then immediately prior to placing the gel in the developer, 37% formaldehyde and sodium thiosulphate were added to concentrations of 0.15% and 0.0002% respectively. As soon as bands became clearly resolved, the fixative solution from the first step was poured directly into the developer and agitated for 3 min. The gel was then rinsed twice in dH_20 and the glass plate with gel was allowed to dry..

Data analysis

Cluster analysis: The PCR products (bands) amplified by each primer were scored. Presence of a band (fragment) was scored as a 1 and its absence as 0. The Statistica program was used for cluster analyses. The program performed percent match (%M) analyses on the 56 cultivars. %M=Nab/Nt, where Nab is the total number of matches(i.e., both bands absence or presence) and Nt is the total number of fragments scored. The percent match method counts both the shared presence and the shared absence of a band as a match. Matrices based on the percent match were transformed into dendrograms using unweighted pair-group method (UPGMA) (Sneath and Soakal, 1973). Cluster analysis was done by the software STATISTICA.

Principal Component: Principal component analysis is used to explain observed similarities or dissimilarities (distances between investigated lines or % of matches between lines). These similarities are expressed in the correlation matrix. Multidimensional scaling using matrix based on RAPD bands (41 bands scored in the 56 chickpea lines) was used. This analysis was performed using Statistica software.

Spearman correlation coefficient: Spearman correlation coefficient is a measure of association between two variables. This association is derived from the ranks of these variables. It is therefore a nonparametric measure which doesn't assume a normal distribution. It assumes only that the values within 2 variables can be ranked.

For RAPD data versus reaction to ascochyta blight each value of bands and disease score was replaced by their ranks (0 or 1). The reaction to ascochyta blight is assumed 1 when the line is resistant or 0 when it is susceptible and RAPD bands were ranked 1 when the band is present and 0 when it is absent. SAS software was used for this analysis.

RESULTS

After testing several concentrations of $MgCl_2$ (1.0, 1.5, 2.0 and 2.5µl /sample) , template DNA (12.5, 25, 50, 100ng /reaction mix) and Taq polymerase (0.25 and 0.75 units per reaction), the optimum reaction mixture which gave clear bands for some primers was 2µl $MgCl_2$, 25ng of template DNA and 0.25 unit of Taq polymerase per reaction. These values gave consistent results for some primers with clear and readable bands. Other primers gave only faint bands which might have been related to the primer itself which may not have true homology with the DNA template.

Quality and quantity of DNA extracted

DNA quantification and quality was determined by spectrophotometry. Most samples tested had sufficient amount and quality of DNA (Fig. 4.1). For example, the DNA spectra indicated high purity for lines 28 and 25 (OD260/280 of 1.91 and 1.89 respectively for 25 and 28 (Table 4.3)). Lines 26, 27 and 29 showed excessive contamination or inpurity as indicated by their spectra and their ratio of OD260/280 (1.94, 1.81 and 1.8 respectively (Fig. 4.1 and Table 4.3).

Most of the samples had ratios of OD260/280 between the values of 1.73 and 2.03. Since a ratio below 1.86 shows contamination with protein and a ratio above 1.91 shows contamination with RNA, the majority of DNA's presented an OD260/280 between 1.87 and 1.91 appeared to be of high quality. The quantity of DNA of the accessions tested are listed in Table 4.3. These results demonstrated the relative high yield of DNA from the samples using very young leaves (15 days old). However, the extraction method # 1 resulted in smears on some gels. This problem may be related to DNA degradation. The second extraction made using procedure #2 outlined above improved slightly this result (less smears on the gels) while the occurrence of faint bands for some primers persisted. This may be related to the ratio between primer and DNA template, the concentration of magnesium chloride used, temperatures in thermocycler and also dNTP's concentrations (Balinger-Crabtree et al., 1990; Black, 1993; Black et al., 1992).

Further optimization for RAPD are unnecessary for chickpea since it has been demonstrated that the level of polymorphism was very low even for those primers that yielded bands. However, other new techniques need to be tested to clarify this low variability of this

species found at DNA level especially the Single Stand Conforation Polymorphism (SSCP) and oligonucleotide fingerprintings.

Level of polymorphism

Two hundred decamer-primers were tested for the 56 chickpea lines. Only 30% of the primers yielded bands with only 15% producing scorable bands while 15% produced very faint bands. The remaining 70% failed to produce any observable bands. This has occured in other species for example *Pheseolus vulgaris* where only 142 primers out of 400 yielded readable discrete bands (Skroch et al., 1992)

Most of the primers yielding scorable bands produced monomorphic patterns (75% of the 15%) which were not useful in the identification of chickpea (primer 489 for example, Fig. 4.2). Amplified DNA fragments (bands) of different sizes and numbers were produced by several primers amplifying chickpea DNA (Fig. 4.3 to 4.7). The number of amplified DNA fragments produced by the primers ranged from 1 as illustrated by primer 440 (Fig. 4.9) to 10 bands produced by primer 489 (Fig.4.2). The fragment size produced by most of the primers ranged from 150 to 1180 Bps. A total of 41 fragments with sizes ranging from 150 to 1180 base-pairs were scored for each of the 56 accessions. Primers were not able to distinguish among all chickpea lines as observed in Fig.4.3 through Fig. 4.7. The six primers yielding polymorphism could differentiated among some lines but not all of them. Only a few bands were polymorphic for the six polymorphic primers (2 for primer 429 to 5 polymorphic bands for primer 428 with an average of 3.3 polymorphic bands per primer, Table 4.4).

A total of 19 polymorphic DNA fragments was produced by primers 402, 415 , 428, 429, 430 and 469 (Table 4.4). Primers 402, 415, 428, 469 and 430 differentiated among several lines relatively more clearly than 429 since they provided greater numbers of polymorphic bands (3 to 5 versus 2 only for 429).

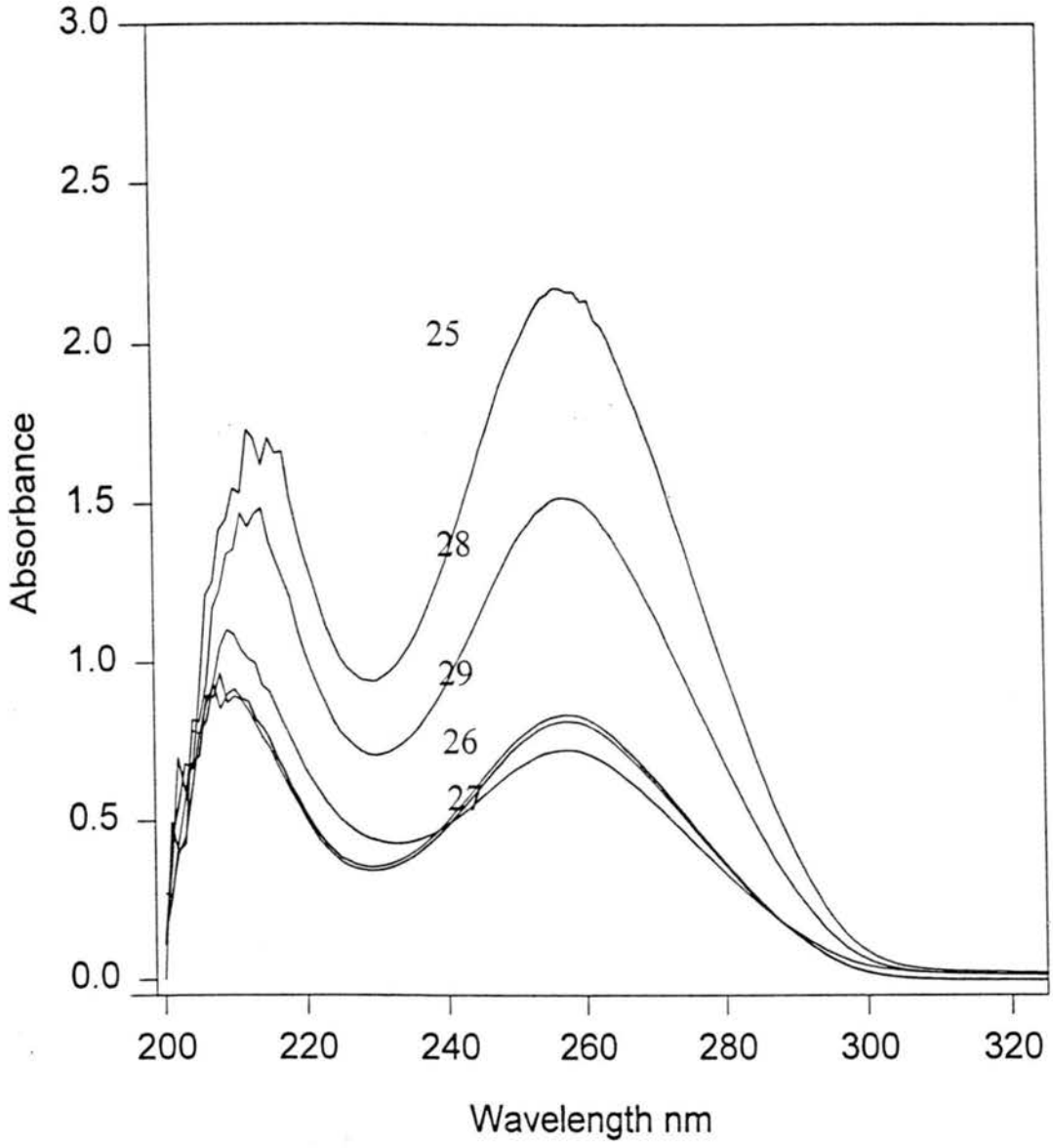


Fig. 4.1 DNA sample absorption spectra of 5 chickpea lines 25 (ILC 195), 26 (ILC 482), 27 (ILC 5924), 28 (ILC 181) and 29 (PI 456883)

Table 4.4. Number of RAPD amplification products generated with 6 oligonucleotide primers in chickpea.

Primer*	Total no. Of RAPD products/primer	Number of polymorphic bands/primer
402	7	3
415	6	3
428	9	5
429	7	2
430	7	3
469	5	3
Total	41	19

* Primers are from set 5 of the University of British Colombia

Bulked DNA analysis

Bulked DNA analysis was performed to determine if RAPD markers were associated with resistant lines. For this objective, two kinds of pooled DNA were used. The first two bulks were made of 2µl of DNA from each of 3 resistant lines (F92-72C, ILC 200 and F92-139) and from 3 susceptible lines (PCH 34, Pch 46 and Local). A second set of two bulks, resistant and susceptible were composed of 2µl of each DNA of six resistant lines (F92-72C, ILC 200, F92-139, F92-78C, ILC 5924 and ILC 72) and six susceptible lines (PCH 34, Pch 46, Local, F90-56C, ILC482 and ILC 195).

A total of one hundred and twenty ten-mer primers were tested against bulked samples set 1 (composed of only 3 resistant or 3 susceptible DNA) and set 2 (composed of 6 resistant or six susceptible DNA). Sixty-eight per cent of the primers tested yielded no amplification. Of the remaining primers which yielded amplified bands, 31% showed one or

two bands, 37% showed three or four bands, and the remaining 32% yielded 5 or more amplified bands (Appendix 3: Table 4.4).

The fragment sizes ranged from an estimated 600 bp to 3,500 bp with the majority falling in the 800-2000 bp range.

Primers that yielded bands didn't show clear differences between the resistant bulked DNA and the susceptible bulk (Fig. 4.8 and 4.9). In general, RAPD patterns for both bulks were similar except for some primers which produced patterns missing one or two bands in one of the two DNA bulks (note primer 388 and 389in Fig. 4.8). Other primers produced bands from resistant bulks and no bands at all in the susceptible bulks as in the cases of primer 438,440, 450 and 456 (Fig. 4.9). These primers were tested for the second time but they failed to yield the same results. These bands would be a potential markers for resistance to ascochyta blight in chickpea if further research demonstrated repeatability. As tested they could not be useful as markers for resistance to ascochyta blight.

RAPD procedure using acrylamide gel and silver staining

The six polymorphic primers noted in the initial RAPD study, were tested against 6 resistant lines (F92-72C, ILC 200, ILC72, F92-139C, ILC 5924 and F92-78) and six susceptible (F84-87C, ILC 482, PCH 37, ILC 3279, S85027 and F92-18C) using the PCR products separated on large acrylamide gels for 18 hours and silver stained according to the procedure outlined above. RAPD's with acrylamide appeared to separate the bands better (Antolin et al., 1996). More polymorphisms were revealed using polyacrylamide gel relative to the agarose gel. An average of 5.6 polymorphic bands per primer were observed using this procedure versus 3.3 using RAPD on agarose (Table 4.6). This is normal since the RAPD products can be separated better on large acrylamide gels since the procedure allows greater time (18 hours vs 3h 30min for agarose).

Table 4.6 . Number of amplification products generated with 6 primers using RAPD with acrylamide gel.

Primer	Total no. Of RAPD products/primer	Number of polymorphic bands/primer
402	9	6
415	8	7
428	13	8
429	7	5
430	8	5
469	8	3
Total	5 3	3 4

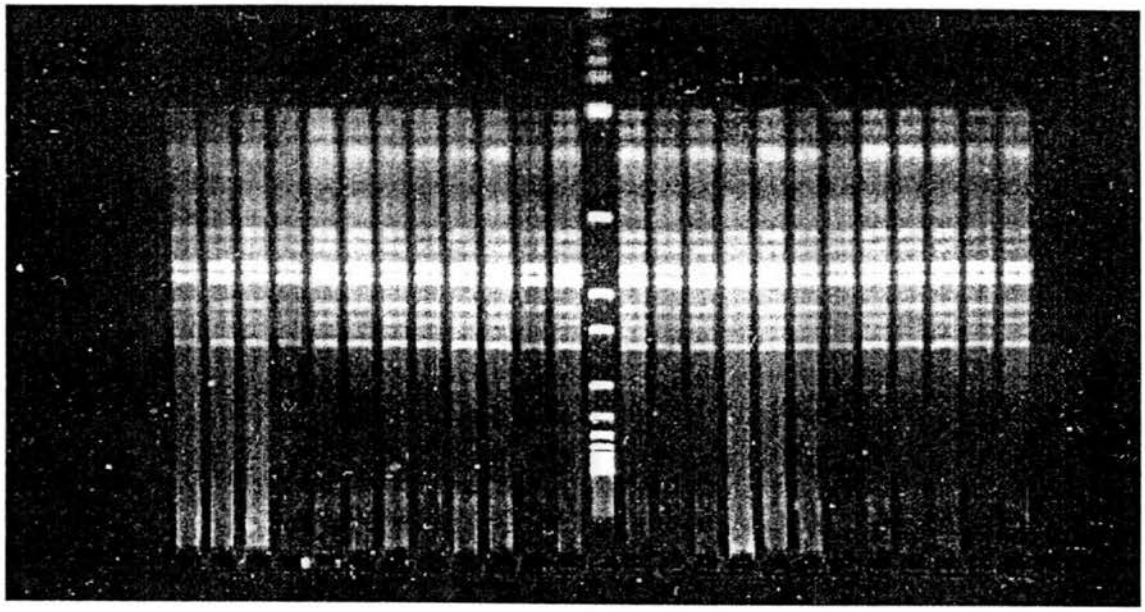


Fig. 4.2 RAPD profiles generated by primer 489 with 28 chickpea lines. The lane in the middle is the DNA ladder.

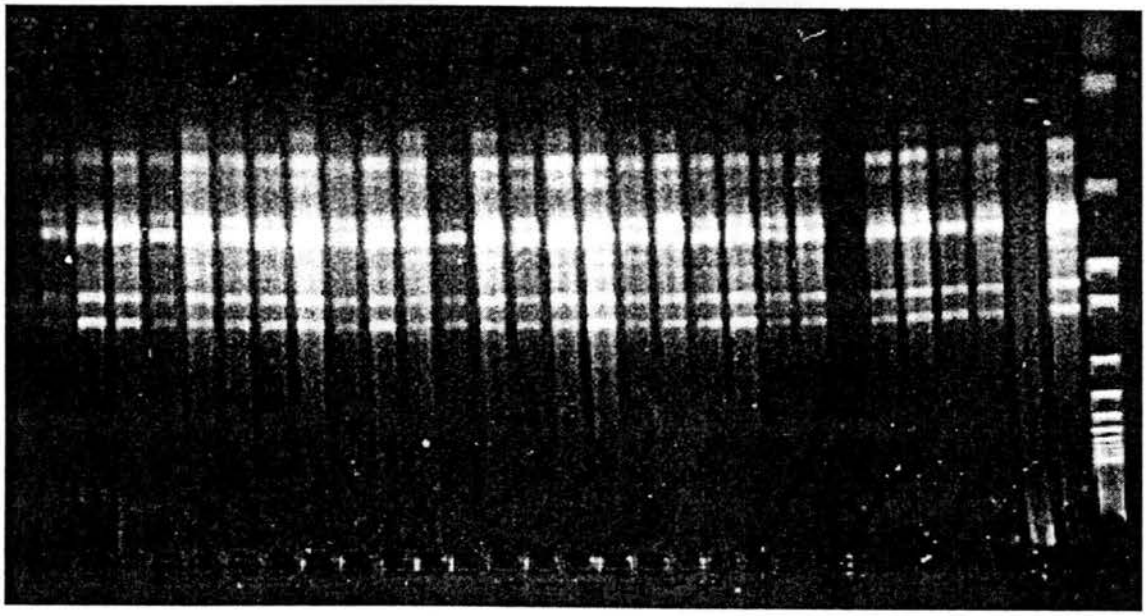


Fig. 4.3 RAPD Patterns generated by primer 429 with 29 chickpea lines. The first lane on the right is the DNA ladder.

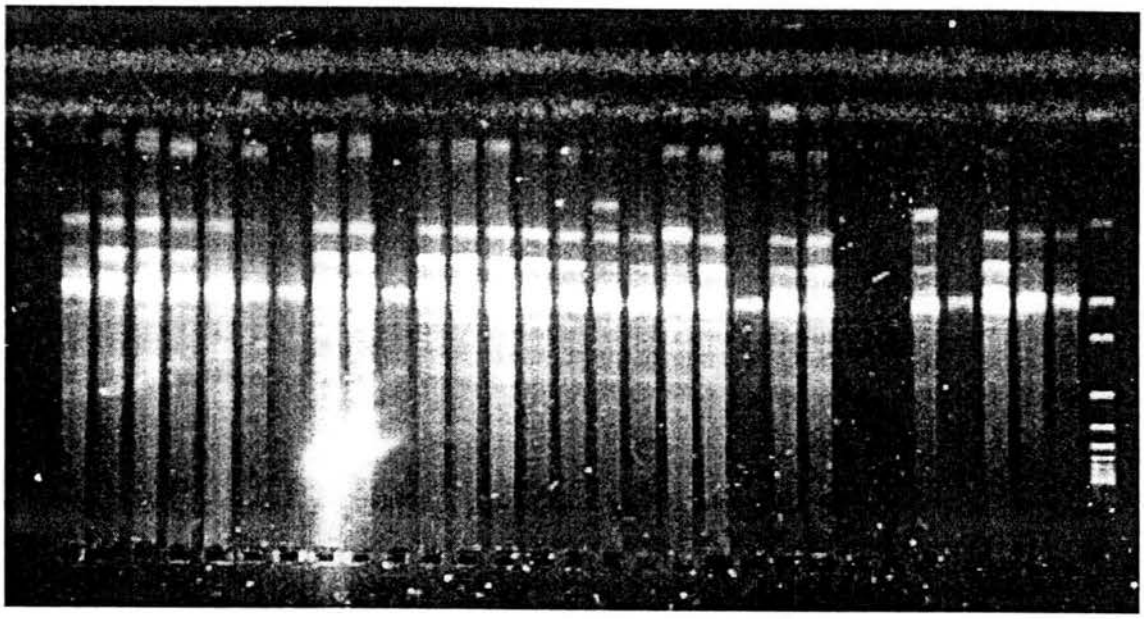


Fig. 4.4. RAPD patterns generated by primer 430 of 28 chickpea lines. The first lane on the right is DNA ladder.

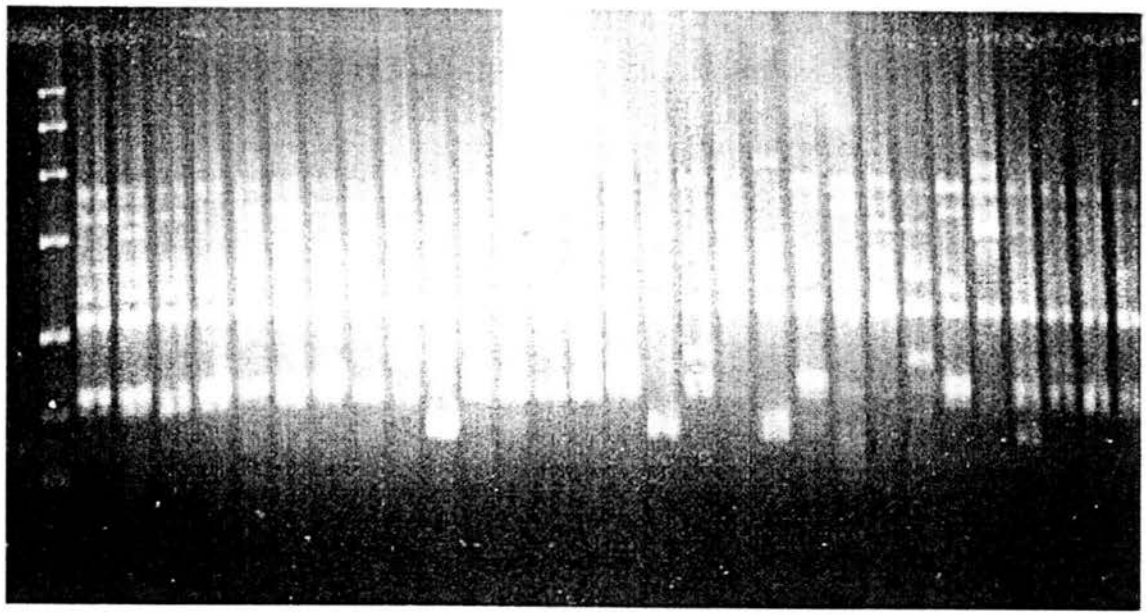


Fig. 4.5. RAPD patterns generated by primer 402 with 29 chickpea lines. The first lane on the left is the DNA ladder.

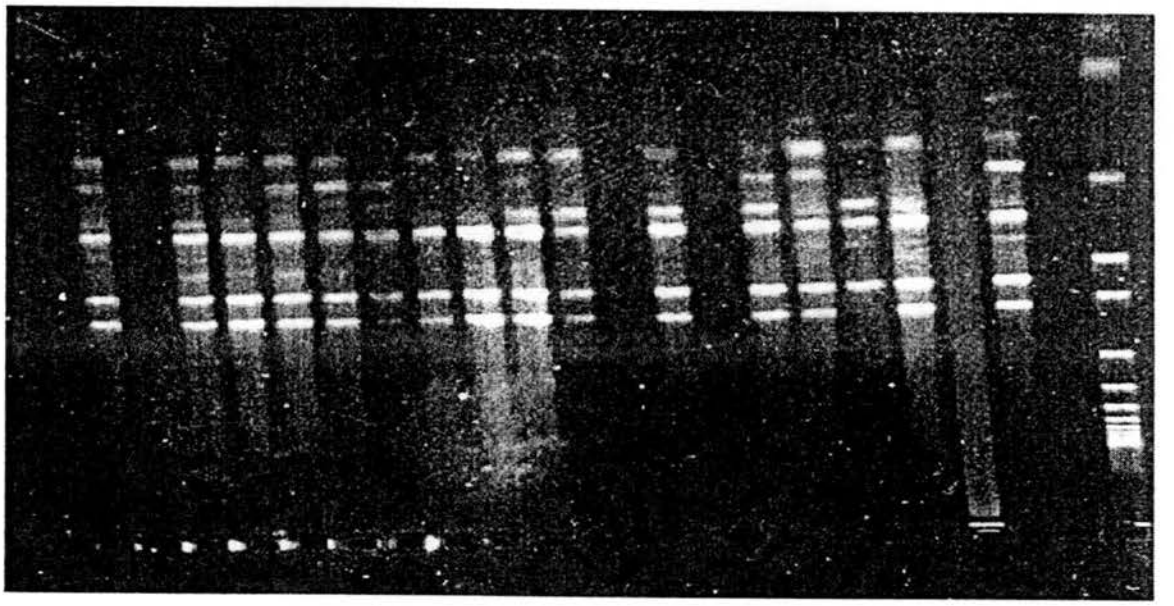


Fig. 4.6 RAPD patterns generated by primer 469 with 20 chickpea lines. The first lane on the right is the DNA ladder.

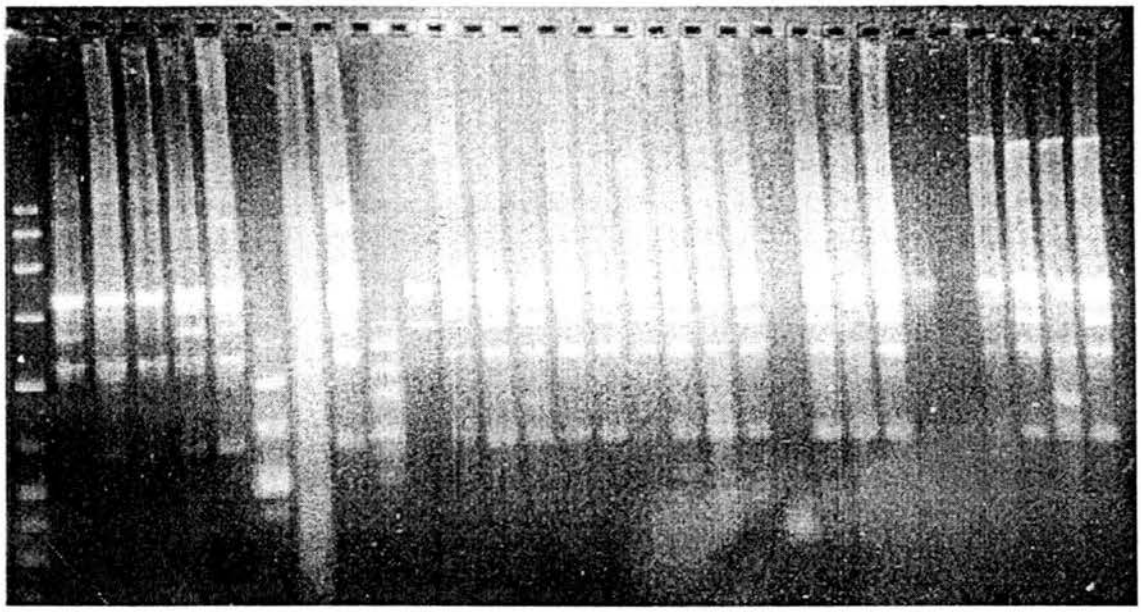
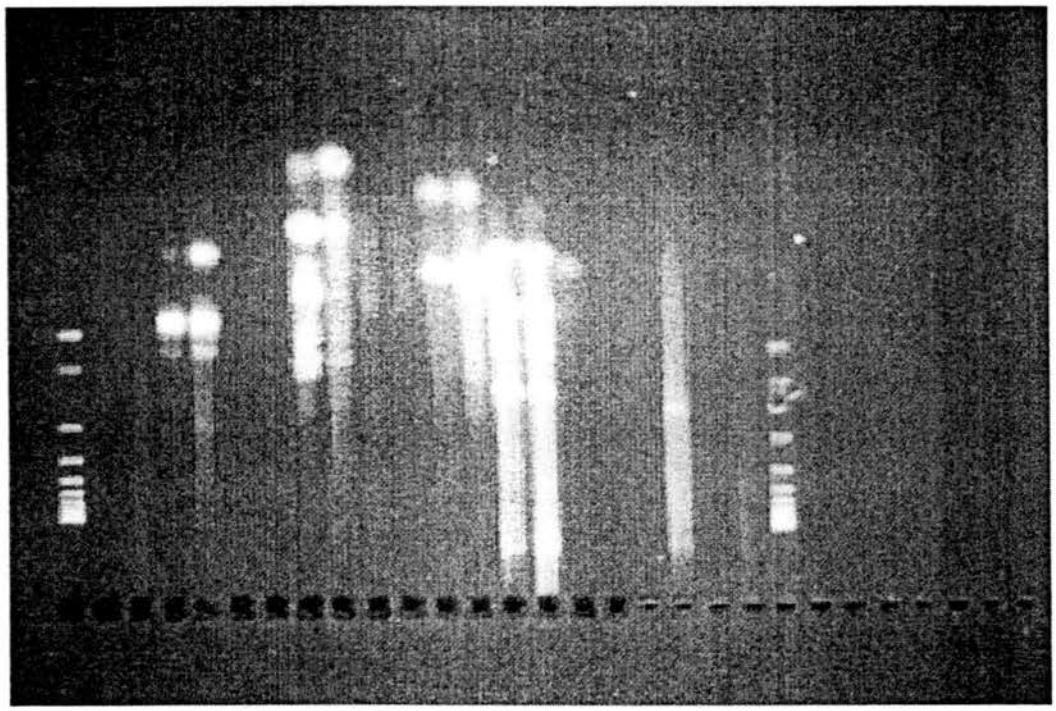
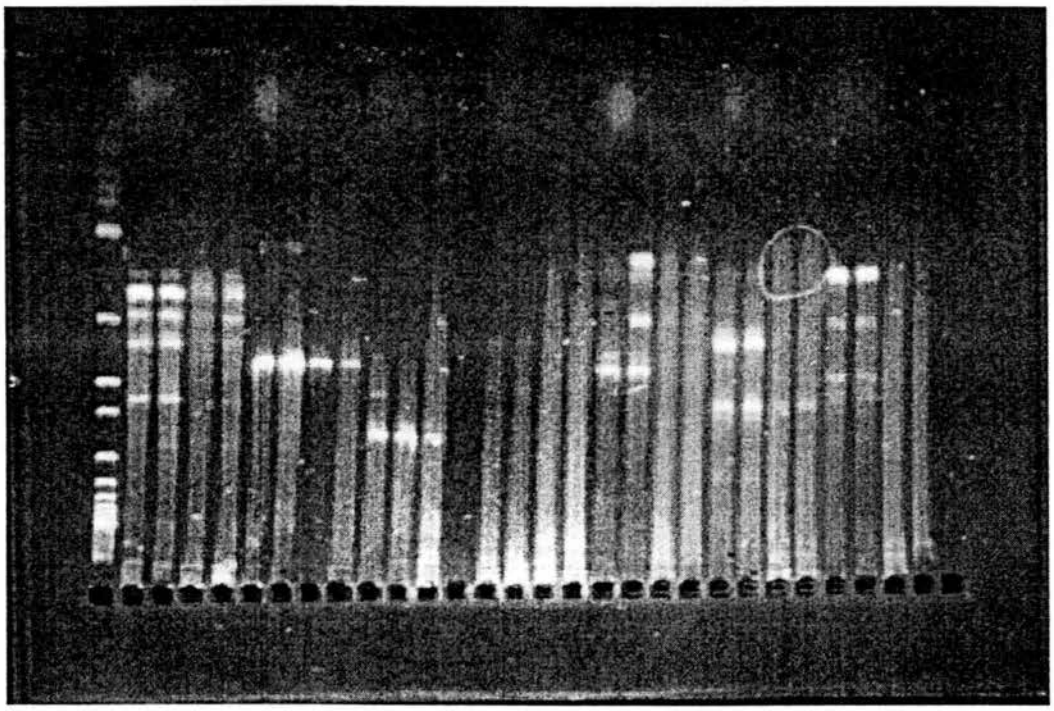


Fig. 4.7 RAPD patterns generated by primer 428 with 28 chickpea lines. The first lane on the left is the DNA ladder.



387 388 394 389 380 391 384 393 378 390 DNA ladder
Primers

Fig. 4.8. RAPD profiles generated by 10 primers (from the left to the right: 387, 388,394, 389, 380, 391, 384, 393, 378 and390). The first pattern from the left and the last one are the DNA ladders. The patterns consist of 2 lanes, the first bulked from 6 resistant lines and the second from susceptible lines, which is repeated across the 10 primers.



---438--- ---339--- ---440--- ---446--- ---447--- ---450--- ---456---

Fig 4.9 RAPD profiles generated by 7 primers (from the left to the right: 438, 339,440, 446, 447, 450 and 456). The first pattern from the left is DNA ladder. The rest of the paterns consist of 2 lanes of bulked DNA from resistant lines followed by 2 lanes of bulked DNA from susceptible lines for each primer.

Discrimination of 56 chickpea lines based on RAPD with agarose data using cluster analysis and principal components analysis

Cluster analysis: The dendrogram was generated by cluster analysis based on single linkage for the 56 lines using the 41 bands generated by the 6 polymorphic primers. It revealed 4 large clusters with the group presenting the greatest similarities (% matches) comprising three unrelated resistant cultivars F9272 (V1), ILC 200 (V8) and ILC 72 (V11) (Table 2.1, chapter 2). Most lines didn't cluster according to their origin or their type (desi or kabuli). The dendrogram constructed on the basis of shared fragments (Fig 4.10) didn't match screening data on the reaction of the tested chickpea lines. In general, it was not possible to separate the two distinct groups of chickpea (susceptible and resistant) since the 7 resistant lines were clustered with the susceptible lines. Among them, 3 resistant lines(V1, V8 and V11) clustered together with the susceptible lines in the cluster showing the greatest similarity (Fig. 4.10). Thus, the two distinct groups were not separated based on RAPD data as demonstrated by cluster analysis, perhaps due to the low polymorphism encountered.

Principal component analysis: The relationships among tested lines were examined using principal component analysis. The first two coordinates which represent 12 and 15% of the variation respectively for the first and second coordinate, plotted the lines as a group and didn't permit discrimination according to their origin or reaction to the disease. But the 3 resistant cultivars which clustered together in the dendrogram (Fig. 4.10) ((V1, V8 and V11) were also plotted close to each other (Fig. 4.11) as reflected in the dedrogram (Fig.4.10). This shows that they share common bands.

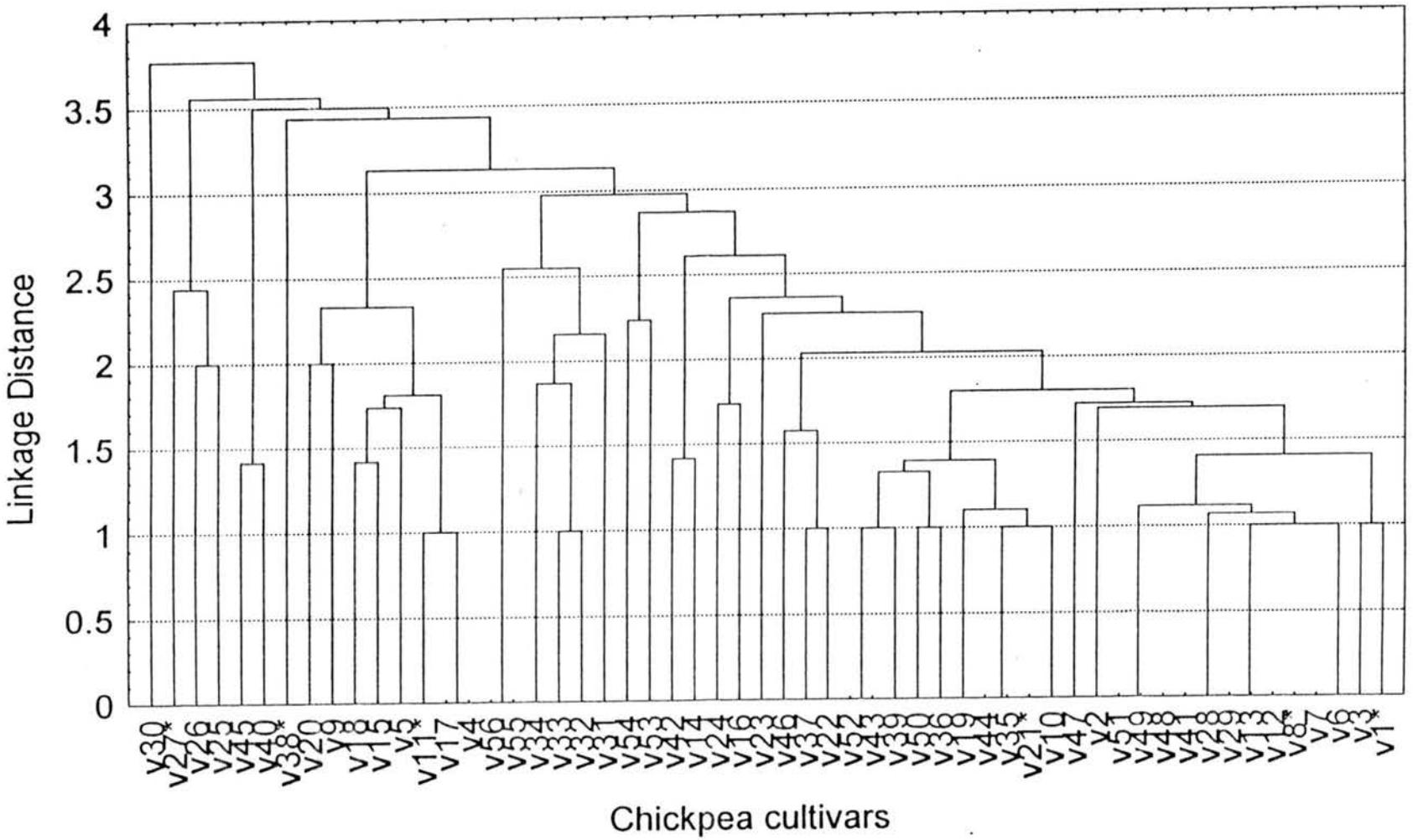


Fig. 4.10. Dendrogram of the 56 chickpea lines based on Euclidean distance, single linkage using RAPD data. Vertical axis is linkage distance values. Horizontal axis is chickpea lines from 1 to 56 (Table 2.3)
(*) represent resistant lines 1, 8, 11, 21, 27 and 38 (Table 2.3)).

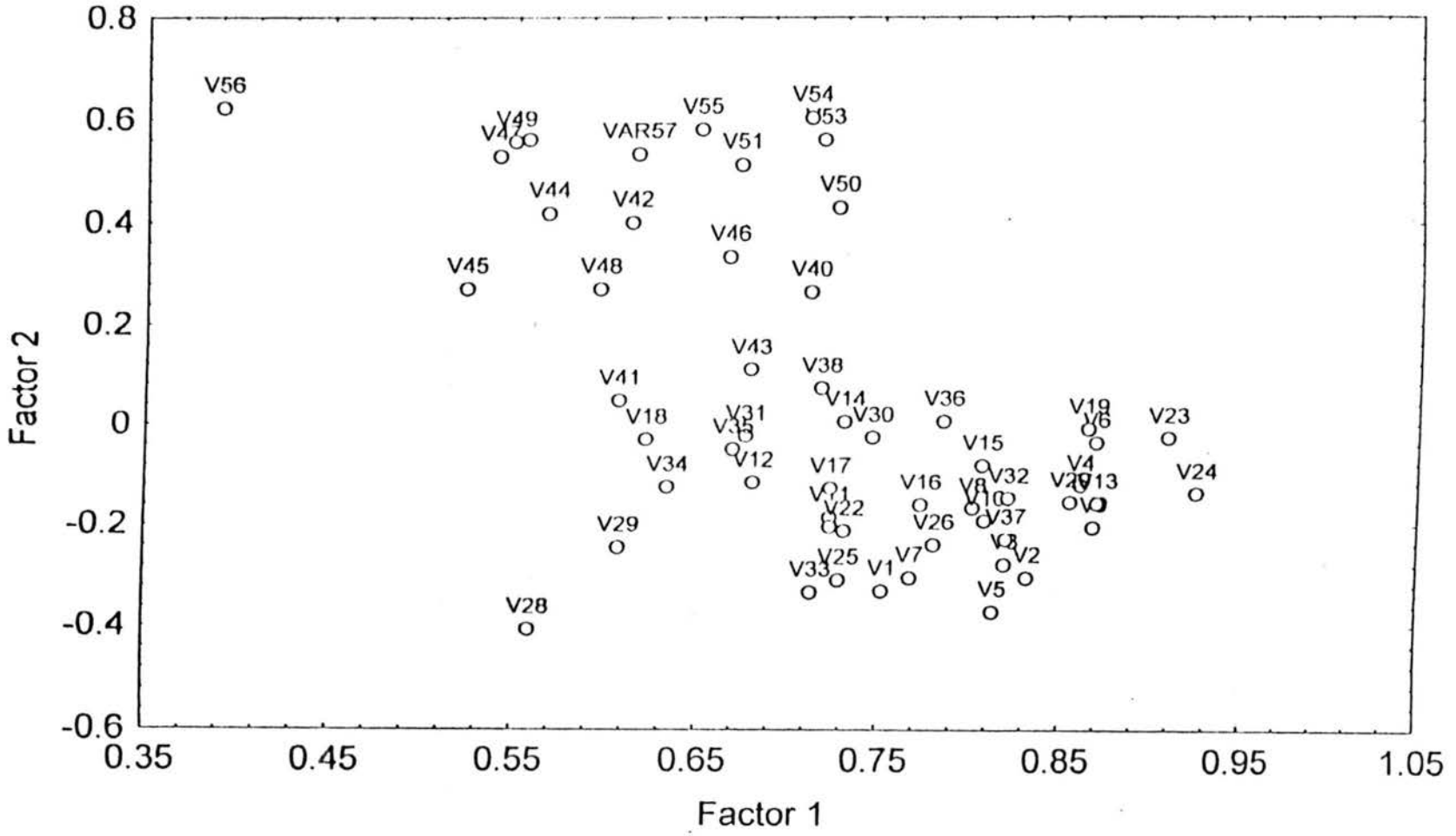


Fig. 4.11. Principal coordinate analysis of the 56 chickpea lines. Factor 1 is the second principle coordinate. Factor 2 is the first principle coordinate.

Var 1 to Var 56 represent chickpea lines tested (Table 2.3)

Discrimination of 12 cultivars by cluster analysis and principle component analysis using RAPD with agarose and RAPD with acrylamide gel data: The dendrogram of 12 cultivars (6 resistant and 6 susceptible) using single linkage for RAPD with agarose data (Fig 4.12) showed that the six resistant cultivars clustered together and appeared to share similar bands. Two of these lines (1 and 8; F9272C and ILC 200 respectively) have one common parent and originated in India. The 3 other lines (21, 27 and 38; F92139C, ILC5924 and F9278C respectively) merge at an intermediate level with a second group of susceptible cultivars and appeared to share some bands with the susceptible lines and with the resistant as well. Cluster analysis was thus able to differentiate somewhat between resistant and susceptible.

However, the dendrogram generated by UPGMA, cluster analysis based on RAPD with acrylamide gel data of these 12 cultivars (Fig. 4.13) reflected a different image than that generated with RAPD with agarose data (4.12). This dendrogram indicated that the resistant cultivars were similar as they clustered together with one susceptible lines (2; F8487C). Two resistant lines (1 and 8; F9272C and ILC200 respectively) appeared to share similar bands since they represent the greatest similarity (Fig. 4.13). Cluster analysis discriminated the resistant and susceptible lines based on RAPD with acrylamide gels (Fig. 4.13) better than RAPDs with agarose (Fig. 4.12).

To examine the relationships among the 12 cultivars, principal coordinate analysis was undertaken. The first two coordinates, which account for 15 and 12% of variation respectively for the first and the second coordinate (Fig. 4.14) effectively discriminate between the resistant and susceptible lines. Four resistant lines (1, 8, 11 and 21) were plotted together and appeared to share the greatest number of bands while two resistant lines (27 and 38) were dissimilar to this group, thus sharing fewer common bands. Three susceptible lines (26, 31 and 48) were plotted on the opposite side of the resistant lines while two susceptible lines (45 and 54) didn't appear on the figure 4.14, as they were plotted out of it. Line 2 was plotted at an intermediate level with resistant and susceptible lines.

Relationships between RAPD amplification products and the reaction to ascochyta blight and RAPD products and the origins of the lines

Association of RAPD amplification products with the origins and reaction to ascochyta blight of the entries

The 41 bands provided by the 6 polymorphic primers were scored 1 when the band was present and 0 when it was absent. The origin of the lines ranked 1 originating from India and 0 when it originated elsewhere. The reaction to the disease was also ranked 1 when the line is resistant and 0 when it was susceptible. These data were submitted to Spearman correlation analysis using SAS procedure.

Spearman correlation coefficient calculated with SAS procedure showed no correlation was found between RAPD run on agarose or RAPD run on polyacylamide gels data and the origin of cultivars. The hypothesis Ho:rho=0 means no correlation, was accepted since P was very high (0.50 and 0.78, Table 4.7). Therefore, no significant correlation between RAPD bands and origin of chickpea lines was found. However, with the reaction to ascochyta blight, four bands (B1 and B2 belonging to primer 428, B14 to primer 430 and B36 to primer 415) were associated with reaction to the disease (Table 4.7).

Table 4. 7 Spearman coefficient of correlation between RAPD amplification products and origin and reaction to ascochyta blight (SAS procedure).

		B1*	B2*	B14*	B36*
Origin	ϱ*	-0.053	-0.053	-0.053	0.132
	P*	0.787	0.787	0.787	0.501
Reaction	ϱ	-0.480	-0.480	-0.480	0.485
	P	0.009	0.009	0.009	0.008

B1*, B2*, B3* and B4*: RAPD amplification products.

ϱ* (rho): coefficient of correlation and P*: probability

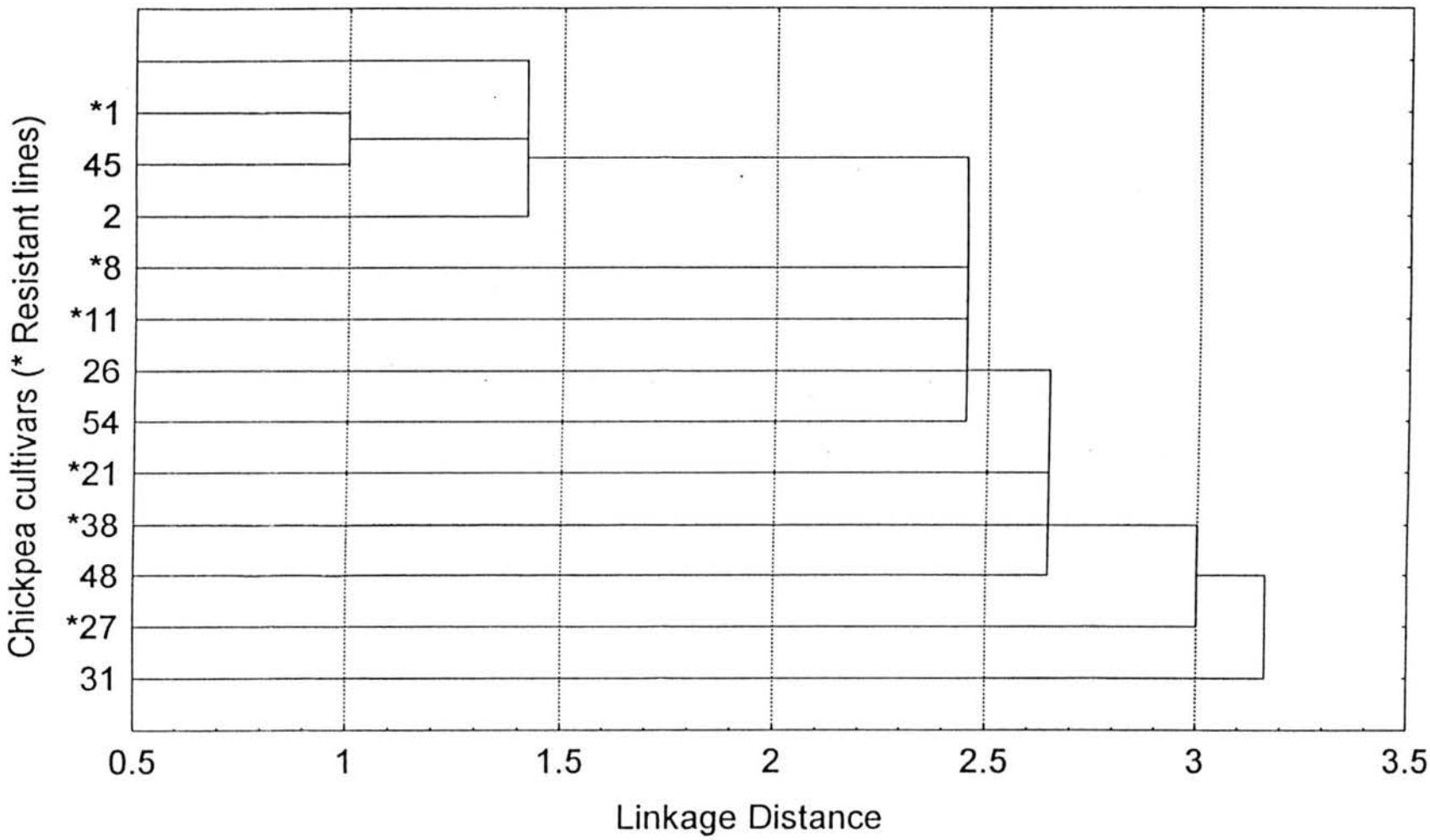


Fig. 4.12. Dendrogram of the 12 chickpea lines (6 resistant* and 6 susceptible) based on Euclidean distance, single linkage using RAPD data. Vertical axis is linkage distance values. Horizontal axis is chickpea lines.
(*resistant lines: 1, 8, 11, 21, 27 and 38; susceptible lines: 2, 26, 31, 45, 48 and 54, Table 2.3)

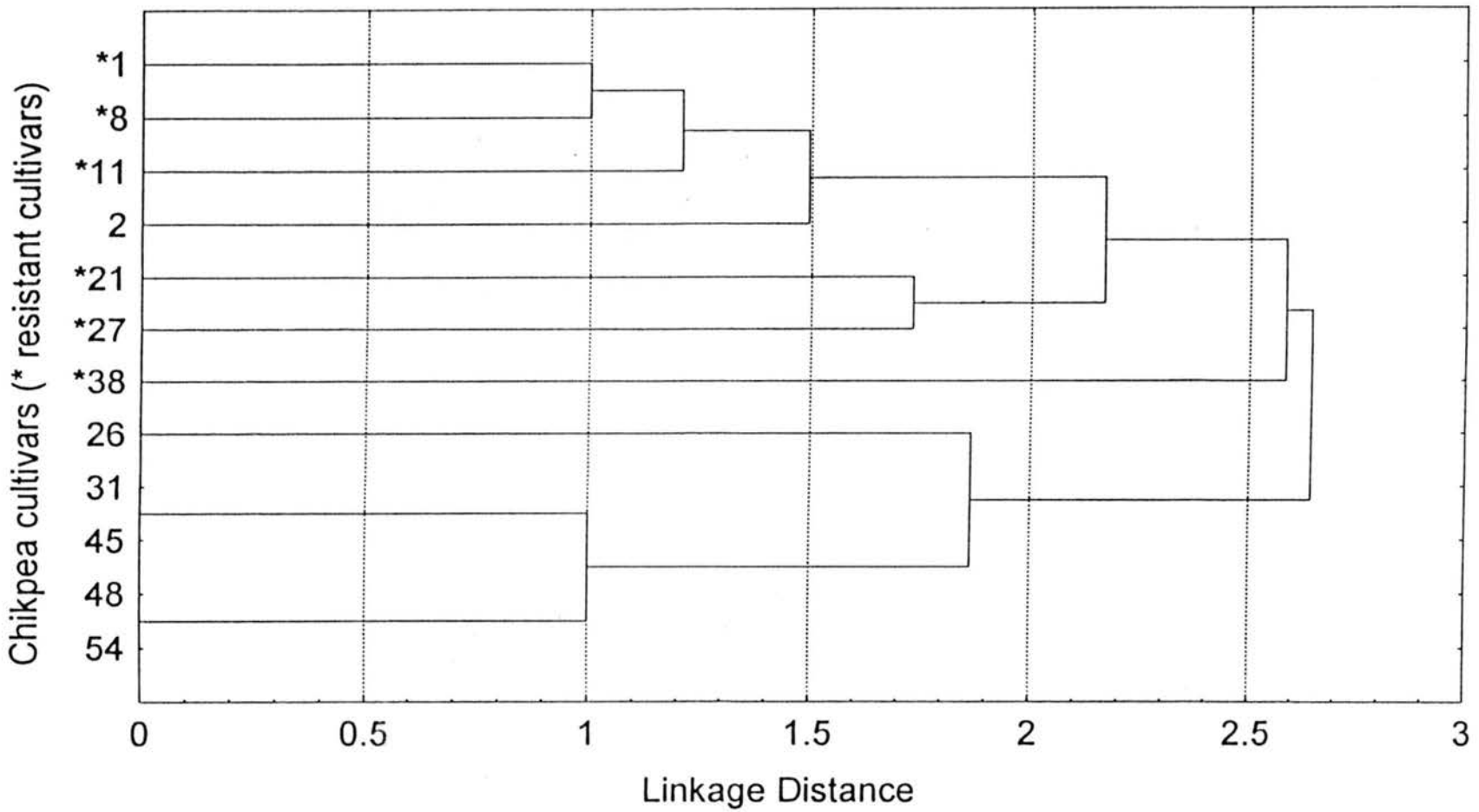


Fig 4.13 Dendrogram of the 12 chickpea lines based on Euclidean distances, single linkage using RAPD with acrylamide gel data. Vertical axis is linkage distance values. Horizontal axis is chickpea lines (* resistant lines 1, 8, 11, 21, 27 and 38; susceptible lines 2, 26, 31, 45, 48 and 54 Table 2.3)

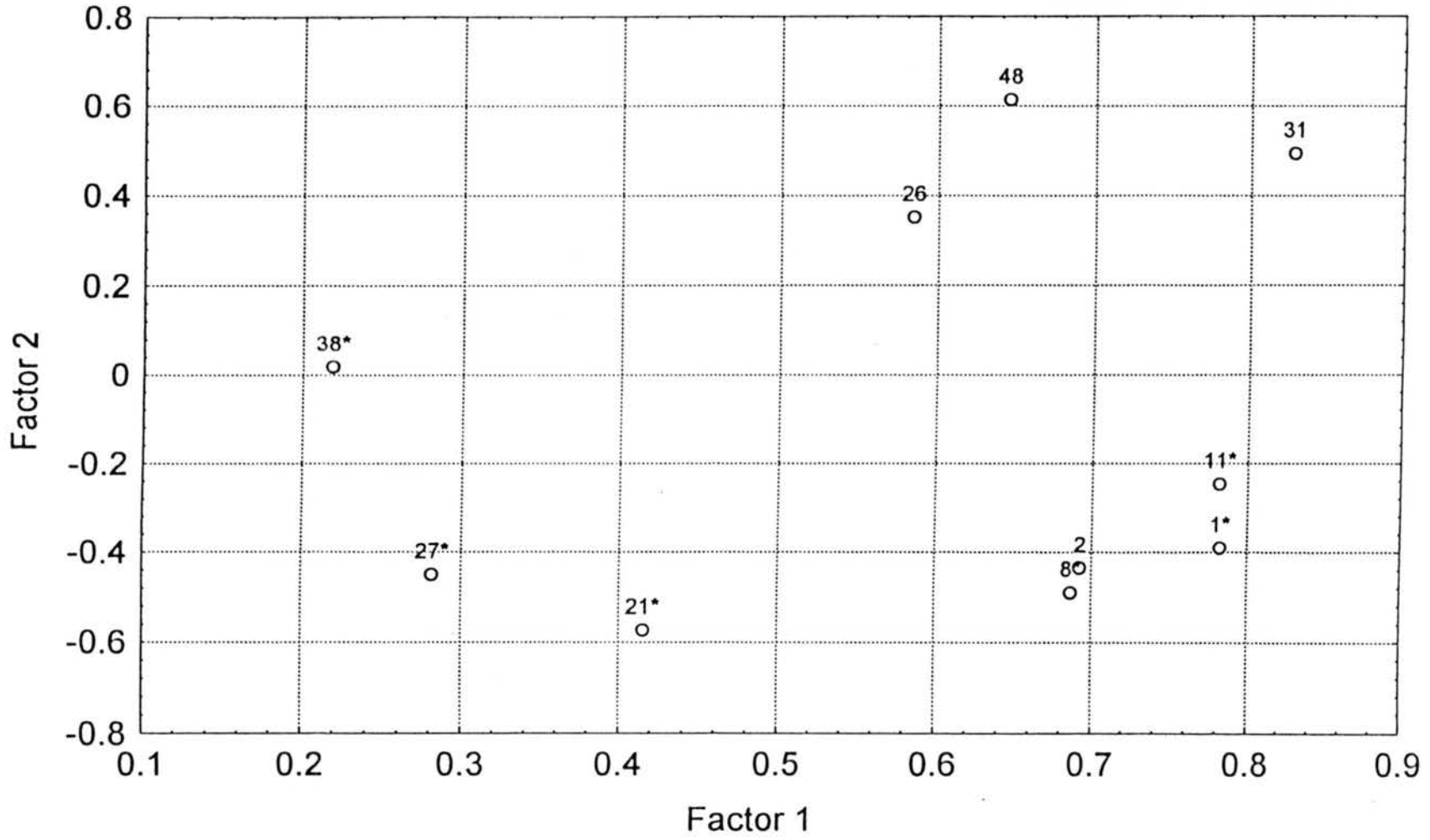


Fig. 4.14 Principal coordinate analysis of the 12 chickpea lines (6 resistant* and 6 susceptible) using RAPD with acrylamide gel data. Factor 1 is the second principle coordinate. Factor 2 is the first principle coordinate.

DISCUSSION

Some primers gave no amplification products, indicating that they lacked homology with chickpea DNA. Six polymorphic primers allowed discrimination of some lines despite the high level of monomorphism detected by RAPD. The majority of lines used in this study were nearly monomorphic for most of the primers used (Fig. 4.2). This low level of polymorphism is consistent with the commonly accepted view of a narrow genetic base in chickpea.

The results using RAPD analyses are similar to those of the isozyme assays used in this study and those of isozymes reported by Tuwafe et al.(1992) and Kusmenoglou et al. (1992) as well as RFLPs used by Udupa et al.(1993) and RAPDs by Sharma and Mohapatra (1996). These chickpea accessions and cultivars were less variable than expected in comparison to phenotypic differences among some lines; (black seed for Desi lines named ICC (Table 2.1) and white seed for the Kabuli lines (FLIP, ILC, RH and PCH), seed size (small size for ILC, RH and some FLIP accessions and Desi lines), color of petals (pink for desi and RH and white for the others). This narrow variability may also be related to the samples tested being breeding lines (F6), some of which are closely related (i.e. 50% of the 32 kabuli breeding lines have one or two common parents, Table 2.1, section 2).

This low genetic variability observed in chickpea lines tested herein could be mainly explained by the high self- pollination of the species. Van der Mesen (1972) reported that all *Cicer* species are almost exclusively self-pollinating. This may also be do to the limited number of accessions of the wild species available for research purposes at both ICRISAT and ICARDA (Tayyar and Waines, 1996). Another explanation of the low degree of intraspecific variation in the cultivated chickpea species that during domestication it passed through a genetic bottleneck. These RAPD results are similar to those of other authors who recently used RAPDs to differentiate chickpea types Kabuli and Desi. The study suggested the presence of narrow polymorphism as revealed by RAPD. However, when that study was extended to the wild species *Cicer reticulatum*, RAPD polymorphism could be detected with only four random primers (Sharma and Mohapatra, 1996).

Although some species show considerable polymorphism when analyzed by RAPDs, chickpea cultivars do appear to be an exception with low polymorphism. Low levels of

polymorphism have been recorded in other crop species analyzed with RAPDs. In *Lycopersicon esculentum,* for example, the level of monomorphism for both wild and cultivated accessions was 62.5% (Williams and St. Clair, 1993). Another case of a similar example is the low level of polymorphism detected with molecular markers in *Arachis hypogae,* as well as with the use of isozymes (Lacks and Stalker, 1993; Stalker et al., 1994), RFLPs (Kochert et al., 1991; Garcia et al., 1996) and RAPDs (Halward et al., 1991, 1992; Lanham et al.,1992). Recently, RAPD analysis carried out on 52 accessions of *Solanum melongena* (egg plant) and related weedy forms showed a high level of similarity among tested plants. These results were similar to the results of an electrophoretic isozyme analysis performed on the same accessions (Karihaloo et al., 1995). RAPD techniques also detected, a surprisingly low level of genetic variation within *Butomus umbellatus (Butomacea) (*Fernando and Cass, 1996) and red pine (Kessler et al.,1992).

Cluster analysis based on the 41 bands generated by RAPD analysis using 6 polymorphic primers didn't discriminate between all resistant and suceptible lines. RAPD with acrylamide data allowed the clustering of all resistant lines with one susceptible. Four resistant lines having one common parent and thus similar origin (India), ILC 9272 (ILC 571XF85122), F92152 (F85122XF85137), F92139 (F85122X F8443) and F92-78 (F85122 X ILC6055) clustered as a group with the other resistant lines, ILC 200 and ILC 5924 which have different parentage and origin (ILC200 selection from Stepnoj-1 from USSR, ILC 5924 (6709) from Bulgaria) and the 5 other susceptible lines (ILC 3279, from former USSR, selection from Stepnoj-1; F8487C from ICARDA/ICRISAT, ILC72x ILC 215; ILC482 from Turky ACC. N26780-68; PCH 37 from Morocco, ILC3815 and F92-18 C from ICARDA/ICRISAT, F85122C x F85137C (Table 2.1) clustered together. This clustering is simialr to the breeding knowledge of the 4 resistant lines as they clustered as a group according to RAPD run on polyacrylamide gels.

Correlation between RAPD data and the reaction to ascochyta blight was found using Spearman correlation coefficient. Four bands were correlated with ascochyta blight disease. This result suggests that these might be potential markers for resistance to ascochyta blight. If verified, these markers may speed up chickpea breeding for ascochyta blight while reducing the cost of screening techniques.

The bulked DNA analysis tested showed that some primers yielded one or two bands with the resistant bulk and no band with the susceptible bulk. However none of these bands was reproducible when tested a second time with the same sample of bulked DNA. This problem may have been due to some technical problem related to the RAPD procedure. There have been similar problems observed in other species relative to reproducibility (Kesseli et al., 1992). According to the literature, many factors are critical to get repeatable results with RAPDs. For example, Erlich et al.(1991) reported that temperatures and magnesium chloride concentrations were found to cause a problem. They reported also that Taq polymerase concentration might be a problem in the repeatability of RAPD results. Kesselli et al. (1992) also found that RAPDs were extremely sensitive to reaction conditions and that magnesium concentration and template-primer ratio may reduce repeatability. In contrast with these findings, Weeden et al. (1992) found that primer and magnesium concentration could vary 2-5 fold without affecting the pattern of RAPD products and template DNA could vary 10 fold (3-30ng per 25µl reaction volume) without affecting RAPD pattern. The most critical element influencing the reproducibility of their results was the degree of purity of the template DNA. DNA which contained only small amounts of impurities often produced blurred or faint RAPD images (Kesseli et al., 1992). This problem may be also related to the primer itself as patterns of some primers were easily scorable and reproducible(case of the six polymorphic primers) and for some other primers were not (primers tested with bulked DNA). In this study, contamination of DNA during thawing and freezing may have influenced the reproducibility of some results.

CONCLUSION

In the present study, RAPD assays using random decamer primers were used to investigate the similarity or difference among 56 lines of chickpea. There was a low variation in RAPD markers within the tested lines of chickpea. Despite this low genetic variability, the RAPD method detected four bands that correlated with ascochyta resistance which might be potential markers for resistance to ascochyta blight provided that the DNA extraction procedure is improved and/or Taq DNA polymerase and dNTPs concentrations are optimized. The cluster analysis of the chickpea cultivars using the unweighted pair group method average (UPGMA) did not show any correlation between RAPD profiles and geographic origin because of the low variability encountered. However, RAPD with acrylamide gel was more informative since generally it distinguished between the two groups of lines, resistant and susceptible with the exception of 1 susceptible clustering with the resistant lines. This procedure should be investigated further in the future with improved DNA extracts.

GENERAL CONCLUSION

The screening tests carried out in fields in Morocco, in the zone of high production of chickpea where ascochyta blight disease was highly prevalent, indicated a high degree of infestation of ascochyta blight in these regions (Jemaa de Shaim and Sidi El Aydi) during 1995-1996. The screening test at the greenhouse, which was performed to compare the severity of the fungus under natural and artificial conditions, showed the scores of the disease at Sidi El Aydi and the greenhouse were significantly correlated and these scores were higher at the podding stage relative to the early stage in the three locations. Four groups of cultivars and accessions were discerned (7 resistant, 7 tolerant, 7 moderately susceptible and 35 highly susceptible). The screening tests showed a high level of the disease as visual damage scores were high and some resistant lines as screened in Syria were scored as tolerant under these conditions and all Moroccan cultivars were highly susceptible.

Little genetic polymorphism was detected by isozymes in the accessions tested. This was surprising because most of the lines were morphologically different, especially the kabuli and desi which have different seed color and size and the overall diversity was well represented in our materiels (56 accessions and cultivars). Four polymorphic isozymes (CA, EST, ACPH and PR-EST) yielded a few variants (2 to 5). These results are similar to those of a recent study (Tayyar and Waines, 1996) on genetic relationships among annual species of *Cicer* using isozyme in 63 accessions of 10 species of Cicer and the cultigen *Cicer arietinum*. These most recent results revealed that 96% of the allelic diversity was found among rather than within species. Another similar study on the genetic diversity among one hundred and thirty nine accessions of nine annual *Cicer* species revealed high levels of polymorphism in all eight wild annual Cicer species. However, for the cultigen among the 14 loci assayed, only two were polymorphic (Labdi et al., 1996).

RAPD analysis was expected to provide more information on the variability of this group of chickpea accessions , as most of the lines originated in India, the center of their diversity. However, the RAPD results were generally very similar to that of isozymes. Despite this low polymorphism, RAPD revealed 4 promising markers for resistance to ascochyta blight which may help in the identification of resistant lines. However, further study is necessary to clarify the problem of DNA degradation encountered herein.

Commonly used markers, such isozymes (Oram et al.,1987, Ahmad et al.,1992, Tayyar and Waines, 1996 and present study) restriction fragment polymorphisms (RFLP Udupa et al., 1993) and RAPDs (Sharma and Mohapatra, 1996 and present study) have not to date demonstrated high levels of polymorphism in chickpea. It is therefore suitable to test other molecular techniques which may reveal polymorphisms in chickpea. Sharma et al. (1995) studied polymorphisms in chickpea by oligonucleotide fingerprinting using 14 restriction enzymes and found 38 different simple-sequence repeat motifs in only four accessions. This demonstrated that considerable variation can be detected by this technique and suggests the suitability of simple-sequence repeat probes as molecular markers for chickpea identification and their potential use as marker genes in ascochyta blight resistance.

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APPENDIX 1

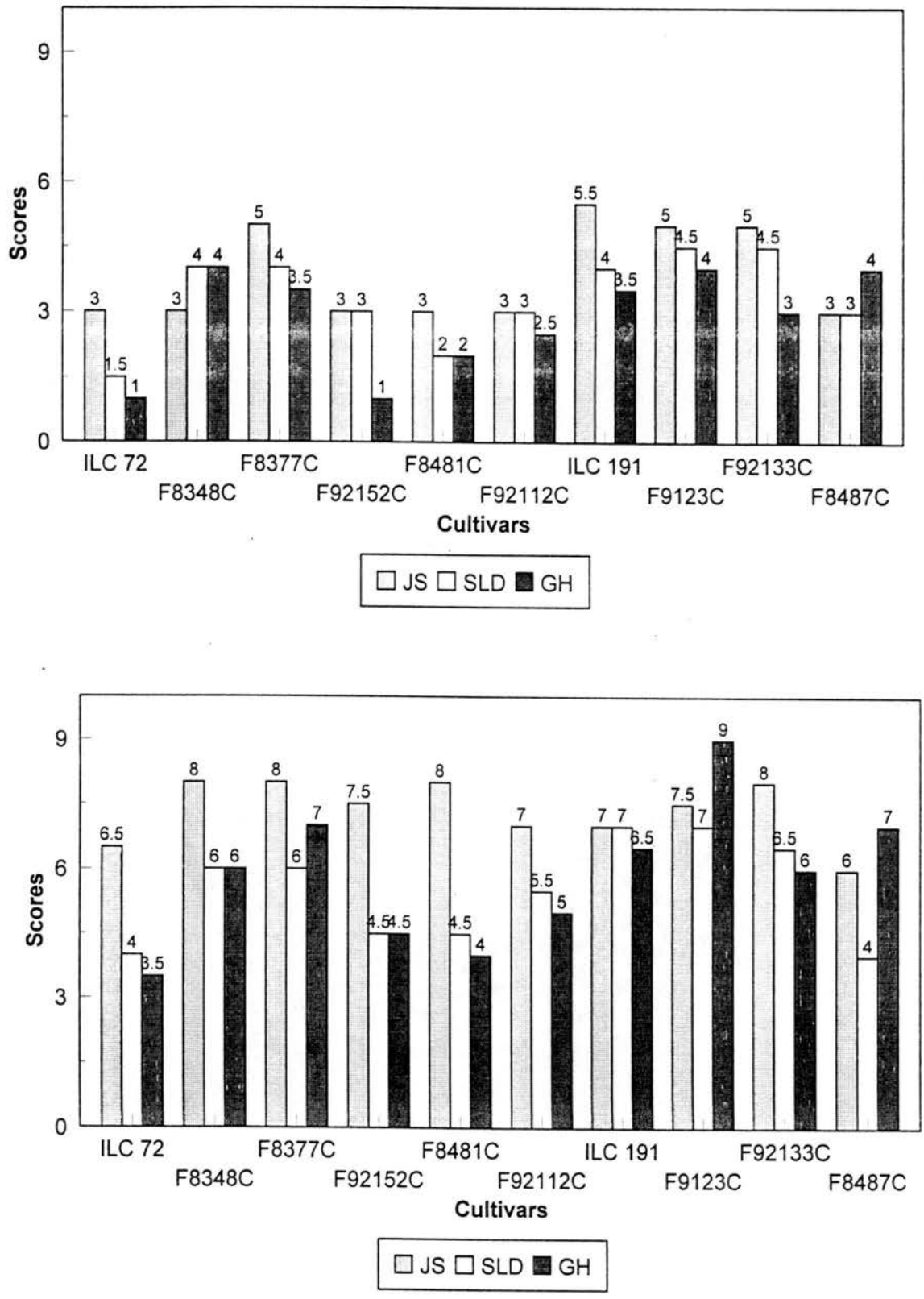
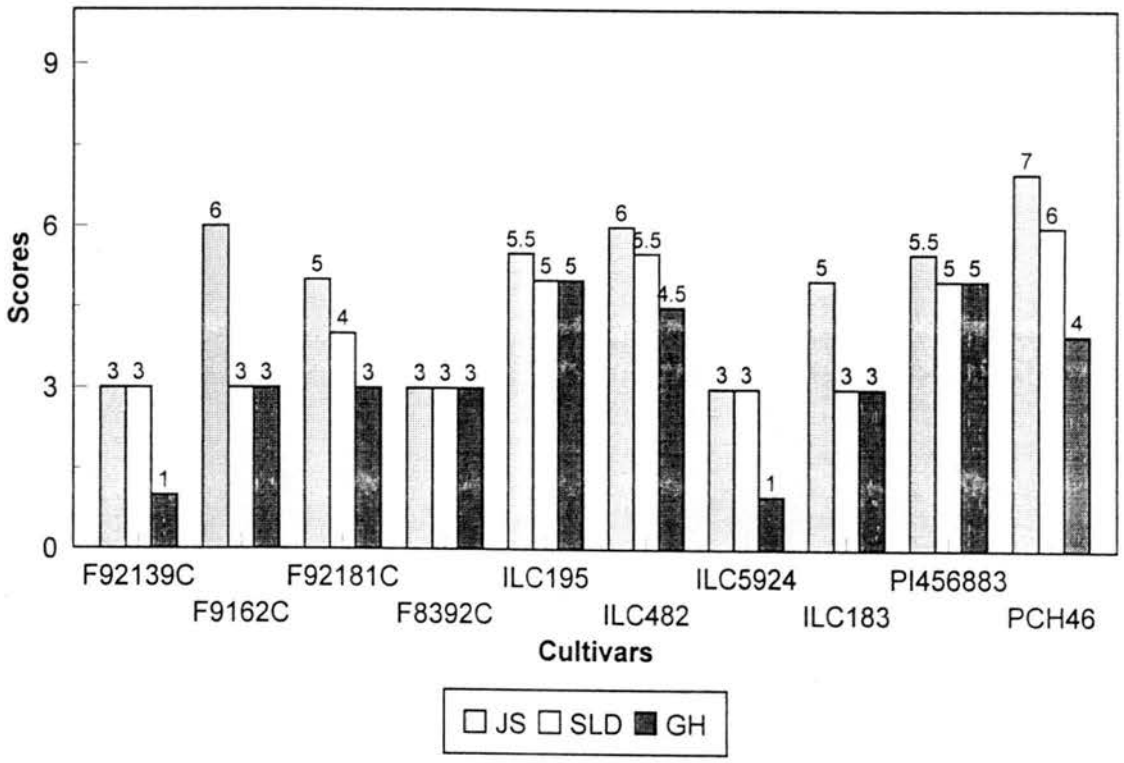


Figure 2.1. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations



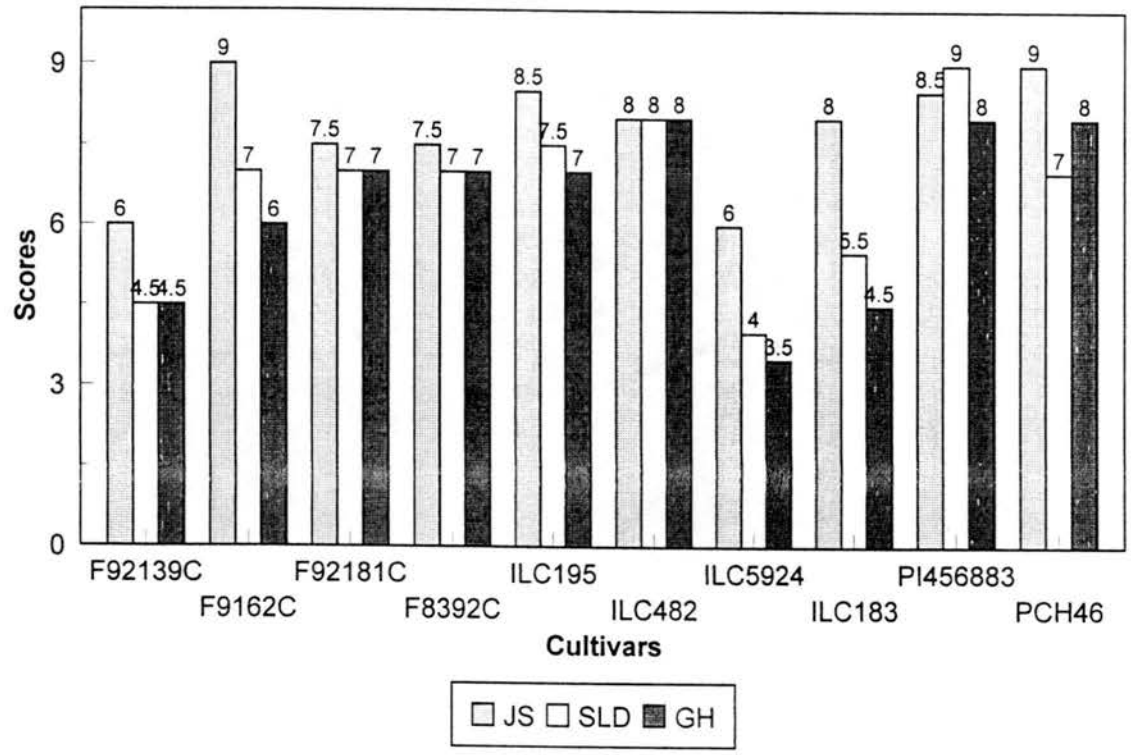


Figure 2.2. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations

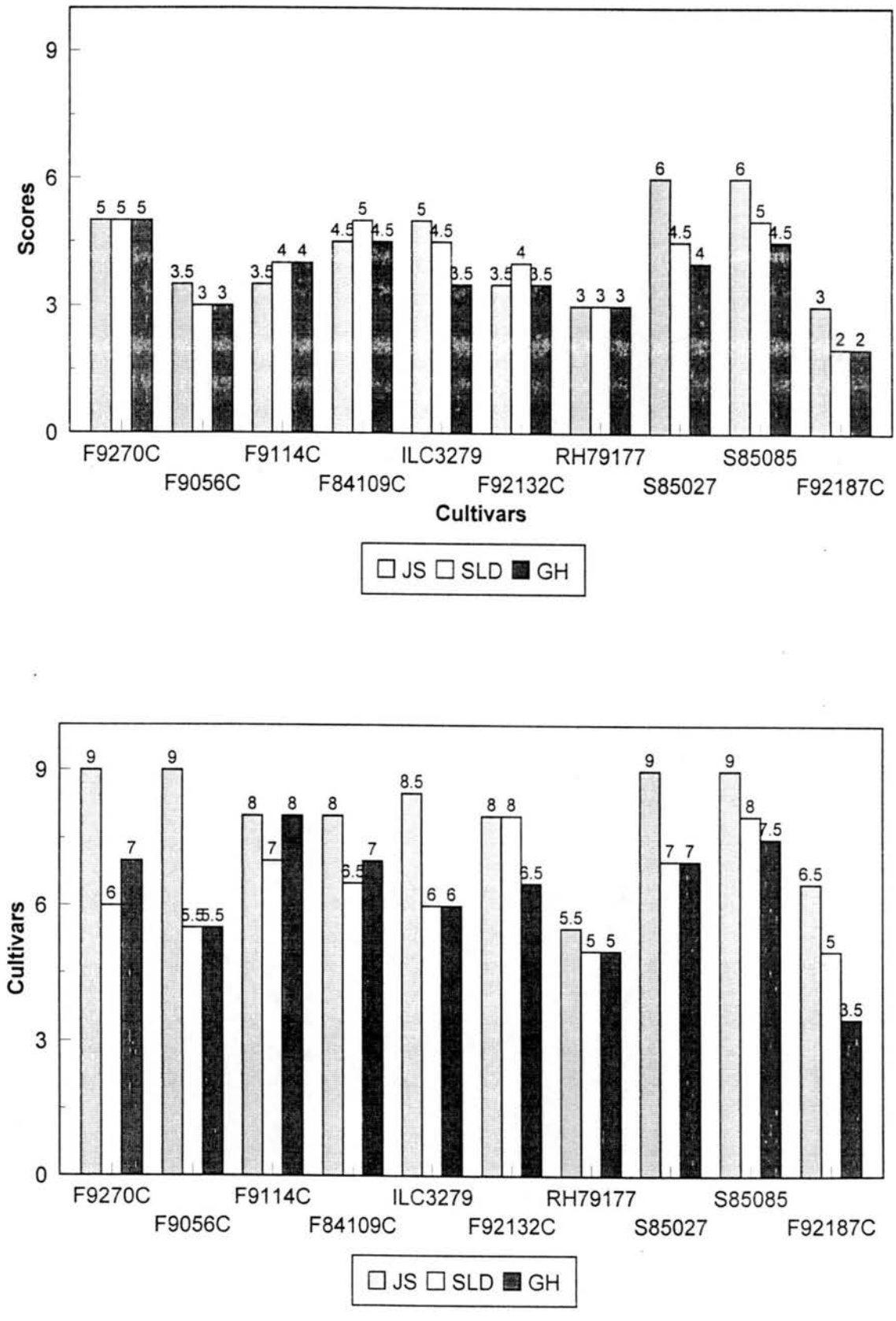
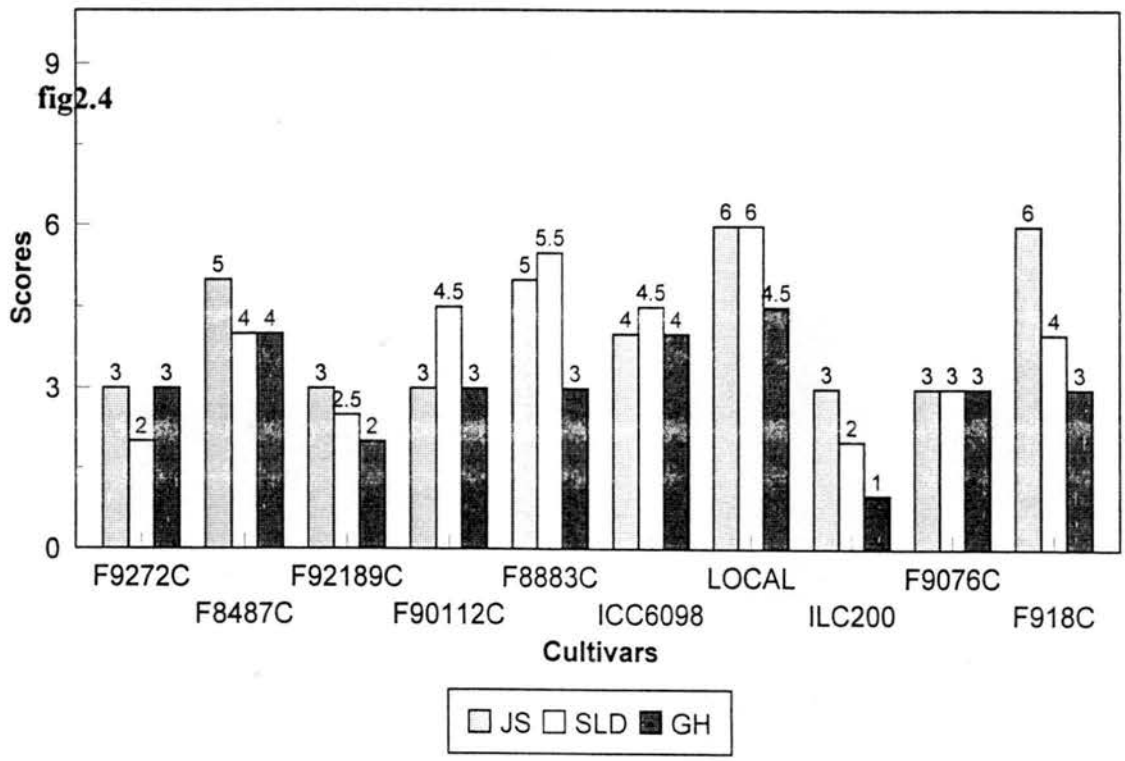


Figure 2.3. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations



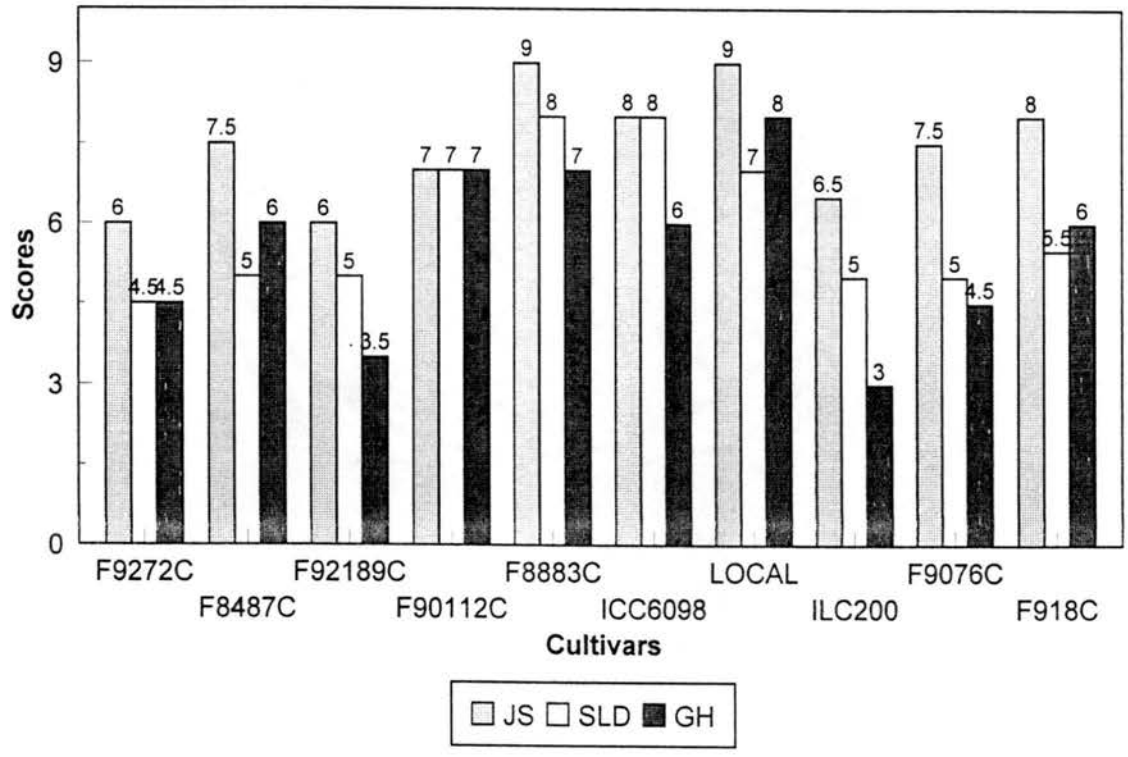


Figure 2.4. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations

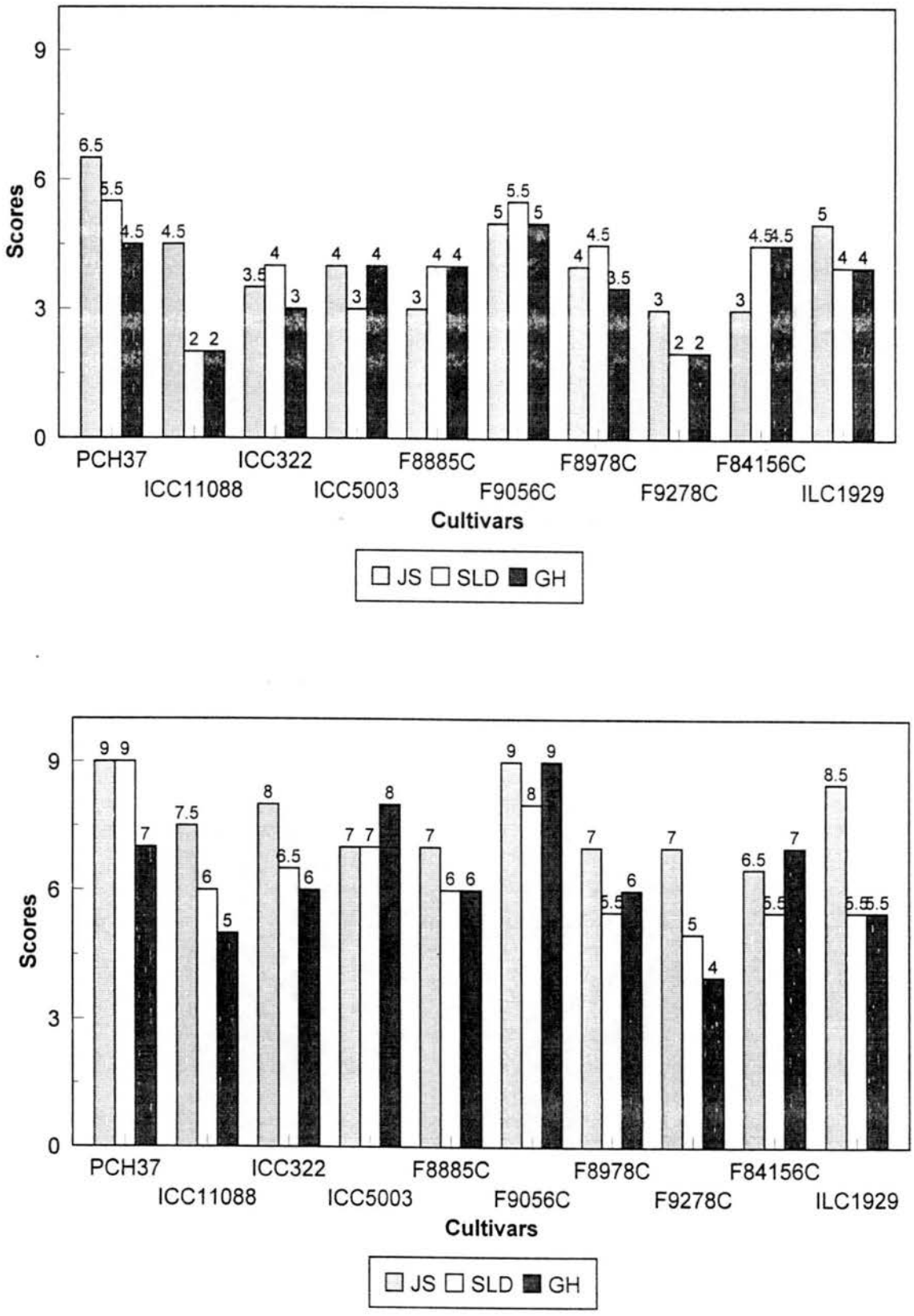


Figure 2.5. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations

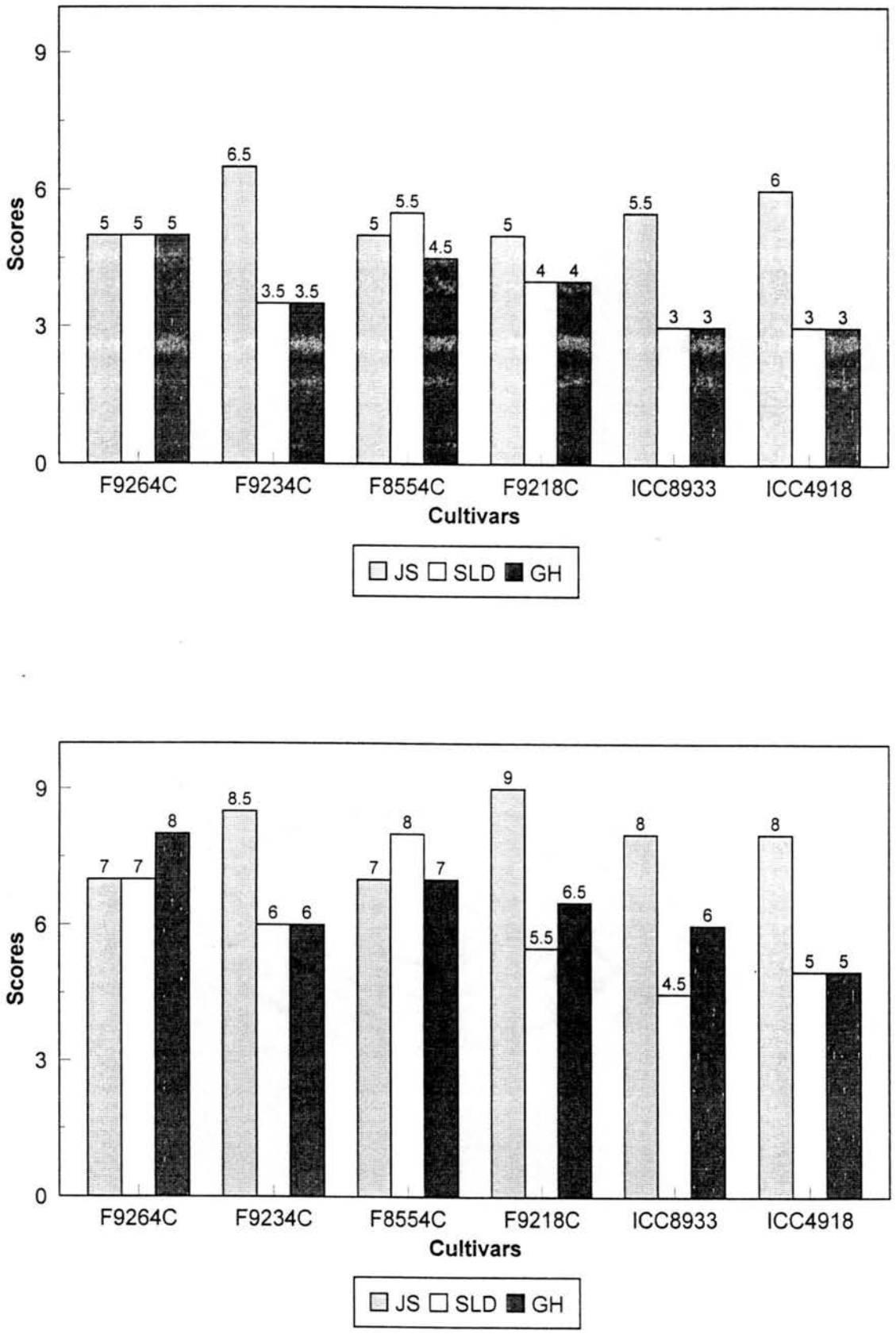


Figure 2.6. Ascochyta blight severity at early (top) and late stage (bottom) in Jemaa Shaim (JS), Sidi El Aydi (SLD), and greenhouse (GH) locations

APPENDIX 2

Table 3.5. Staining solutions and incubation conditions

Stain (Reference)	Ingredients for 100ml Gel of Solution	Concentration	Electrode Buffer	Incubation conditions
Esterase (Arulsekar & Parfitt,1986)	Fast blue RR salt 40mg in 40ml distilled H_20 Phosphate solution A 50ml Phosphate solution B 10ml 0.1% a-naphtylacetate 2ml	6%	Tris-glycine	30°C Dark 30-90 minutes
Aspartate Amino-transferase (AAT) (NSSL)	Fast Blue BB Salt 100mg per 100 ml Substrate Solution: α-ketoglutaric acid 36.5mg, L-aspartic acid 500mg, EDTA 50mg, Na_2HPO_4 1.42mg, Dissolved in 100ml H_20	6%	Tris-glycine	37°C Dark 30 minutes
6-Phosphoglu-conate dehydrogenase (6-PGD) (Soltis & al.1983)	1M Tris HCl pH 8.0 10ml Distilled H_20 90ml 6-phosphogluconic acid 40mg 1M $MgCl_2$ 2ml, NADP 10mg, MTT 10mg, PMS 2mg	6%	Tris-glycine	37°C Dark 30 minutes

Table 3.5. Staining solutions and incubation conditions (Cont.'d)

Stain (Reference)	Ingredients for 100ml Gel of Solution	Electrode Buffer	Incubation conditions
	Concentration		
Alcohol dehydrogenase(ADH)(Schwen nesen & al. 1982)	Nitro blue tetrazolium 20mg, 6% NAD and 5 mg phenasine methosulfate were dissolved in 100 ml 0.10M tris HCl of pH 8.5 with 2ml 95% ethanol	Buffer B	35°C Dark 2 hours
Carbonic anhydrase (CA) Hamdaoui (1987)	Tris HCL buffer 0.1M pH 7.4 6% 100ml, Fast Blue RR salt 200mg β Naphtyl acetate 4mg dissolved in 10ml of 3% acetone.	Tris HCL	37°C Dark 30 minutes
Propionate esterase (PR-EST))	Sodium acetate buffer 0.05M at 6% pH 5 100ml, αNaphtyl propionate 100mg dissolved in 10ml of 50% acetone Fast Garnet GBC 70mg	Sodium acetate	37°C Dark 30 minutes

Table 3.5. Staining solutions and incubation conditions (Cont.'d)

Stain (Reference)	Ingredients for 100ml Gel of Solution	Concentration	Electrode Buffer	Incub. cond.
β-Amylase (β-AMY)	0.5M acetate buffer 100ml Hot water 90ml+starch 2g+ solution D 10ml.	6%	Acetate	37°C dark 15mn
Glucose dehydrogenase(GDH) Soltis & al. 1983	1M tris HCL buffer pH 8.0 10ml 1 M glutamic acid (monosodium) ph 8.0 20ml NAD 20mg; MTT 10mg; PMS 2mg	6%	lithium borate	37°C Dark, 40 minutes to 3 hours
Fumarase (Fu) Hamdaoui (1987)	Phosphate dibasic buffer pH 6.5 100ml, NBT 30mg, NAD 30mg EDTA 90mg, α glycerophosphate dehydrogenase 400mg, PMS 4mg	6%	Phosphate dibasic	37°C Dark 40 minutes

Stain (Reference)	Ingredients for 100ml Gel of Solution	Concentration	Electrode Buffer	Incub. cond.
Malic Enzyme (ME) (Soltis & al. 1983)	1 M tris HCl pH 8.0 10ml Distilled water 80ml 2 M DL-malic acid(pH 8.0 with NaOH pellets)10ml 1 M $MgCl_2$ 2ml, NADP 20mg MTT 20mg, PMS 2mg	6%	Lithium Borate	37°C Dark 30 minutes
Phospho-glucomutase (PGM) (Soltis & al.1983)	1 M Tris HCl pH 8.0 10ml Distilled H_20 90ml, Glucose-1-phosphate disodium salt 50mg, Glucose-6-phosphate dehydrogenase 40unites, NADP 10mg, MTT 10mg, PMS 2mg	6%	Lithium Borate	37°C Dark 30 minutes

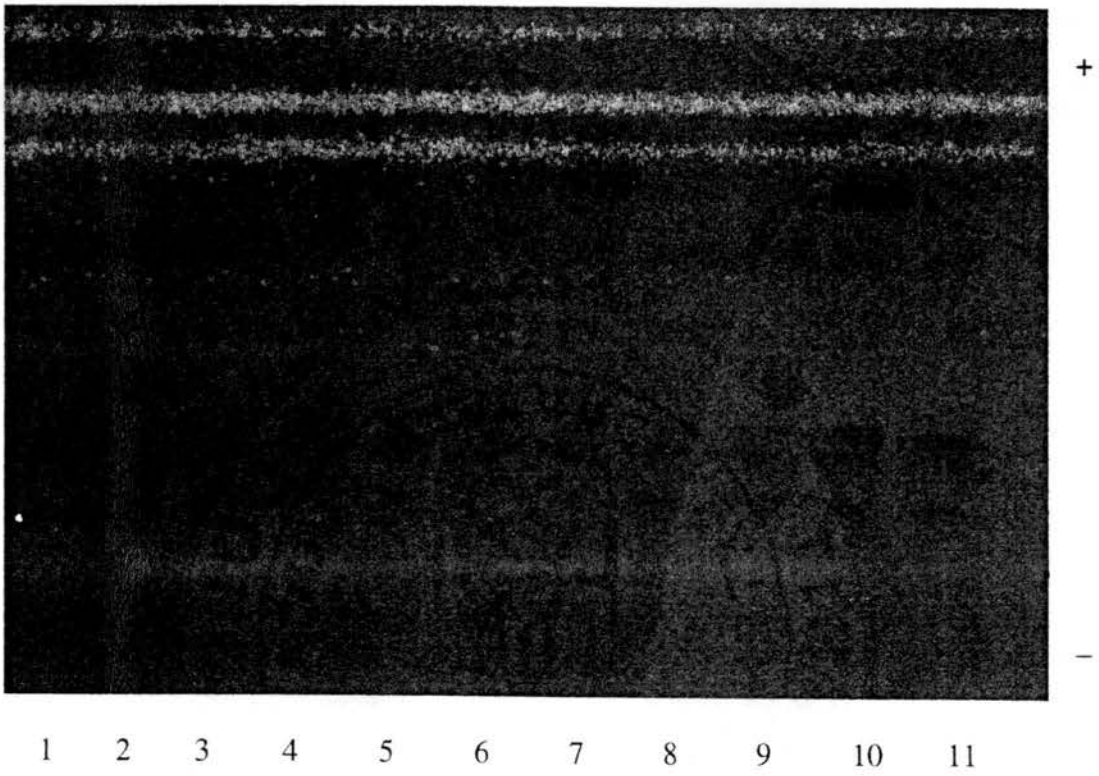


Fig. 3.1 Electrophoretic patterns of esterase isozymes of 11 chickpea accessions. Phenotypes from left to right: (1) F92-72C, (2) PCH 46, (3) ILC72, (4) ILC200, (5) F92-189C, (6) F92-112, (7) F92-139C, (8) ICC4935, (9) RH79177, (10) S85027 and (11)ICC4918.

fig3.2

PGM

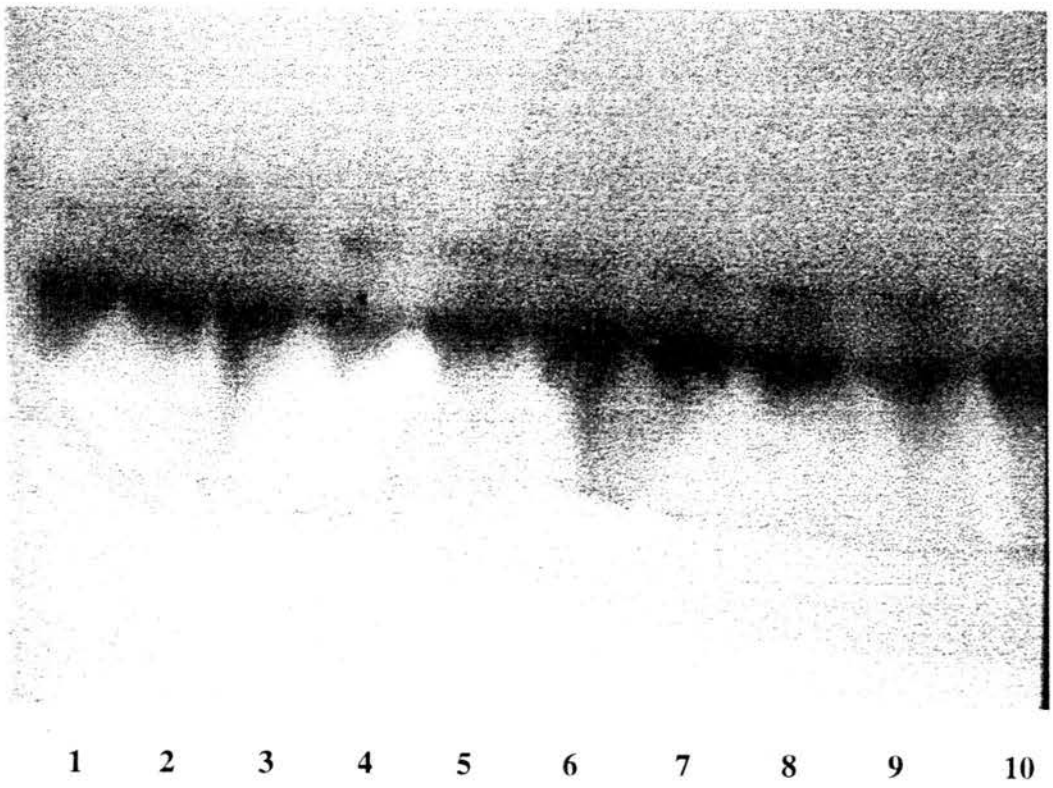


Fig. 3.2 Electrophoretic patterns of phosphoglucose mutase of 10 chickpea germplasm lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ILC 191, (4) F91-23C, (5) F83-92C, (6) F92-78C, (7) F84-156C, (8) F90-56C, (9) F91-14C and (10) F92-132C.

PGI

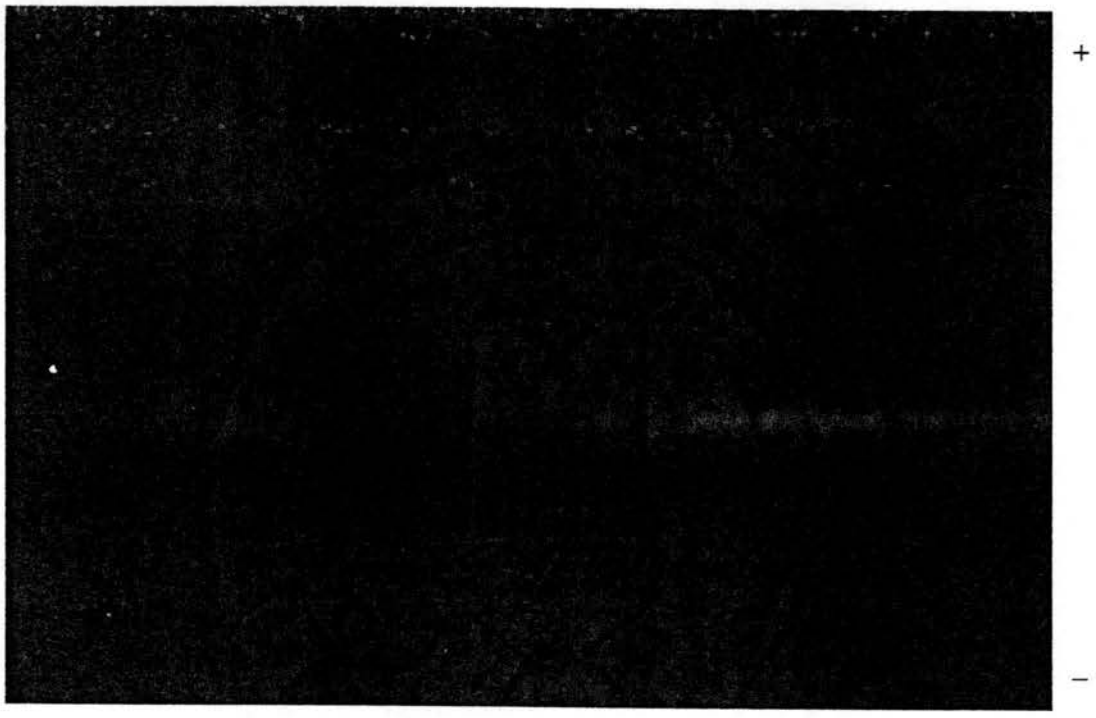


Fig. 3.3. Electrophoretic patterns of phosphoglucose isomerase isozymes of 12 chickpea germplasm lines. Phenotypes from left to right: (1)F88-83C, (2) F84-87C, (3)F84-87C, (4)ILC191, (5)ILC482, (6)PCH46, (7)ICC322, (8) F84-156C, (9)ILC1929, (10) F91-14C, (11)RH79177 and (12)F92-34C..

AAT

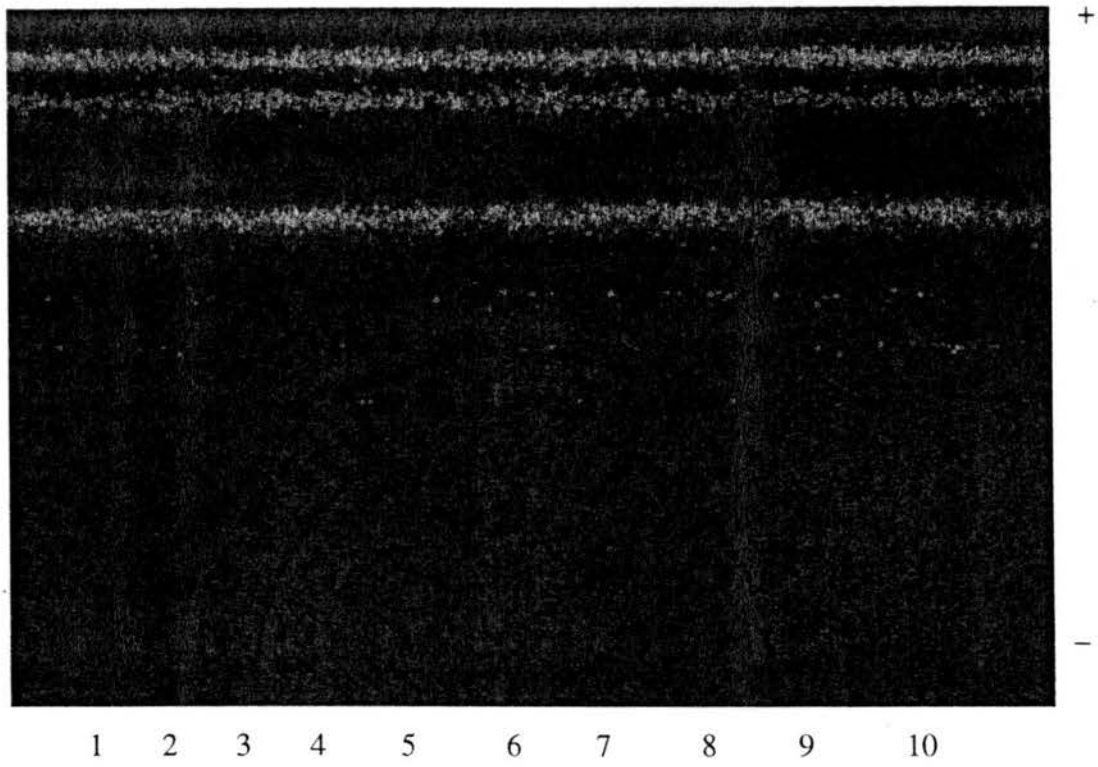


Fig. 3.4 Electrophoretic patterns of Asparate amino-transferase isozymes of different germplasm lines of chickpea. Phenotypes from left to right: (1)F88-83C, (2) F92-112C, (3) F91-8C, (4)ILC 482, (5) ILC 195, (6)ILC 200, (7) ICC 6098, (8) ICC 322, (9) PCH34 and (10) PCH 37.

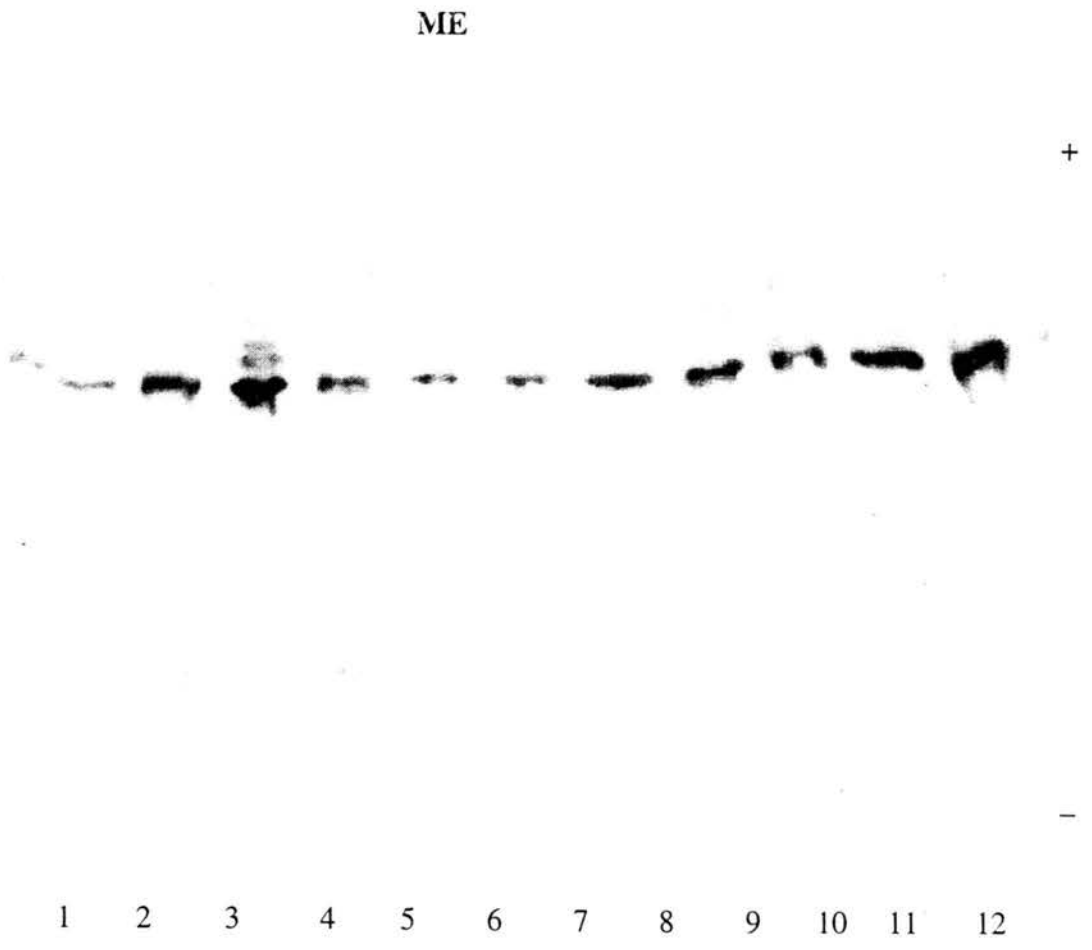


Fig 3.5. Electrophoretic patterns of malic enzyme isozymes of 11 chickpea germplasm lines. Phenotypes from left to right: (1)F88-83C, (2) ILC191, (3)F92-139C, (4) ILC5924, (5) PCH37, (6)ICC5003, (7) F88-85C, (8)F92-70C, (9)F90-56C, (10) S85085, (11) F92-187C and (12)F92-64C.

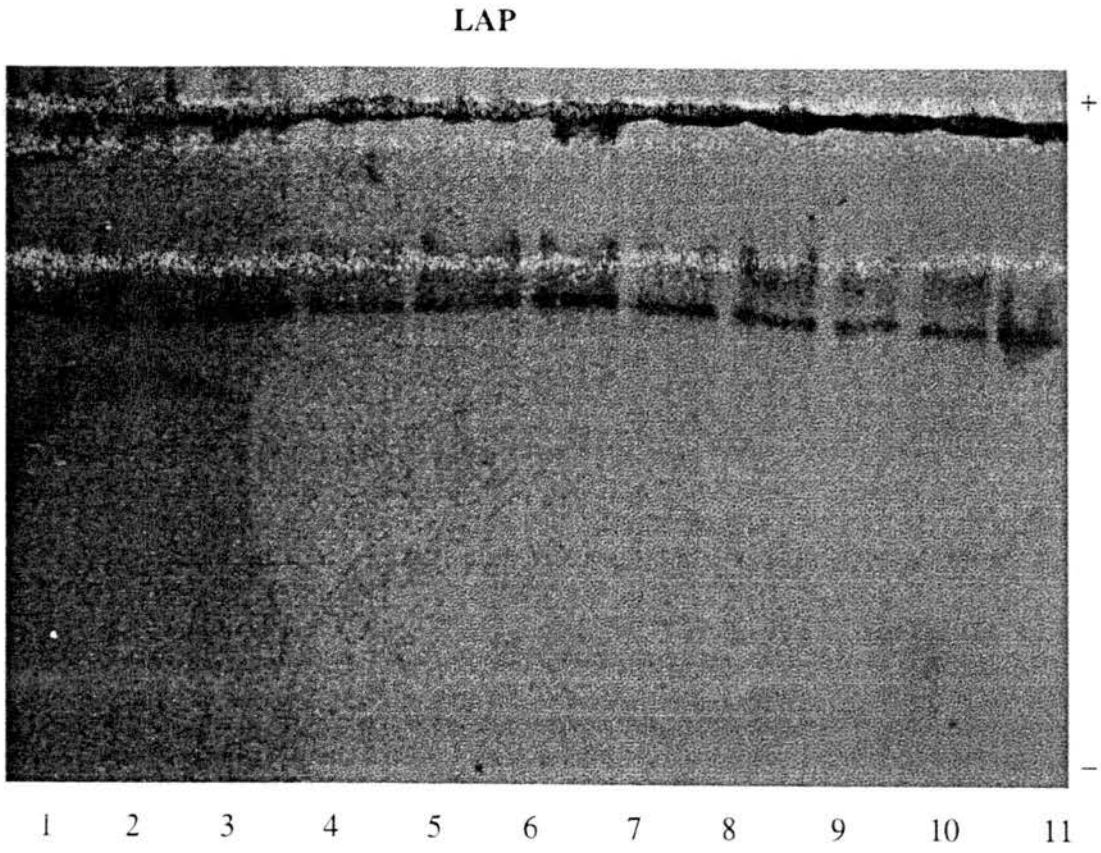


Fig. 3.6 Electrophoretic patterns of leucine aminopeptidase isozymes of 11 chickpea germplasm lines. Phenotypes from left to right: (1) F83-48C, (2) F83-77C, (3) ILC191, (4)F91-23C, (5)F83-87C, (6)F92-78C, (7) F84-156C, (8)F90-70C, (9)F91-14C, (10) F92-132C and (11)F85-54C.

6-PGD

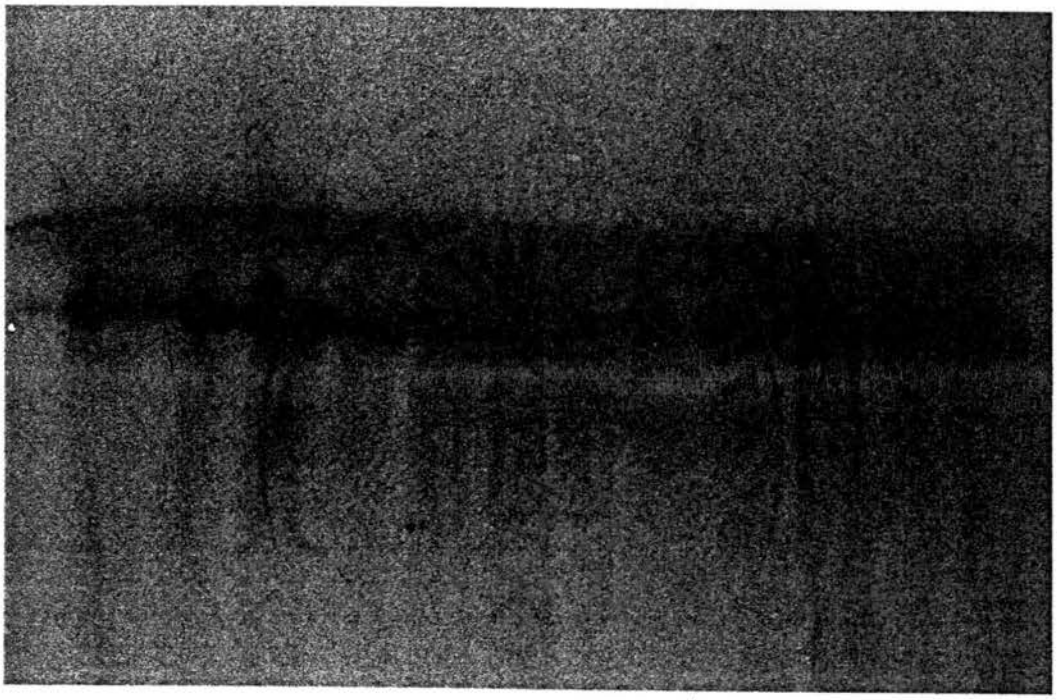


Fig.3.7 Some electrophoretic patterns of 6-PGD isozymes of different germplasm lines of chickpea. Phenotypes from left to right: (1) F92-72C, (2) F83-77C, (3) ILC 200 (4) ILC 482,(5) ILC 195, (6) ICC 322, (7) ICC 5003, (8) ILC 1929, (9) ILC183, (10)PCH 37,(110 Local and (12) F 83-92C.

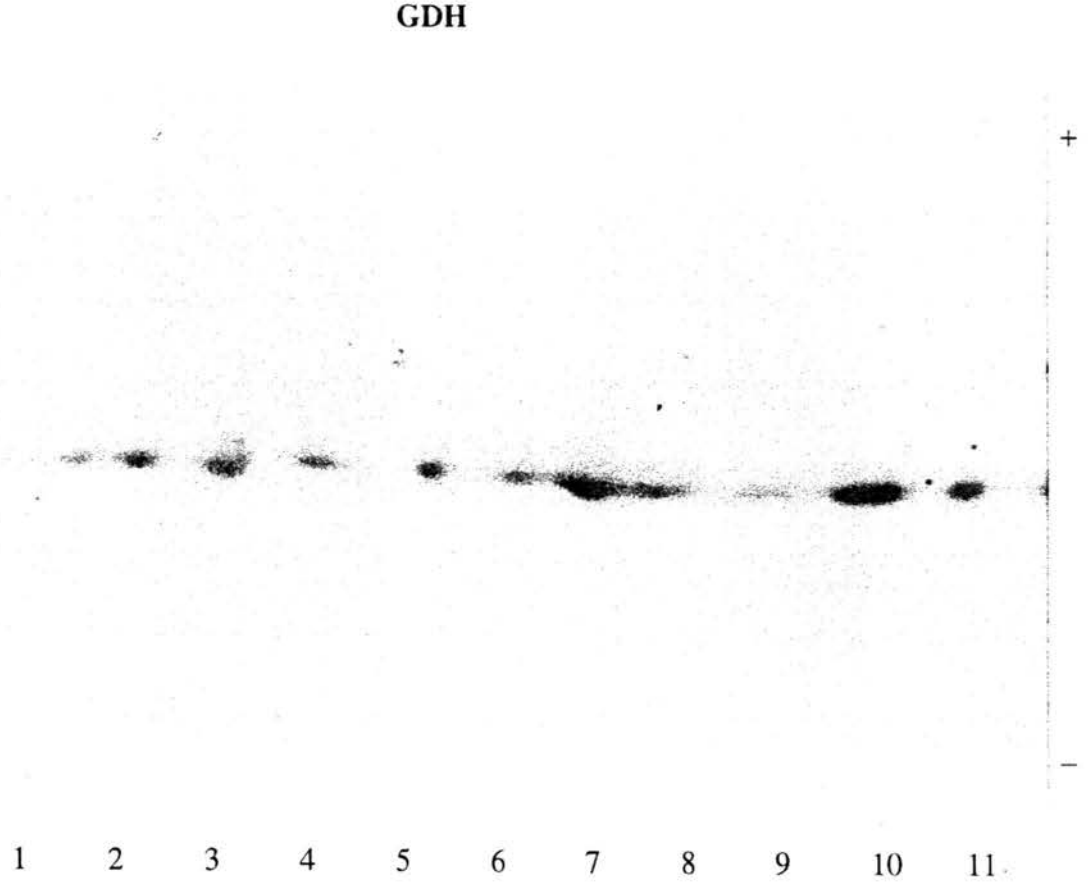


Fig. 3.8. Electrophoretic patterns of glucose dehydrogenase of 11 chickpea germplasm lines phenotypes from left to right: (1)F88-83C, (2)LOCAL, (3) ILC72, (4)F84-81C, (5) F91-23C, (6)F92-181C, (7)ILC5924, (8)PI4568, (9)PCH37, (10) F90-56C and (11)F92-78C.

ADH

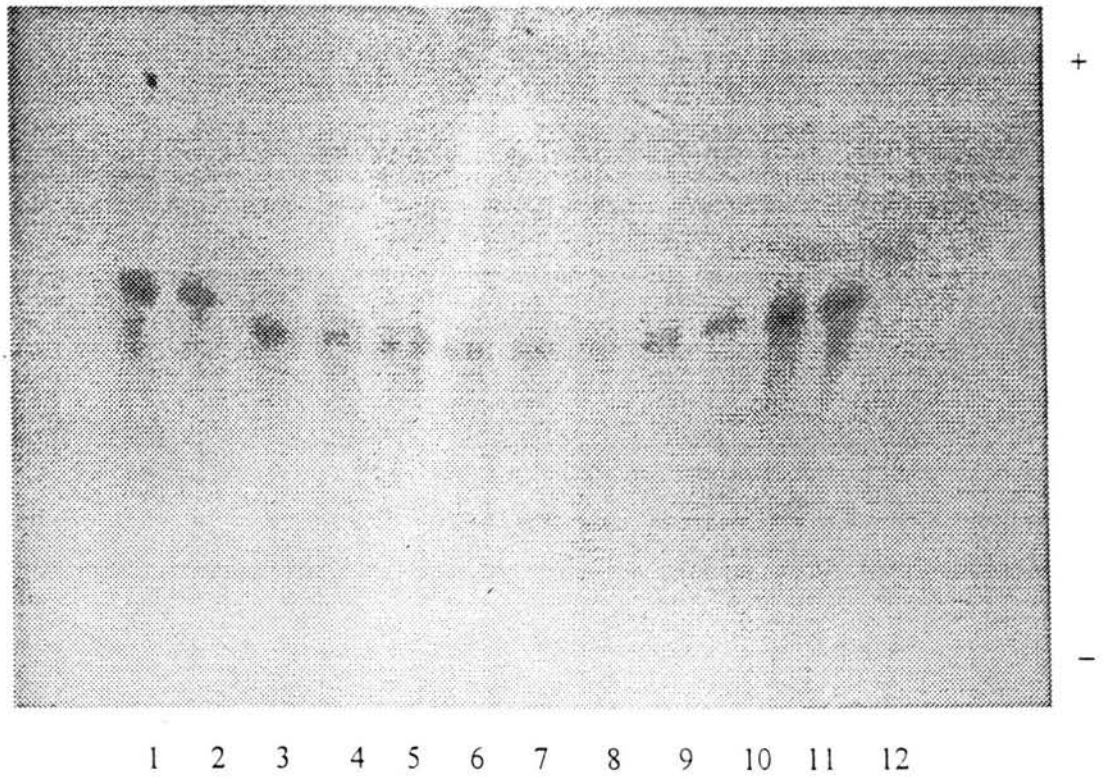


Fig. 3.9 Electrophoretic patterns of alcohol dehydrogenase isozymes of 12 chickpea lines. Phenotypes from left to right: (1) F88-83C, (2) F84-87C, (3) F83-77C, (4)ILC191, (5) ILC482, (6)PCH46, (7)ICC322, (8)F84-156C, (9)ILC 1929, (10)RH79177, (11) S85085and (12)F85-54C.

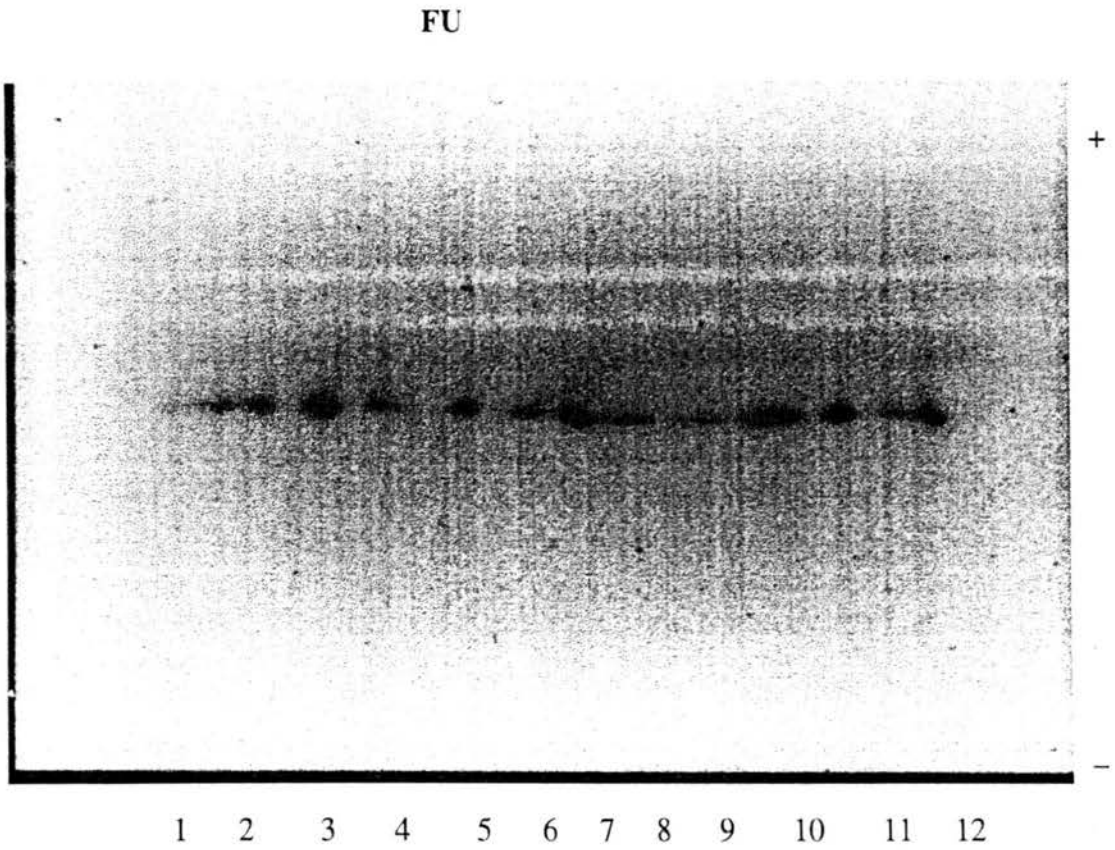


Fig. 3.10. Electrophoretic patterns of fumarase isozymes of different germplasm lines of chickpea. Phenotypes from left to the right:(1) F92-189C, (2)LOCAL, (3)ILC191, (4)PCH37, (5)ICC322, (6)F90-56C, (7)F91-14C, (8)ILC3279, (9)RH79177, (10)F92-187C, (11) F92-34C and (12) ICC4918.

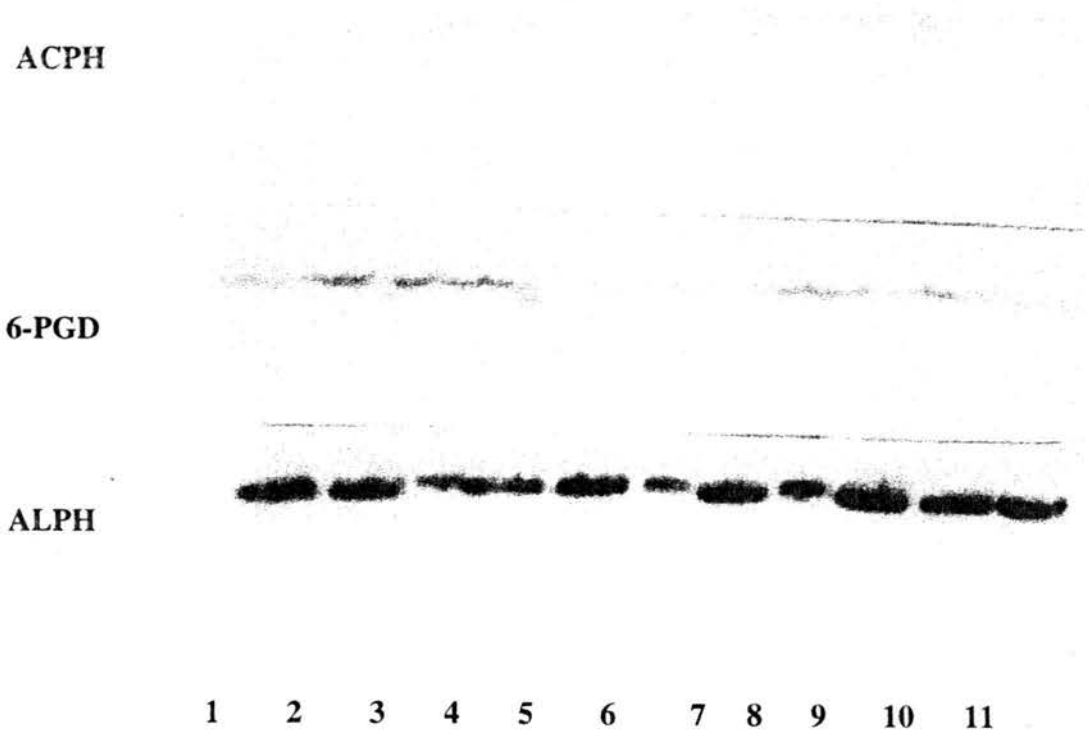


Fig. 3.11 Electrophoretic patterns of acid phosphatase, glucose phosphate dehydrogenase and alkaline phosphatase of 11 chickpea lines. Phenotypes from left to right: (1) F92-72c, (2)ILC 200, (3) ICC322, (4) ILC 3279, (5) PCH 37, (6)RH79177, (7) F92-187, (8) F92-139, (9) ILC1929, (10) PCH34 and (11) ILC 72.

APPENDIX 3

Table 4.3 Primers and their nucleotide sequences

Primer #	Nucleotide sequence	Primer #	Nucleotide Sequence
301	CGG TGG CGA A	351	CTC CCG GTG G
302	CGG CCC ACG T	352	CAC AAC GGG T
303	GCG GGA GAC C	353	TGG GCT CGC T
304	AGT CCT CGC C	354	CTA GAG GCC G
305	GCT GGT ACc C	355	GTA TGG GGC T
306	GTC CTC GTA G	356	GCG GCC CTC T
307	CGC ATT TGC A	357	AGG CCA AAT G
308	AGC GGC TAG G	358	GGT CAG GCC C
309	ACA TCC TGC G	359	AGG CAG ACC T
310	GAG CCA GAA G	360	CTC TCC AGG C
311	GGT MC CGT A	361	GCG AGG TGC T
312	ACG GCG TCA C	362	CCG CCT TAC A
313	ACG GCA GTG G	363	ATG AcG TTG A
314	ACT TCC TCC A	364	GGC TCT CGC G
315	GGT CTC CTA G	365	TAG ACA GAG G
316	CCT CAC CTG T	366	CCT GAT TGC C
317	CTA GGG GCT G	367	ACC TTT GGC T
318	CGG AGA GCG A	368	ACT TGT GCG G
319	GTG GCC GCG C	369	GCG CAT AGC A
320	CCG GCA TAG A	370	TCA GCC AGC G
321	ATC TAG GGA C	371	TCT CGA TTG C
322	GCC GCT ACT A	372	CCC ACT GAC G
323	GAC ATC TCG C	373	CTG AGG AGT G
324	ACA GGG AAC G	374	GGT CAA CCC T
325	TCT AAG CTC G	375	CCG GAC ACG A
326	CGG ATC TCT A	376	CAG GAC ATC G
327	ATA CGG CGT C	377	GAC GGA AGA G
328	ATG GCC TTA C	378	GAC MC AGG A
329	GCG AAC CTC C	379	GGG CTA GGG T
33O	GGT GGT TTC C	380	AGG AGT GAG A
331	GCC TAG TCA C	381	ATG AGT CCTG
332	AAC GCG TAG A	382	ATA CAC CAG C
333	GAA TGC GAC G	383	GAG GCG CTG C
334	ATG GCA AAG C	384	TGC GCC GCT A
335	TGG ACC AcC C	385	ACC GGG AAC G
336	GCC ACG GAG A	386	TGT AAG CTC G
337	TCC CGA ACc G	387	CGC TGT CGC C
338	CTG TGG CGG T	388	CGG TCG CGT C
339	CTC ACT TGG G	389	CGC CCG CAG T
340	GAG AGG CAC C	390	TCA CTC AGA G
341	CTG GGG CCG T	391	GCG AAC CTC G
342	GAG ATC CCT C	392	CCT GGT GGT T

Table 4.2 Primers and their nucleotide sequences(Cont'd)

Primer #	Nucleotide sequence	Primer #	Nucleotide Sequence
343	TGT TAG GCT C	393	TTC CAT GCC T
344	TGT TAG GCA C	394	TCA CGC AGT T
345	GCG TGA CCC G	395	TCA CTT GAG G
346	TAG GCG AAC G	396	CGM TGC GAG G
347	TTG CTT GGC G	397	GGG CTG TGC C
348	CAC GGC TGC G	398	CAG TGC TCT T
349	GGA GCC CCC T	399	TTG CTG GGC G
350	TGA CGC GCT C	400	CCC CTG ATAT
401	TAG GAC AGT C	451	CTA ATC TCG C
402	CCC GCC GTT G	452	CTA ATC ACG G
403	GGA AGG CTG T	453	AGT ACA AGG G
404	TCT CTA CGA C	454	GCT TAC GGC A
405	CTC TCG TGC G	455	AGC AAG CCG G
406	GCC ACC TCC T	456	GCG GAG GTCC
407	TGG TCC TGG C	457	CGA CGC CCT G
408	CCG TCT CTT T	458	CTC ACA TGC C
409	TAG GCG GCG G	459	GCG TCG AGG G
410	CGT CAC AGA G	460	ACT GAC CGGC
411	GAG GCC CGT T	461	CCC GTA TGT C
412	TGC GCC GGT G	462	CAT AGC GGC A
413	GAG GCG GCG A	463	AGG CGG MG C
414	AAG GCA CCA G	464	CAC AAG CCT G
415	GTT CCA GCA G	465	GGT CAG GGC T
416	GTG TTT CCG G	466	TTC TTA GCG G
417	GAC AGG CCA A	467	AGC ACG GGC A
418	GAG GM GCT T	468	ACG GAA GCG C
419	TAC GTG CCC G	469	CTC CAG CAA A
420	GCA GGG TTC G	470	AGG AGC TGG G
421	ACG GCC CAC C	471	CCG ACC GGAA
422	CAC CTG CGG G	472	AGG CGT GCA A
423	GGG TCT CGA A	473	ATC CCC AAGA
424	ACG GAG GTT C	474	AGG CGG GAAC
425	CGT CGG GCC T	475	CCA GCG TAT T
426	TCT CCC GGT G	476	TTG AGG CCC T
427	CTA ATC GAC G	477	TGT TGT GCC C
428	GGC TGC GGT A	478	CGA GCT GGT C
429	AAA CCT GGA C	479	CTC ATA CGCG
430	AGT CGG CAC C	480	GGA GGG GGG A
431	CTG CGG GTC A	481	GTA ATT GCG C
432	AGC GTC GAC T	482	CTA TAG GCC G
433	TCA CGT GCC T	483	GCA CTA AGA C
434	TCG CTA GTC C	484	CTG GCA AGG A

Table 4.2 Primers and their nucleotide sequences(Cont'd)

Primer #	Nucleotide sequence	Primer #	Nucleotide Sequence
435	CTA GTA GGGG	485	AGA ATA GGG C
436	GAG GGG GCCA	486	CCA GCA TCA G
437	AGT CCG CTGC	487	GTG GCT AGG T
438	AGA CGG CCGG	488	TTC GCT TCT C
439	GCC CCT TGAC	489	CGC ACG CAC A
440	CTG TCG AACC	490	AGT CGA CCT T
441	CTG CGT TCTT	491	TCC TGT CAAG
442	CTA CTC GGTT	492	GTG ACT GCT C
443	TGA TTG CTCG	493	CCG AAT CAC T
444	GCA GCC CCAT	494	TGA TGC TGT C
445	TAG CAG CTTG	495	CTT TCC TTC C
446	GCC AGC GTTC	496	CCT TTC AAG G
447	CAG GCT CTAG	497	GCA TAG TGC G
448	GTT GTG CCTG	498	GAC AGT CCT G
449	GAG GTT CMC	499	GGC CGA TGA T
450	CGG AGA GCCC	500	TTG CGT CAT G

These primers were from set 4 and 5 of the University of British Colombia.

Table 4.3. Relative quality and quantity of DNA from 56 chickpea accessions

Cultivar	OD260/OD280	DNA Concentration (µg/µl)
1) F 92-72C	1.88	2383.53
2) F 84-87C	1.86	3052.80
3) F92-189C	1.90	4302.30
4) F 90-112C	1.75	2960.40
5) F88-83C	1.92	2906.40
6) ICC 6098	2.00	1125.00
7) LOCAL	1.85	2227.14
8) ILC 200	2.01	1067.16
9) F 90-76C	2.17	2789.73
10) F 91-8C	1.90	3107.70
11) ILC 72	1.87	1725.60
12) F83-48C	1.88	4722.00
13) F83-77C	1.86	1272.00
14) F92-152C	1.87	7433.50
15) F84-81C	1.88	4026.90
16) F92-112C	1.89	3065.97
17) ILC 191	1.76	4329.39
18) F83-78C	1.82	1375.50
19) F91-23C	1.73	4578.60
20) F84-88C	1.90	4723.60
21) F92-139C	1.96	4509.60
22) F 91-62C	1.98	4724.10
23) F 92-181C	1.63	4672.11
24) F 83-92C	1.89	4427.99
25) ILC 195	1.91	1372.50
26) ILC 482	1.94	2522.70
27) ILC 5924	1.81	2190.50
28) ILC 183	1.89	4598.80
29) PI 456883	2.00	2472.90
30) PCH 46	2.03	2130.40
31) PCH 37	2.01	2473.44
32) ICC 4935	1.67	1762.90
33) ICC 322	1.89	3343.50
34) ICC 5003	1.90	3866.80
35) F 88-85C	1.91	8100.00
36) F 9056C	1.87	1566.00
37) F89-78C	1.97	4490.00
38) F92-78C	1.90	4455.00
39) F84-156C	2.01	1371.50
40) ILC 1929	2.00	4068.00
41) F92-70C	1.86	5486.09

Table 4.3 Relative quality and quantity of DNA from 56 chickpea accessions (Cont.'d).

Cultivar	OD 260 / OD 280	DNA Concentration (µg / µl)
42) 90-56C	1.90	5118.85
43) F91-14C	2.03	349.20
44) F84-109C	2.13	1130.40
45) ILC 3279	1.88	2567.50
46) F92-132C	1.89	2567.50
47) RH79177	2.10	3904.25
48) S850227	1.88	1224.90
49) S85085	1.90	1224.90
50) F92-187C	1.87	4048.50
51) F92-64C	1.99	5091.80
52) F92-34C	1.92	3510.00
53) F85-54C	1.96	6627.10
54) F92-18C	1.88	2171.00
55) ICC8933	2.01	1396.00
56 ICC4918	1.87	4557.00

Table 4.4. RAPD bands produced by primers screened against bulked DNA samples.

Primer#		Total bands/primer	Bulk #	Primer #		Total bands/primer	Bulk #
	490	9	5 & 6		440	1	1,2,3,4
gel	495	0	"	gel	446	0	"
1	496	0	"	7	447	3	"
	497	0	"		450	2	"
	498	3	"		456	4	"
	499	6	"				
	500	2	"	gel	363	1	5 & 6
				8	360	0	"
gel	466	0	5 & 6		359	0	"
2	467	2	"		355	0	"
	468	3	"		361	0	"
	469	0	"		362	0	"
					376	0	"
gel	387	5	5 & 6		365	0	"
3	388	3	"		366	0	"
	394	0	"		369	0	"
	389	6	"		374	0	"
	380	0	"		367	0	"
	391	2	"				
	384	4	"	gel	364	0	7 & 8
	393	0		9	356	5	"
	378	0	"		368	0	"
	376	0	"		352	4	"
					372	4	"
gel	390	0	5 & 6		357	6	"
4	395	4	"		358	0	"
	377	0	"		370	0	"
	397	0	"		351	0	"
	381	0	"		371	0	"
	379	2	"		375	0	"
	392	3	"				
	382	0	"	gel	426	4	1,2,3,4
	400	0	"	10	427	5	"
					428	9	"
gel	390	0	5 & 6		429	7	"
5	383	2	"		430	8	"
	399	2	"		436	0	"
	398	0	"		437	0	"

Table 4.4. RAPD bands produced by primers screened against bulked DNA samples (Cont.'d).

Primer#	Total bands/primer		Bulk #	Primer #	Total bands/primer		Bulk #
gel 6	479	1	5 & 6	gel 11	406	0	1,2,3,4
	480	2	"		407	0	"
	486	0	"	gel 15	408	0	"
	487	5	"		409	1	"
	488	0	"		410	1	"
	489	4	"		416	3	"
	485	3	"		417	2	"
gel 12	438	4	5 & 6	gel 16	402	3	5 & 6
	439	1	"		405	0	"
gel 13	437	1	5 & 6		460	0	5 & 6
	447	3	"		401	0	"
	412	5	"		422	1	"
	456	7	"		425	4	"
	404	0	"				
	467	0	"	gel 17	418	0	1,2,3,4
	415	0	"		419	0	"
	423	0	"		420	0	"
	424	0	"		457	0	"
	409	0	"		458	0	"
	446	0	"		459	0	"
	437	0	"		460	0	"
	403	0	"				
gel 14	470	1	5 & 6				
	476	0	"				
	477	0	"				
	478	0	"				

DNA bulks from 3 susceptible lines.
Bulked DNA 5 and 6: DNA bulks from 6 resistant lines (5) and (6) was a DNA bulk of six susceptible lines.