CHAPTER 1

INTRODUCTION

Production of cotton, the second largest agricultural foreign currency earner after tobacco and source of employment for millions of Zimbabweans, is severely limited by Verticillium wilt caused by *Verticillium dahliae* Kleb. The disease can cause substantial yield loss on susceptible varieties and yield loss of 10-100% has been recorded in some trials at Cotton Research Institute (Cotton Research Institute, AREX, unpublished data 2000). The fungus, which is soil borne, belongs to the fungal class Deuteromycetes (Fungi Imperfecti), (Gomez-Alpizar, 2003).

There has been an increase in the spread of the disease in Zimbabwe since it was first reported in Mazowe valley in 1966 (Chinodya, 1996). Verticillium wilt poses a serious threat to commercial cotton production because of difficulties encountered during the process of controlling the disease. A survey by Mapope (2001) revealed that 75% of the respondents in the Mazowe farming area had the wilt problem. It has become widespread in areas in the Lowveld, Kadoma, Glendale and Bindura. Disease severity varied from mild to severe in the Mazowe farming region depending on the variety being grown. Once the fungus has been introduced into a field, it can persist long periods thus making it difficult to eradicate and control (Foreman, 2001). The use of fungicides is not effective against the pathogen. However the use of tolerant cultivars may reduce yield loss (Foreman, 2001). Hence, resistant cultivars are of great importance in controlling the disease. Breeding for Verticillium wilt tolerance has become a major goal at the Cotton Research Institute (CRI) in Zimbabwe (Hillocks, 1991).

In screening trials carried out by the Cotton Research Institute over the years (Cotton Research Institute, 1989,1990, 1993 and 1994) variable results were obtained across the sites that were used. Results were not similar across the sites and they were not consistent. This could be due to a number of reasons including the possible existence of different strains of *Verticillium dahliae* in the country. The control of Verticillium wilt in Zimbabwe through the use of tolerant cultivars (which is a cheap way of controlling the disease) may be negated by the emergence of these strains. These strains will make breeding for resistance difficult and all material in the breeding programme may have to be exposed to these strains to check their reaction prior to release.

This study will seek to establish whether there are different strains of *Verticillium dahliae* in Zimbabwe and if so what their effect on some of the varieties grown is. A DNA amplification technique known as (Polymerase Chain Reaction (PCR). The characterisation of pathogen population according to variation in virulence is important for disease management strategies (Radisek *et al*., 2003) and results will help researchers to partition the country into zones and screening promising lines against predominant strain with the view of releasing the new varieties that are tolerant to the strain in these areas.

1.1 OBJECTIVES

The specific objectives of this study were:

1. To characterise *Verticillium dahliae* isolates from representative sites in Zimbabwe using Polymerase Chain Reaction (PCR).
2. To compare the virulence of isolates on five selected cultivars in the field and the green house

1.2 HYPOTHESIS

The hypotheses that were tested were:

1. There are no genetic differences in *Verticillium dahliae* isolates found in Zimbabwe
2. The virulence of the isolates on each of the selected varieties is the same.

CHAPTER 2

LITERATURE REVIEW

2.1 CLASSIFICATION OF *Verticillium dahliae*

V. dahliae is a soil borne fungus that belongs to the imperfect fungi. It is classified as follows (Agrios, 1988):

Sub division: Deuteromycotae

Class: Myphomycetales

Order: Thyphomycetales

Family: Dematiaceae

Sector: Nigrescentia

Genus: *Verticillium*

Species: *dahliae*

2.2 DESCRIPTION OF STRAINS

Isolates of *V. dahliae* frequently show variations in morphology and virulence in different plant species (Bell, 1992). Serological and autotrophic variants have been described elsewhere. The fact that different levels of virulence to cotton occur among strains of *V. dahliae* has been proved (Bell, 1992). Virulent strains have been designated as P-1 in the United States of America (USA) and less virulent strains have been designated as the P-2 strains (Bell, 1992), he also asserted that variants may arise as a consequence of mutation, parasexual (mitotic) recombination or heteroploidy.

Spontaneous mutations frequently occur in morphological types. The frequency of mutations can be greatly increased with ultraviolet light or toxic chemicals. In Zimbabwe, Chinodya (1996) observed physiological differences in terms of colony diameters and temperature optima for growth between *V. dahliae* isolates from three locations. The host range, virulence and other characteristics of these strains vary considerably. Some strains have a very broad host range while others are quite host specific, whereas some have a host range that overlap (University of Guelph, 2003). This suggested that these isolates might be different from each other.

2.3 VERTICILLIUM WILT DISEASE

The disease was first reported in 1914 on Upland Cotton (*Gossypium hirsutum* L) in California, USA (Bell, 1992). The disease is spread through infected soil or infected trash and sometimes contaminated seed (Elena, 1999). It was exacerbated by the cotton monoculture, which led to the build up of the inoculum in the soil. The fungus can infect roots causing several wilts after cotton is planted but does not usually spread through the vascular system until flowering has been initiated (Wheeler *et al*., 2000). In Zimbabwe, the disease normally appears around the sixth week after planting or towards the end of January and beginning of February for early-planted crops due to the drop in temperature around this period and falling rains. These conditions are favourable for the expression of the disease (Chinodya, 1996). Generally cool conditions during flowering period result in more severe wilt symptoms (Wheeler *et al*., 2000) than when dry conditions prevail during the same period

Primary infections of the disease are often caused by microsclerotia, which are capable of surviving in soil, in the absence of the host for many years (Wheeler *et al*., 2000).

In the world verticillium wilt has become a major disease in all major cotton growing countries (the United States of America, China and the former Soviet Union) and in Africa the disease is a problem in countries like South Africa, Sudan and Zimbabwe (Bell, 1992). Losses from the disease are greatest in the former Soviet Union, with 25-30% yield loss being recorded. In Zimbabwe the disease can cause a yield loss of almost 100% in susceptible varieties like the SZ 9314 and FQ 902 (Cotton Research Institute, unpublished data).

2.4 ABOVEGROUND SYMPTOMS

Verticillium wilt symptoms vary with cultivar, the virulence of the fungal isolate, stage of crop growth and the environment especially temperature (Bell,1992). Symptoms normally appear any time from six weeks after planting to boll split (Chinodya, 1996). The first symptom to appear is slight reddening or yellowing at the edge of a leaf. Chinodya (1996) observed that with time the colour of the leaves increases between the veins and more leaves develop symptoms. The leaves then become dry between the veins and are often shed from the plant.

The presence of the disease can be confirmed by peeling off the outer layers of the stem below where the characteristic brown staining would be observed. The disease causes stunting, wilting, early senescence and total plant death (Gilat Research

Centre, 2005). Plants may be stunted if the infection occurs early before flowering. Severely infected plants may shed leaves and bolls. Depending on prevailing conditions, plants that are infected early (before flowering) usually die or fail to produce any crop, but older plants (that have flowered) can tolerate the infection although with reduced yield. Extended periods of overcast and rainy periods may result in the severe wilt symptoms. Generally cool conditions during the flowering period result in severe wilt symptoms (Wheeler *et al*., 2000). Although plants may recover to some extent during periods of hot weather the yield would have been affected already due to the reduction of photosynthetic area as a result of leaf shedding or death.

2.5 ROOT INFECTION

Microsclerotia of *V. dahliae* from field soil consist of a few to 30 or more cells, which usually germinate when favourable conditions like cool temperatures and high relative humidity prevail (DeVay and Pullman, 1984). According to Saeed *et al.* (2003), infection of plants by *V. dahliae* begins with the germination of the dormant propagules in the soil. Germination of the propagule, is triggered by the the exudates from the roots of the host plant (Saeed *et al.,* 2003). Disease severity can be enhanced by the presence of nematodes like *Pratylechus spp* in the soil (Gilat Research Centre, 2005; Saeed *et al.,* 2003). Wounded roots may either stimulate the germination of the dormant microsclerotia of the fungus by increased root exudation or facilitate access for the fungus to the vascular cylinder. These two processes may occur together (Saeed *et al*., 1997). The germ tube or infection hyphae which emerges from the microsclerotia about 16 hours after germination begins to interact with the host roots and tend to penetrate directly uninjured areas of young roots. A growing hypha of the

fungus invades the root by passing through the epidermis cortex and the endodermis. Eventually the fungal hyphae enter the stele and colonise the plant systemically by growing and proliferating through the xylem elements of the vascular system (Saeed *et al.,* 2003). Alternatively the fungus may enter the xylem through the undifferentiated tissue near the root tip. The fungus colonises the xylem elements by means of mycelium and conidia. These are later transported upward by the transpiration stream (Garber, 1973). Xylem colonisation by the fungus increases the resistance to water flow within the plant, thus resulting in the leaf water deficits that lead to the reduction in leaf photosynthesis, transpiration rate and the leaf longevity (Adams *et al*., 1987). The hyphae grow both inter and intracellularly through the root cortex and eventually penetrates the endodermis and become established in the xylem tissue.

2.6 ECOLOGY

Most of the microsclerotia that serve as the inoculum in the soil are formed in the undecomposed plant residues. The pathogen over winters in the soil as microsclerotia (Agrios, 1988). The development and longevity of *V. dahliae* survival structures which include microsclerotia greatly depends on soil temperature, soil or plant tissue moisture and population of other soil borne microorganisms like *Trichoderma virens* (Devay & Pullman, 1984 and Hanson, 2000). Populations of microsclerotia of *V. dahliae*, although they decline with time may remain stable for more than 20 years in the soil (DeVay *et al*., 1974). DeVay, and Pullman (1982), citing Green (1962) reported that hyphae and conidia are short-lived in soil and persist for less than three to four weeks in contrast to more persistent microsclerotia. However Green (1962) cited by DeVay and Pullman (1982) observed rapid decline of microsclerotia under

low soil matric water potential and high soil temperature (at 28^{o}C microsclerotia remained viable for up to 3 to 4.5 years depending on soil type).

Soil conditions directly affect the pathogen, alter host resistance or change relationships with other microorganisms. Verticillium wilt normally occurs in the neutral or slightly alkaline soil, growth and microsclerotia production by the fungus is inhibited by soil pH of 5.5 or below (Bell, 1992). Bell (1992) urges that the fungus is a soil invader rather than a soil inhabitant, because it is unable to grow appreciably as a saprophyte on tissue other than that which it originally parastised. Practices that increase the activity of saprophytes without other wise favouring the disease often decrease wilt severity.

2.7 EPIDEMIOLOGY OF VERTICILLIUM WILT ON COTTON

Verticillium wilt is a single cycle disease; inoculum levels of *V. dahliae* in the soil at planting time play a critical role in the disease development (Paplomats, Bassett, Broome and DeVay, 1992). Disease incidence is usually assessed as the percentage foliar symptoms or vascular discoloration. The main factors that influence the epidemiology of Verticillium wilt in cotton are the pathotypes and inoculum density of *V. dahliae* in the soil, air temperature, soil moisture, plant density, potassium and nitrogen availability during the growing season (DeVay and Pullman, 1984). Temperature and moisture play a very crucial role, in the development of the disease, the development is greatly reduced by temperatures exceeding 28-30^{o}C. If temperature during the night falls to between 10-15^{o}C the incidence of the disease

increases. Research has proved that the effect of temperature is dependent on moisture availability. Under hot air conditions the foliar symptoms are suppressed. DeVay and Pullman (1984) observed that these factors are directly related to the incidence and time of appearance of foliar symptoms of Verticillium wilt and the time of appearance of the disease has a bearing on the lint yields in cotton. DeVay *et al.* (1974) observed that the incidence as measured by vascular discoloration at the time of harvest was closely related to inoculum density at the time of planting. However, disease incidence as indicated by presence of foliar symptoms was variable and did not always reflect inoculum density when many fields were compared.

2.8 CONTROL

No single method is highly effective in controlling the disease (Bell, 1992). Due to the nature of the disease preventative approaches rather than curative are recommended. Integrated management system is necessary to minimize losses from the disease. Bell (1992) asserts that control begins with the selection of cultivars that have some degree of resistance to wilt, good agronomic practices and adaptation to the geographical location. The combination of cultural practices with good cultivars can help to minimise the losses from wilt.

2.8.1 Resistant Cultivars

The primary method of controlling Verticillium wilt is the use of resistant or tolerant varieties. Resistance to Verticillium wilt is associated with the growth stage of the plant and is maximum during the flowering stage (Garas, Wilhm and Sagen, 1986). Garas *et al*. (1986) observed that resistance to *Verticillium dahliae* is associated with the production of some antifungal compounds that take place immediately after

infection. Methylated sesqueterpenoid phytoalexins have been identified as some of the compounds. Ride (1983) asserts that mechanisms which trap spores are thus likely to be important physically limiting the spread of fungus in the plant. These physical barriers may allow the accumulation of antifungal chemicals (phytoalexins) and the sealing off of the infected vessels to prevent the upward movement of fungal enzymes and the toxins. The rapid formation of gels and tyloses has frequently been associated with the resistance of the plants to vascular pathogens with the susceptibility being correlated with poor or delayed response (Ride, 1983).

Resistance to Verticillium wilt is expressed as a delay in the onset of visible symptoms and decrease in symptom severity especially during the second half of the season. In Zimbabwe, resistant cultivars are those that show a lower percentage of plants with foliar symptoms after the first boll set and when temperature begins to decline usually around February-March period (Chinodya, 1996). Cultivars with moderate to high levels of resistance to Verticillium wilt have been developed in several countries, with Zimbabwe having developed G501 (Hillocks, 1991). Currently BC 853 is recommended for areas with wilt problems. However its yield and Gin Out Turn (GOT) are slightly lower than conventional varieties like SZ9314 (has GOT of 42% compared to BC853 that has a GOT of 35%, Cotton Research Institute, 2004). A greater proportion of breeding time is devoted for the breeding of tolerant cultivars at Cotton Research Institute. Lines (advanced varieties awaiting release) that have tolerance to the disease in the Cotton Research Institute's breeding programme have low GOT and yield 60% lower than the established varieties like SZ 9314 (Cotton Research Institute, 2002 unpublished data).

2.8.2 Cultural Practices

According to Bell (1992) cultural practices used to control Verticillium wilt are aimed at preventing the introduction and establishment of the pathogen in the soil, soil eradicating or reducing inoculum potential in the soil and to optimise the expression and use of resistance.

To achieve these above objectives, there is need to manipulate crop sequence, nutrients, soil moisture, planting practices, tillage practices, chemical and biological control. The benefits derived from different control practices will depend on the cultivars, the inoculum concentration, the prevalence of different strains of *V. dahliae* environmental conditions and the physics, chemistry and biology of the soil (Bell, 1992).

Repeated planting of cotton or tomato on the same field or rotating them with other host plants results in the increase of the population of the wilt fungus in the soil (Buttterfield, DeVay and Garbar, 1978). On average an increase of 13 to 15 propagules per gram soil per year has been recorded in soil that is continuously planted to cotton (Pullman and DeVay, 1992).

Fibre hemp, *Canabis sativa* L., is one of the crops that can effectively reduce the *V. dahliae*. Experiments by Kok, Coenen and de Heji (1994) demonstrated that uprooting the fibre hemp from infected fields before it dies off naturally would inhibit the formation of the microsclerotia thus reducing the population in the soil. Bell (1992) urges that any disruption of continuous culture of susceptible cotton cultivars will reduce the incidence and severity of wilt compared with continued monoculture Rotation of cotton with cereals is recommended as a control measure against

verticillium wilt (Mathre, 1989). One-year rotations do not appreciably reduce the existing inoculum potential. However work by Chinodya (1996) showed that some sorghum varieties reduce the density of V. dahliae in the soil by stimulating germination of microsclerotia in the absence of a host. He suggested that rotation of sorghum with cotton might help to reduce disease incidence in Zimbabwe.

The deficiency of nutrients to growing cotton may exacerbate the wilt problem (Bell, 1989). The deficiency of potassium increases the severity of wilt, presumably by increasing host susceptibility. Research at the Cotton Research Institute demonstrated a linear response to muriate of potash application of 0, 200,400, and 600 kg/ha and it was noted that correcting potassium deficiencies decreased wilt and increased yield (Cotton Research Institute Annual Report, 1993). It was found that fertilising of cotton fields with potassium helped to decrease verticillium wilt. However the percentage of affected plants and the severity of symptoms increased with rate of nitrogen application and this response was amplified with potassium deficiency (Chinodya, 1996).

Decreasing irrigation frequency or amounts especially during flowering help to reduce wilt severity. Increasing plant density by using narrow row spacings in cotton fields has proved to be effective in reducing wilt (Cotton Research Institute Annual Report, 1993).

2.8.3 Chemical Control

Benzimidazole fungicides that are systemic in cotton gave complete control of the disease when applied as drenches to infected cotton. Bell (1992) was able to have complete control and yield restoration with 50-100kg/ha of Benomyl. The adoption of

chemical control of verticillium wilt has been very slow due to the fact that these chemicals are expensive.

Fumigating with chemicals like methyl bromide, ethylene bromide, chloropicrin can reduce the population of *V. dahliae* in the soil. However the chemicals used in the fumigation are very expensive and can therefore be used in high value crops and not cotton.

2.9 POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is an innovative tool for molecular biology that has had a huge impact. PCR exploits a remarkable natural function of the enzymes known as polymerases, which one found in living things with the job to copy genetic material (Powledge, 2005). PCR can be used to characterise, analyse and synthesise any specific piece of DNA or RNA. The reaction uses two oligonucleotide primers that hybridise to opposite stands and flank the target DNA sequence that has to be amplified. The elongation of the primers, is catalysed by a heat-stable DNA polymerase called Taq polymerase (Application Manual, 1999. Roche Molecular Biochemicals). The enzyme was derived from a bacterium called *Thermus aquaticus*, which survives in hot springs. PCR according to Powledge (2005) requires a template molecule of the DNA or RNA you want to copy and two primer molecules to get the copying process started. The primers are typically short single stranded olinucleotides, which are complementary to the outer regions of the known sequence (Blaber, 1998). They have the 5` end and the 3` end and they are made in pairs with opposing end and anneal at the complimentary ends of the target DNA.

Primer annealing according to Blaber (1998) is an important parameter in the success of the PCR experiment. The annealing temperature is characteristic for each oligonucleotide; it is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer. Estimates of annealing temperatures can be calculated and they are a starting point for any PCR but ideal temperatures should be determined empirically. Most Primers anneal at temperatures of between 45-55^{o}C.

Primer extensions are usually done at 72^{o}C or the optimum temperature of the DNA polymerase. The length of time the primer extension steps can be increased if the region of the DNA to be amplified is long. However in the majority of PCR experiments an extension time of two minutes is sufficient to get complete extension. The number of cycles usually varies between 25 and 35. More cycles mean a greater yield of product twenty cycles can produce one million copies (Heyer, 2004). Since the extension products of the reaction are complementary to and capable of binding the primer successive cycles of amplification essentially double the amount of target DNA synthesised in the previous cycle. The result is an exponential accumulation of the specific target fragment, theoretically 2^{n}, where n is the number of amplification cycles performed. However with increase in the number of cycles, the greater the probability of generating various artefacts (mispriming products).

The PCR is very simple, quick and exceedingly sensitive (Heyer, 2004). A single molecule of DNA can be sufficient. The one problem of PCR is related to its major asset, its sensitivity. The PCR is prone to contaminations and much care must be taken not to contaminate PCR reactions with extraneous DNA (Heyer, 2004). Factors

such as enzyme, dNTP, primer, magnesium concentrations also affect the PCR product yield and denaturation temperatures affect the efficiency of the PCR. DNTPs provide bases that are used in the extensions, while the magnesium provide conditions that are required for the enzyme to work, so any variations of these parameters can easily influence the results ([1]Sandasi, 2005, Personal Communication). PCR is very sensitive to the levels of divalent cations (especially Mg^{2+}) and nucleotides so conditions for particular application have to be worked out (Heyer, 2004)

The advent of PCR exponentially increased the availability and affordability of different molecular marker techniques, PCR has revolutionised molecular biology because it is simpler, less expensive than the previous techniques for duplicating DNA. PCR has democratised genetic research, putting it within reach of all biologists even those with no formal training in molecular biology (Powledge, 2005).

Fingerprints are possible due to the existence of difference in the nucleotide sequence between individuals (Archak, 2000). A typical fingerprinting generate molecular markers that should be polymorphic enough to discriminate between cultivars but not too polymorphic to throw up the differences within individuals of cultivars. Fingerprinting techniques differ depending on how the DNA fragments are generated and assayed. Fragments are generated by either restriction digestion or by the PCR (Archak, 2000). Fragments are separated by agarose or polyacrylamide gel electrophoresis and detected by various approaches and all fragments on gel are directly visualised by staining with ethidium bromide. Gel electrophoresis takes advantage of the overall negative charge possessed by nucleic acids, due to the phosphates in the backbone (www.emunix.emich.edu, 2005). Because nucleic acids

[1] Sandasi, M., Research Officer, Tobacco Research Board

are negatively charged, they migrate in an electric field from the negative electrode to the positive electrode. If the electric field and the nucleic acids are run in a semisolid matrix (usually a gel) the nucleic acids will migrate at speeds inversely proportional to their size

PCR based marker techniques are grouped based on sequence, length number of primers and size range of the amplified products. Amplification using designed primer PCR loci differences in the fragment length can often be analysed as codominat alleles in diploid genomes. Amplification using designed primers flanking microsatelites (SSRs) display polymorphism in the length and number of amplicons. Polymorphism appears because variation in the number of tandem repeats in a given modification. Randomly amplified polymorphic DNA analysis (RAPD) requires no prior sequence information. A single arbitrary sequence (usually ten nucleotides) is used with formed and reverse primer to generate a set of fragment with with amplification range. The number and length of fragment vary between primers and genotypes. Many PCR assays are minor modification or combination of designed primer and random primer PCR, to increase reproducible results. A rbitirary. Restriction fragment length polymorphism (RFLPs) are based on the detection of variations in the restrictions fragment length of specific DNA sections among individuals as determined by presence or absence of restriction sites. It possible to locate a gene by looking for RFLPs that are almost always inherited with it. Primed PCR (AP-PCR) amplifies discrete patterns by employing single primers of 10 to 50 bases in length for amplification. Anchored PCR is another variation which uses one designed (anchored at 5`or 3` termini) and one arbitirary primer to amplify unknown sequences one more such variation is nested PCR. In this techniques one amplification reaction with two designed primers is followed by a second reaction.

The first PCR product is the template for the second reaction, which uses two designed primer internal to the first set. Nested PCR is Useful for extremely sensitive genotypes.

The use of PCR to study strains has been employed to study the genetic relatedness of *Trichoderma* and *Gliocladium spp* (Bulat *et. al*., 1998). PCR offers rapid and accurate study of population diversity in the *Trichorderma* and *Gliocladium*. In Australia RAPD-PCR has been used in the identification of *Verticillium dahliae* iosolates with differential pathogenicity on cotton (Ramsy *et al*., 1996). They investigated genetic relatedness of the isolates and they also wanted the molecular techniques to act as a basis for identification of the pathogen. Molecular techniques they used failed to pick differences from the isolates (Ramsay *et al*.,1996)

CHAPTER 3

MATERIALS AND METHODS

3.1 FIELD TRIALS

3.1.1 Sites

Five sites were used in this study these were Chinhoyi, Cotton Research Institute, Chisumbanje, Henderson and Rafingora. Chose of the sites was based on their history of Verticillium wilt disease incidence and they have been used in Cotton Research Institute Verticillium wilt screening programme over the past five years. Below is a brief description of the sites.

Cotton Research Institute lies three kilometres northwest of Kadoma on coordinates 18^{o}C 19`S and 29$^{o}$C 53`E at altitude 1156m. The average rainfall at the station is 743mm while the average maximum and minimum temperature over the years has been 28^{o}C and 14.2^{o}C respectively.

Henderson Research Station lies fifteen kilometres North of Harare on coordinates 17^{o}C 35` and 30$^{o}$58` E at altitude 1300m. The average rainfall at the station has been 870mm over the years, with maximum and minimum temperature of 21^{o}C and 12.3^{o}C respectively.

Chinhoyi (Eastrange farm) lies 15km North of Chinhoyi Town The site lies in region 2a and has deep clay loamy soils.

Chisumbanje lies in the South East lowveld in the region 4 and the soils are deep vertisols

3.1.2 Trial Design

At each site the five varieties were laid in a randomised block design, which was used with four replications per treatment. The varieties constituted the treatments and were randomly allocated to plots at each site.

3.1.3 Treatments

The trials were conducted in the 2003/04 season at the following sites Chinhoyi, Henderson Research Institute Chisumbanje, Rafingora and Cotton Research Institute The above sites were selected based on their history of wilt infestation over the last five to ten years. Released and prereleased cultivars were used in the trials. These varieties were chosen based on performance at Cotton Research Institute trials and some are grown commercially and one is awaiting release. The varieties were 563-97-12 (a pre-release), BC 853 (a variety recommended for wilt infested fields), G501 (tolerant variety), SZ 9314 (a commercially grown variety) and FQ902 (commercially available cultivar). Fine tilth was obtained in all the fields. At each site the trials were planted with the first effective rains at the site (The first effective rains fell at the end of November 2003 and all the trials were established around this period. At each site a treatment (a variety) was planted to eight rows that were 5.4m long. The first row and the last row were treated as discards giving a net plot of six rows that were 5.4m long giving a net plot of 32.4 m^2 each treatment in each replication. Spacing was 0.9 m by 0.3 m. Pathways of one metre separated replications. Compound L was applied at

250kg/ha at planting and the 200kg/ha of Ammonium Nitrate was applied at flowering. Pest control was based on scouting a results, spraying was only done when the pests had reached threshold levels. All the trials were established as dryland, irrigation was only applied when there was water stress.

3.1.4 Data Collection

Monthly disease infections were recorded as from January to May 2004 and they were used to derive infection percentages calculated by dividing number of infected plants by total number of plants in the nettplot. Disease severity scores of 1-5 scale by Cotton Research Institute were used for each row per plot and then an average was then derived from these scores. Severity scores were done once per every two weeks as from end of January 2004 up to the end May 2004 at each site. Severity scores were recorded at the following dates Chinhoyi (25/02/04,9/03/04, 6/04/04, 20/04/04, 7/05/04 and 20/05/04), Chisumbanje (10/03/04, 24/03/04, 7/04/04, 21/04/04, 6/05/04 and 19/05/04) Henderson (26/02/04, 11/03/04, 25/03/04, 1/04/04, 15/04/04, 29/04/04 and 13/05/04), CRI (9/02/04, 23/02/04, 8/03/04, 22/03/04, 5/04/04, 20/04/04 and 3/05/04) and at Rafingora scoring was done at the following dates (26/02/04, 11/03/04, 25/03/04, 8/04/04, 4/05/04, 18/05/04 and 1/06/04). At maturity picks for each plot were done using standard picking bags and the weights were recorded.

3.1.5 Data Analysis

Infection percentages were arc sine transformed to normalise the dataand then subjected to analysis of variance using Genstat Statistical package. Severity scores were used to construct disease progress curves. The scores were also used to calculate area under the disease progress curve (AUDPC) with Sigma Plot trapezoidal rule.

Values obtained were then subjected to analysis of variance using the above software to compare effects of the disease on the varieties. Yield records were subjected to analysis of variance using Genstat 3.2. A combined site analysis was carried out on the infection percentages and AUDPC.

3.2 GREEN HOUSE TRIAL

3.2.1 Soil Sampling

Five soil samples were collected from the sites used in the experiment one. Areas to sample within a site were chosen in a random pattern. First a 5-8 cm layer of topsoil was removed from an area that was to be sampled. Using a soil sampling tube, a 25 cm soil core was removed and put in a bag. The soil cores from each sampling area were then bulked and hand mixed. The samples were then air dried in paper bags for 4-6 weeks.

3.2.2 Fungus Extraction

Using the Anderson Sampler 500mg of air-dried soil was distributed evenly through six sieve plates onto six petri dishes containing 20 ml of soil extract agar covered with cellophane pressed to the agar surface. Colonies of *Verticillium dahliae* were recovered form the microsclerotia that germinated after ten days. Repeated sub culturing onto Potato Dextrose Agar (PDA) was used to purify the fungus.

3.2.3 Trial Design

Using the same varieties used in the field experiments and the five isolates, a 5*5 factorial experiment was conducted. The trial was laid in a split plot design with

verticillium isolates as main plots and varieties as subplots. Treatments were replicated three times. A pot constituted a plot (single plant plots).

3.2.4 Treatments

Soil used was sterilised by oven heating to get rid of any potential incoculum. Nine grams of compound L was applied per pot as basal dressing. Temperatutre in the green house was kept at 27^{o}C with fluctuation of plus or minus 0.5 ^{o}C. The trial was regularly watered. The trial was also top dressed with eight grams of Amonium Nitrate at six weeks after germination.

3.2.4 Inoculation

Inoculum was prepared from the shake culture of *Verticillium dahliae* isolate grown on Czapek Dox Broth (Hillocks, 1991). The inoculum for each isolate was adjusted to a concentration of 2*10^{6} conidia/ml using a haemocytometer. At six weeks after emergence, plants were stem inoculated at the base of the plant using sewing needles dipped in the conidia suspension of each isolate.

3.2.5 Data Collection

The severity scoring was according to Hillocks (1991). And below is an illustration of the Scoring System that was used:

1	no symptoms
2	Symptoms confined to vein discolouration or slight chlorosis
3	Several leaves showing chlorosis
4	Many leaves showing chlorosis some with necrotic areas
5	Most leaves chlorotic some with severe necrosis usually some defoliation
6	Whole plant affected, top most leaves necrotic and shedding of lower leaves

Severity scores were done a week after inoculation and then once per week for four weeks.

3.2.6 Data Analysis

Severity scores were used to calculate area under the disease using the Sigma plot. The AUDPC values were then subjected to analysis of variance using Genstat 3.2 statistical software. Severity scores were also used to plot disease progress curves using Microsoft excel

3.3 STRAIN DIFFERENTIATION

3.3.1 DNA Preparation

Five isolates and the standard *Verticillium dahliae* that had been stored on slates were each revived on Potato Dextrose Agar. After three days small pieces of hyphae were inoculated onto Malt Extract Broth (Appendix 18). The flasks with the malt extract were put onto a shaker for 72 hrs at 200 rpm at room temperature (25^{o}C).

3.3.2 DNA Extraction[2]

Broth with the fully grown fungi was squeezed through a sterile mutton cloth to collect mycelium. For each isolate 200mg of mycelia was placed into an eppendorf tube. The eppendorf tube was then washed three times in 1000µl of TE buffer. Before each wash the tube was centrifuged for ten minutes. Mycelia were suspended in 500µl of the extraction buffer, 100µl of 10% Sodium Dodecyl Sulphate (SDS) and 600µl of benzyl chloride. The suspension was heated for thirty minute at 50^{o}C. After heating 300ml of 3M Sodium Acetate was added and the solution was cooled to -20 ^{o}C for fifteen minutes. After cooling, the solution was centrifuged at 12 000 rpm for ten minutes. Supernatant was collected and two-thirds volume isopropanol was added and then stored in the freezer for 2hrs. The precipitate was then centrifuged at 14 000 rpm

[2] Protocol provided by Ms M. Sandasi (Tobacco Research Board)

for 10 minutes and then washed with 70% ethanol and finally centrifuged for five minutes. Pellets were then dried using vacuum for 30 minutes. The pellets were than dissolved in 70µl of TE buffer and then stored at -20 ^{o}C until ready for use.

3.3.3 Analysis of Nucleic Acids by gel electrophoresis[3]

After the extraction of DNA from the isolate, nucleic acids were analysed to check if the extraction was successful. 1.5g of the extraction were weighed into a 50ml flask and 100ml TBE was added into the flask. The flask was cooled and 5µl ethidium bromide was added. Gel was then allowed to set. After the gel had set TBE was added to cover the surface of the gel. 5µl of digest digests was loaded into fresh tube. 5µl f loading dye and 5µl was then loaded. The tubes were then spinned for 2 minutes. Samples were loaded in wells of 1.5% Agarose gel ((prepared by weighing 7.5g of Agarose into 500ml TBE(0.5%) and the adding 25µl of ethidium bromide)) and the gel tank was connected to powerpac. Power was switched on and voltage was adjusted to 100, and run for 30 minutes, after 30 minutes, power was switched off and electrodes were disconnected. Gel was removed carefully from the tank and view on UV light and photograph was taken

3.3.4 PCR Reaction and Gel Electrophoresis

Primers:

The following Primers for RFLP developed by Carder *et al*. (1994) were used

19/22 5’-CGG TGA CAT AAT ACT GAG AG-3’

5’-GAC GAT GCG GAT TGA ACG AA-3’

[3] Protocol was provided by the Tobacco Research Board (2005)

This pair primer can amplify all isolates of *V. dahliae* according to Carder *et al*. (1994)

42/70 5'-GTT TCT TAG CTT GCA ACA T-3

5'-ACG AGA GTG GAA TAA AGC GA-3'

This pair of primers are RFLP primers and they can amplify, diploid isolates of *V. dahliae* (Carder *et al.,* 1994). The primers were manufactured at the University of Cape Town, South Africa based on the sequence we supplied them.

Procedure[4] for probing *V. dahliae* DNA sequences

A master mix was prepared in tube using the order below

Table 5: PCR Reaction Mixture

Reagent	Mixing order	Volume x2 (µl)
Water ultrapure	1	32.75
10x Buffer	2	5
dNTPS (2.5mM)	3	4
$MgCl_2$ (2.5mM)	4	3
Primer 1 (10µl)	5	2
Primer 2 (10µl)	6	2
Taq polymerase (5µl/µl)	7	0.5
DNA Template (20ng/µl)	8	5
Total Volume (µl)		100µl

[4] Procedure provided by Dr. D. Garwe of the Tobacco Research Board

PCR reaction mixture was prepared for each primer pair (19/22 and 42/70). 12µl of PCR reaction mixture was dispensed into PCR tubes. To each tube a template DNA was added to the tubes for each primer pair and the order was noted. PCR was then run using the following conditions for thirty-five cycles

$94^{o}C$ for 2 minutes
$94^{o}C$ for 1 minute
$46^{o}C$ for 1 minute
$72^{o}C$ for 1 minute
$72^{o}C$ for 7 minutes

At the end of the PCR cycles the samples were loaded in wells of 1.5% Agarose gel (prepared by weighing 7.5g of Agarose into 500ml TBE(0.5%) and the adding 25µl of ethidium bromide). A Hyper ladder V marker was used for comparison with the sample. The marker produce bands that range from 25 to 500 base pairs. .The PCR produced bands that were bigger 500bp.

CHAPTER 4

RESULTS

4.1 Field Trials

There were significant differences in infection percentages at all the sites with the exception of Henderson and Rafingora (Table 1). Cross-site analysis of the infection percentages revealed significant differences between the sites ($p<0.001$, Appendix 6). There were also significant differences between the entries and a strong interaction between the sites and the entries (Appendix 6). Significant differences for the area under the disease progress curve were obtained at Chinhoyi but at the rest of the sites it was not significant (Table 2). Although there were no significant differences in the area under the disease progress slight differences on the disease progress curves were observed (Figs 1-4). Across the sites variety G 501 had the slowest disease progress. G 501 had the lowest infection percentages while SZ 9314, 563-97-12 and FQ 902 were the most susceptible across the sites (Table 1). There were no significant differences on the yield of the varieties across four sites, significant differences were only obtained at Chinhoyi where 563-97-12 outperformed all the other varieties under heavy disease infestation. Cross-site analysis revealed strong significant differences between the sites and the entries. There was also strong interaction between the site and the entries were also obtained (Appendix 6).

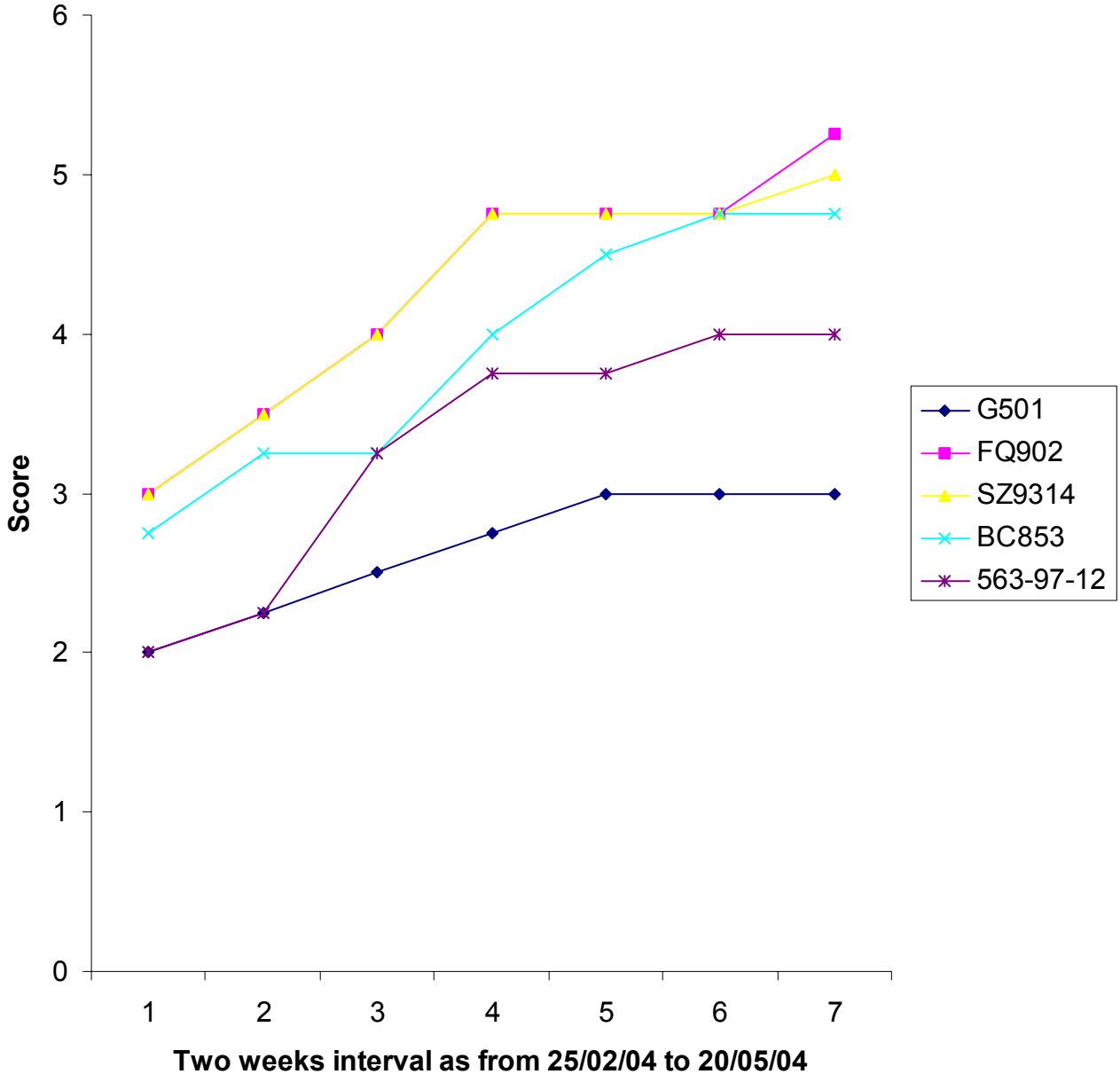

Fig 1: Disease progress at Chinhoyi

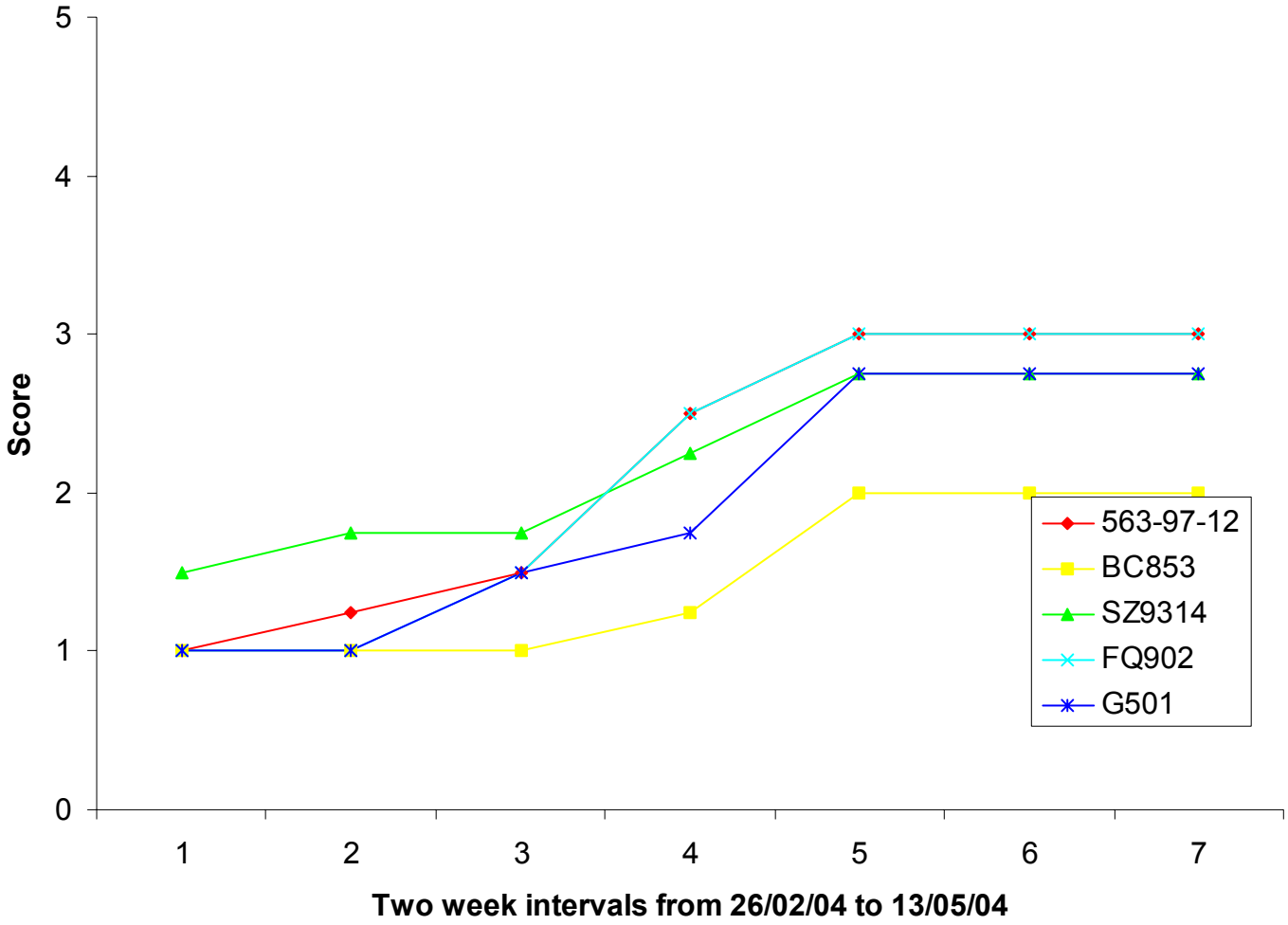

Fig 2: Disease progress at Henderson

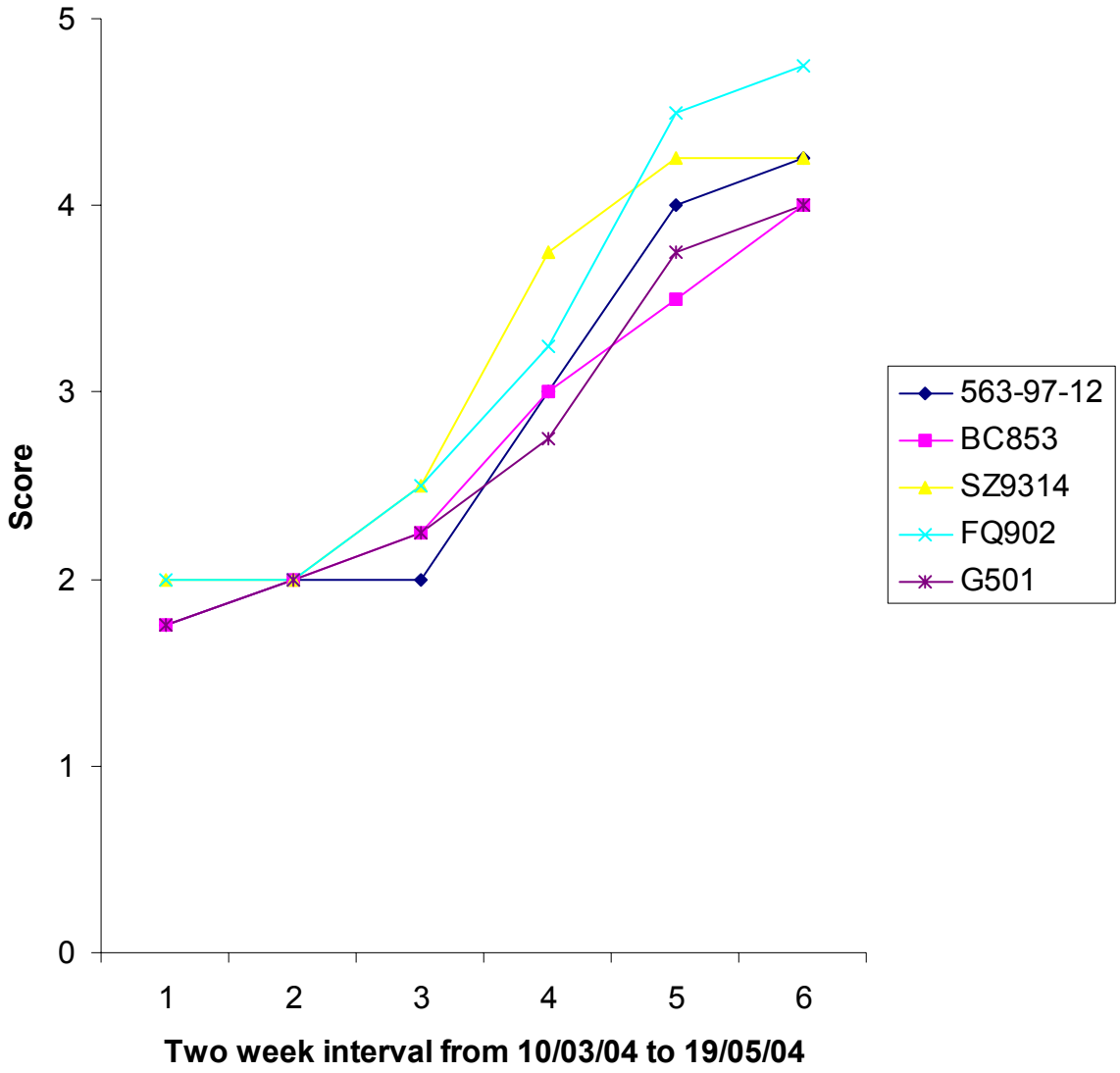

Fig 3: Disease progress at Chisumbanje

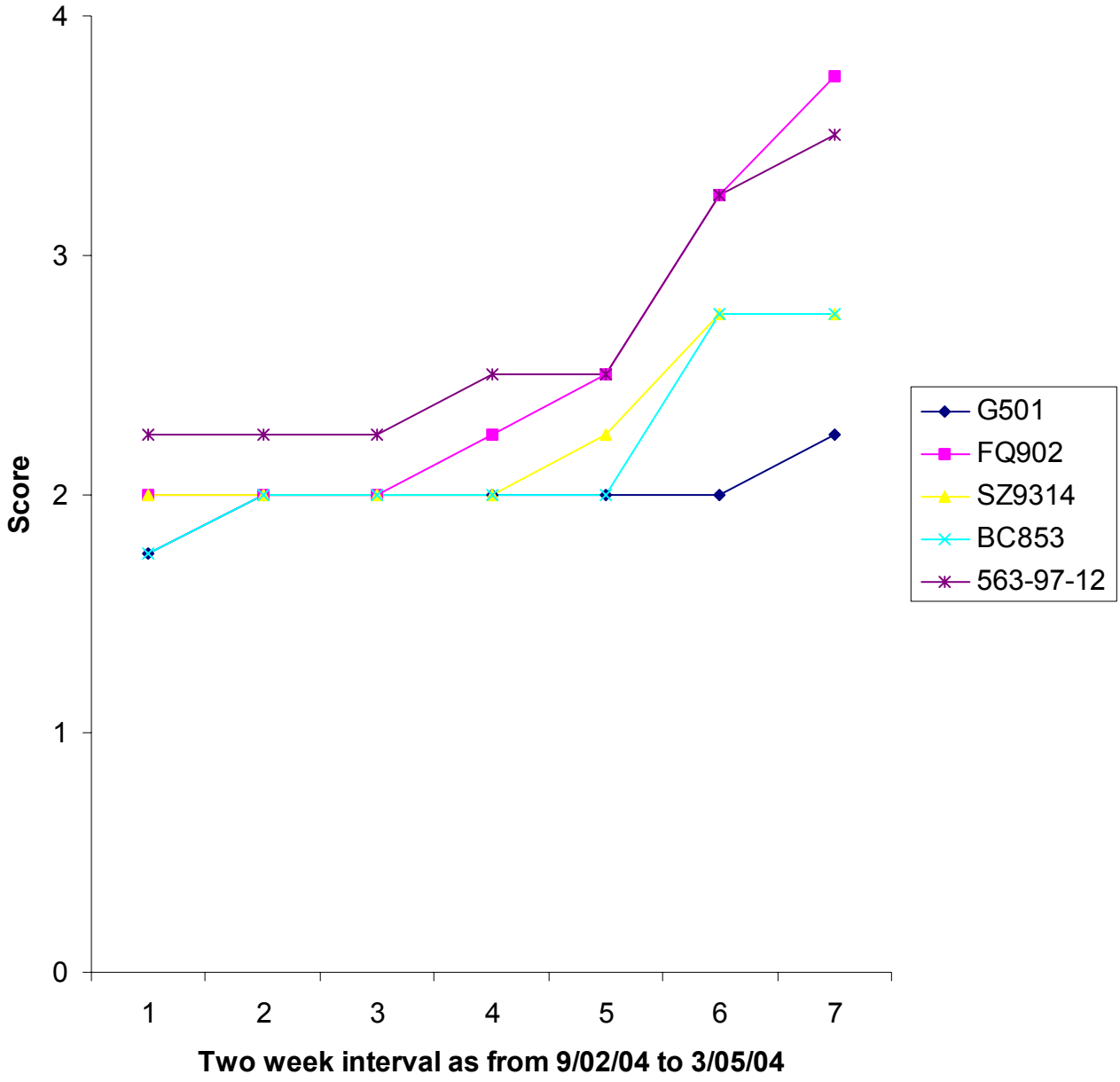

Fig 4: Disease Progress at Cotton Research Institute

Table 1: Infection % (arc sine transformed) for five varieties across four sites

Entry	CRI	Henderson	Chinhoyi	Chisumbanje	Total	Mean
563-97-12	57.9	39.5	35.2	47.8	180.4	45.1
SZ9314	41.3	36.6	50.3	57.6	185.8	46.5
G501	31.7	16.9	31.4	45.2	125.2	31.3
BC853	48.7	27.8	44.7	49.8	171.0	42.8
FQ902	65.6	29.3	46.2	55.8	196.9	49.2
Mean	49.1	30.0	41.6	51.3	171.9	42.9
Significance	**	Ns	**	**		
S.e.d	9.36	9.95	4.29	3.84		
CV (%)	27.0	46.9	14.6	10.6		
Lsd (0.05)	20.40	(21.68)	9.35	8.36		

Key:
Ns – not significant
** -Significant differences

Table 2: AUDPC for five varieties across four sites

Entry	CRI	Henderson	Chinhoyi	Chisumbanje
563-97-12	31.25	26.5	40	28
SZ 9314	26.75	26.8	51.5	31.25
G 501	24	23.3	32	27.25
BC 853	26	17.5	47	27.25
FQ 902	29.75	26.5	51.7	31.25
Mean	27.55	24.1	44.5	29
Significance	Ns	Ns	***	Ns
S.e.d	2.761	7.4	3.67	1.81
CV (%)	14.2	43.4	11.7	8.8
Lsd (0.05)	(6.015)	(16.11)	8	(3.943)

Key:
Ns – not significant
** -Significant differences

Table 3: Yield (t/ha) of five varieties across five sites

Entry	Site				
	CRI	Henderson	Chinhoyi	Rafingora	Chisumbanje
563-97-12	0.706	4.57	2.072	1.32	2.979
SZ9314	0.664	3.54	1.846	1.51	2.787
G501	0.724	3.88	1.73	0.7	2.188
BC853	0.865	3.76	2.177	0.68	2.837
FQ902	0.742	3.15	1.762	1.26	2.496
Mean	0.7402	3.78	1.9174	1.094	2.6574
Significance	ns	Ns	***	Ns	Ns
S.e.d	0.0971	1.301	0.0919	0.471	10.3058
CV (%)	18.6%	48.7	6.8%	61%	16.3
Lsd (0.05)	0.2116	2.834	0.2003	1.027	0.6663

Key:
Ns – not significant
** -signficant differences

4.2 Green House Trial

There were significant difference between the isolates ($p<0.001$) and there were also significant differences between the varieties ($p<0.001$). Interaction between the isolates and the varieties was also significant ($p<0.001$). Across the isolates BC853 and G501 had lower AUDPC whilst SZ9314 and FQ902 had the highest AUDPC (Table 3). Isolates from Chinhoyi and CRI were more virulent whilst isolate from Henderson and Rafingora were less virulent (Table 3). All the isolates are equally virulent on the varieties in the green house as shown on Figure 5-8 G501 and BC853 had the lowest disease progress curves across all the isolates whilst SZ9314 and FQ902 had the highest disease progress curves across the isolates.

Table 4: Table of means for AUDPC of five varieties across five isolates

Isolates	Varieties				
	563-97-12	BC 853	FQ 902	G501	SZ 9314
Chinhoyi	10.0	7.5	12.3	9.0	12.3
Chisumbanje	9.0	5.8	7.7	9.5	8.0
CRI	11.2	6.8	10.5	6.7	10.8
Henderson	5.5	5.0	7.3	6.2	8.0
Rafingora	5.5	5.3	7.8	6.5	8.5
Mean	8.2	6.1	9.1	6.8	9.5
s.e.d	0.4	0.4	0.4	0.4	0.4

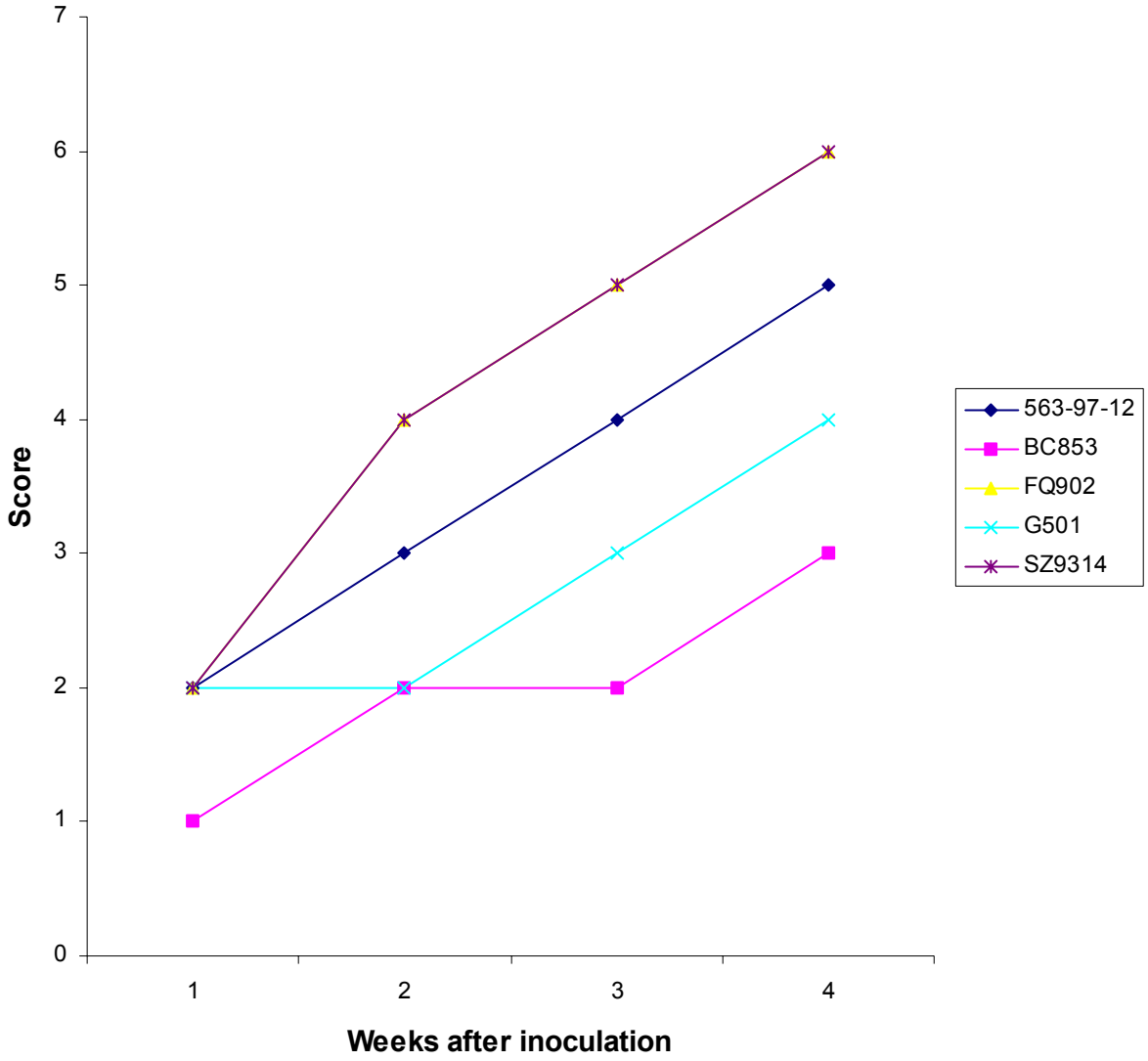

Fig 5: Disease Progress on varieties inoculated with Chinhoyi Isolate

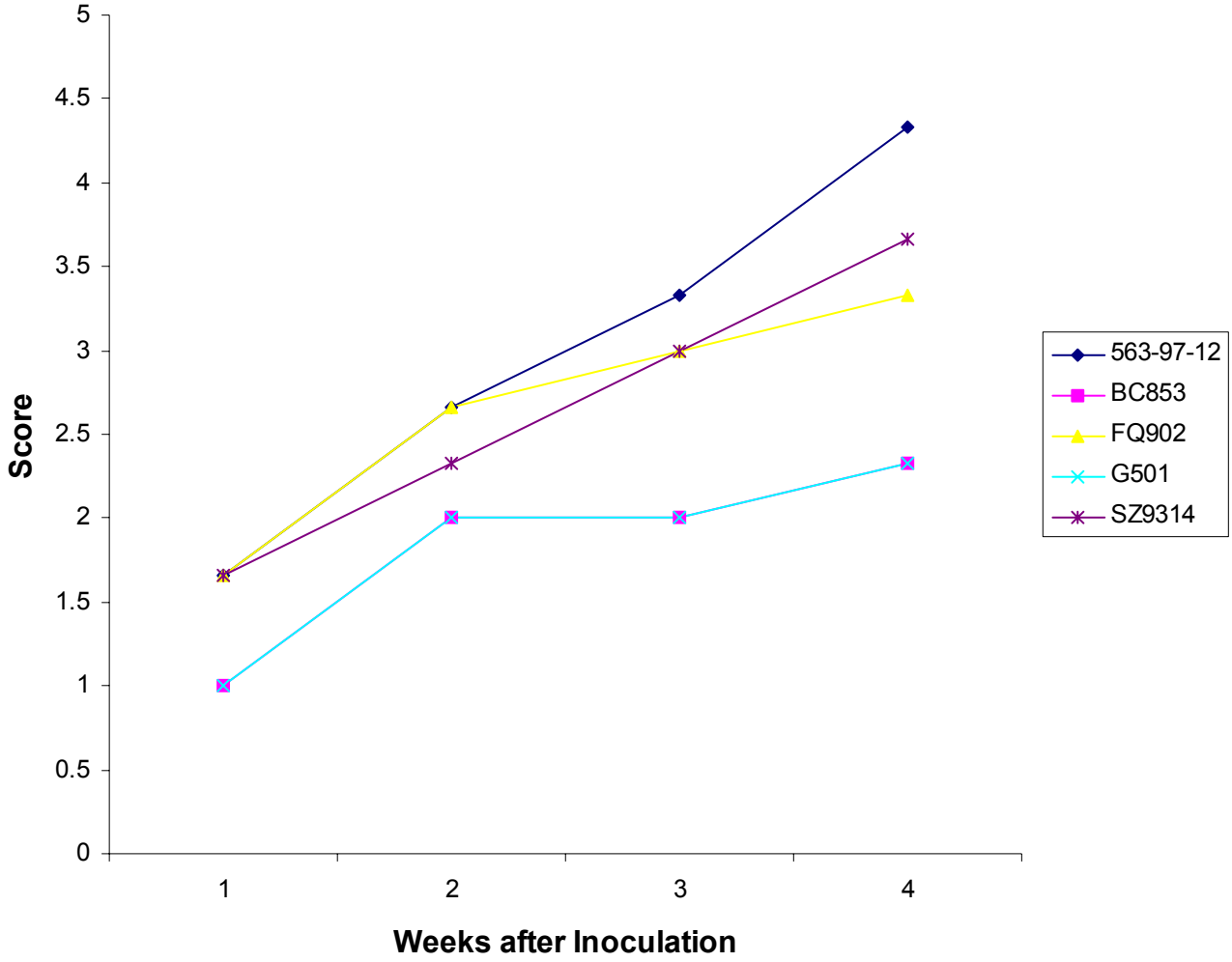

Fig 6: Disease Progress on varieties inoculated with Chisumbanje isolate

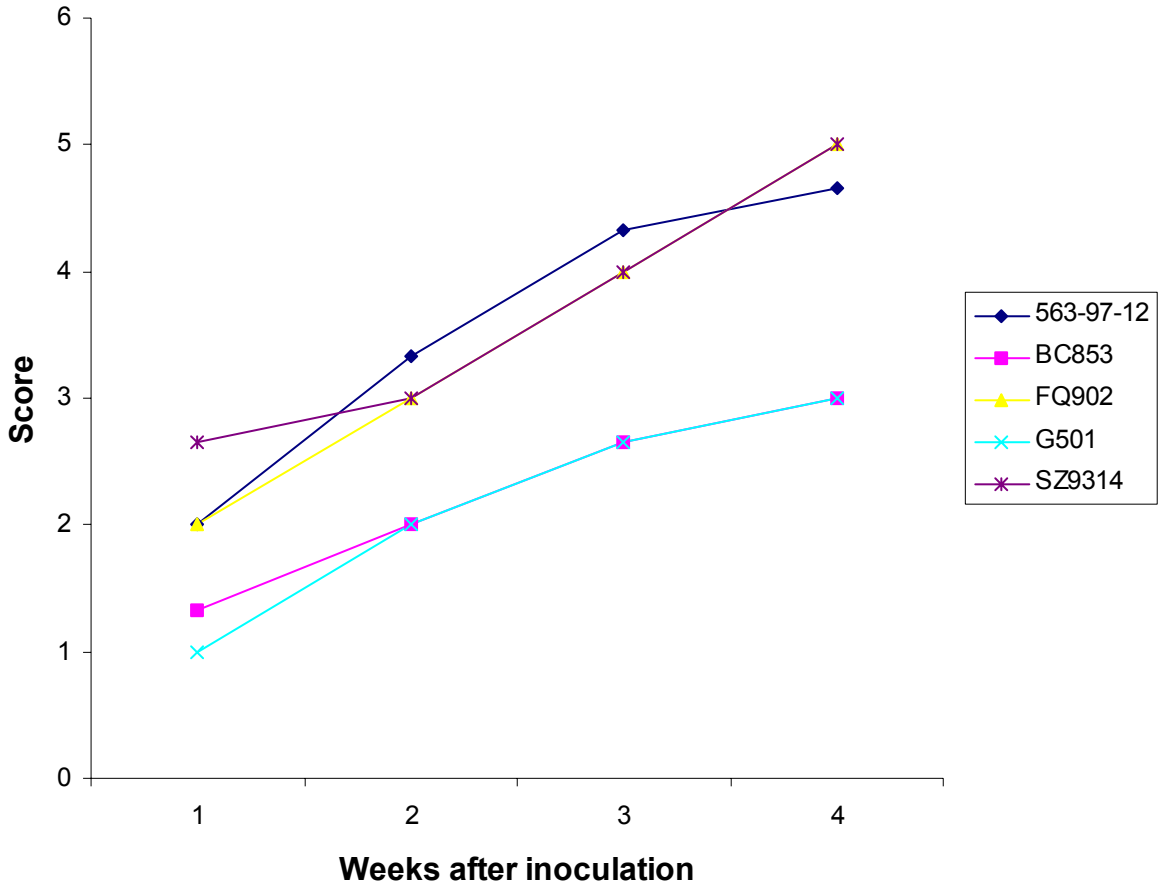

Fig 7: Disease Progress on varieties inoculated with CRI isolate

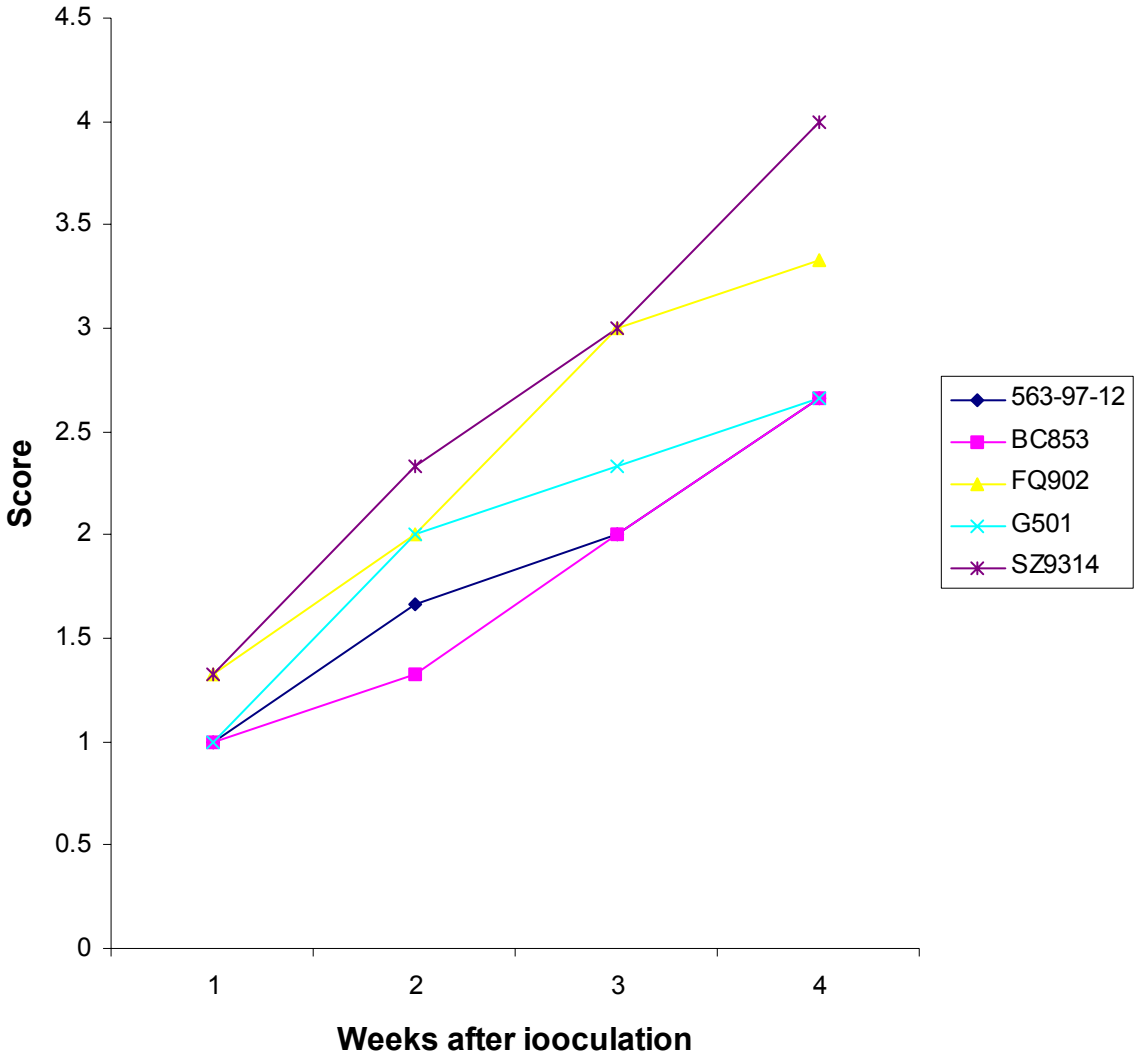

Fig 8: Disease Progress on Varieties inoculated with Henderson Isolate

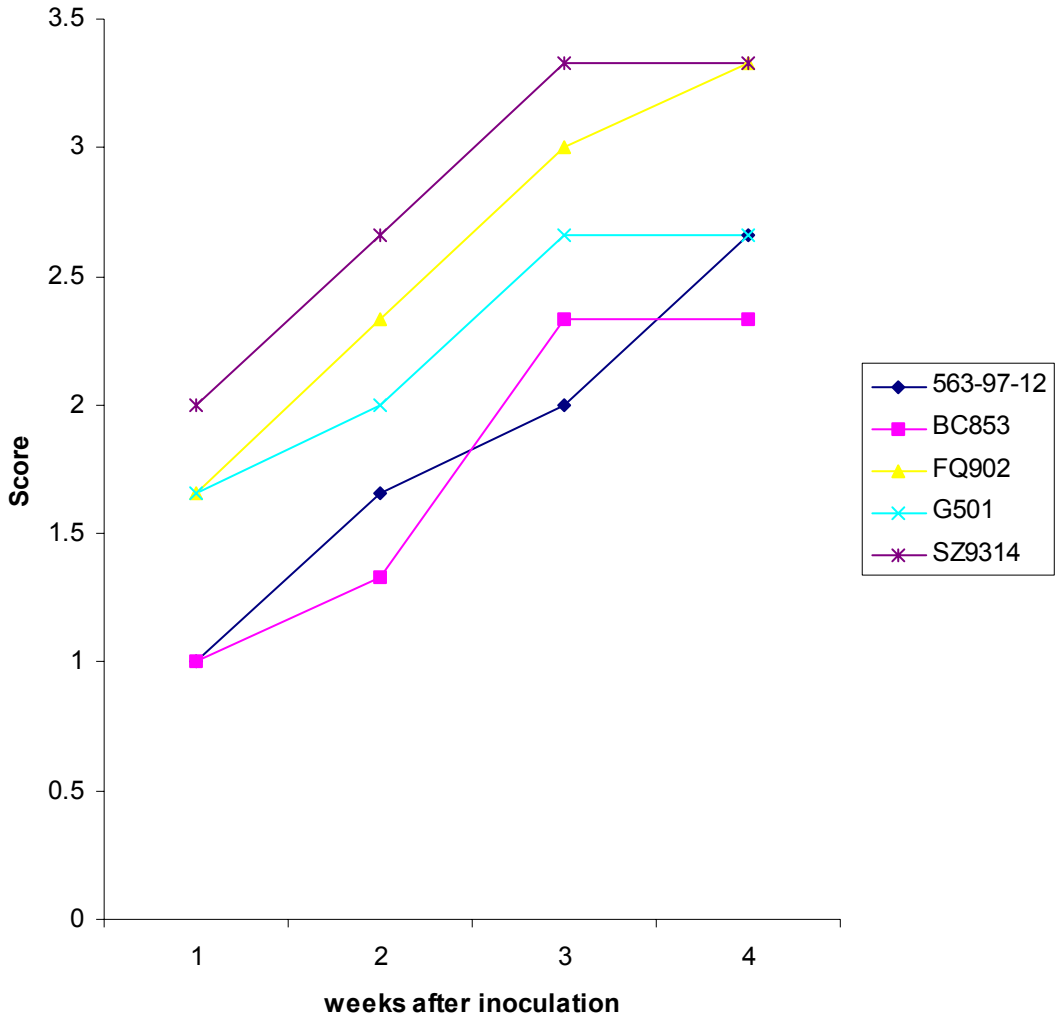

Fig 9: Disease Progress on Varieties inoculated with Rafingora Isolate

4.3 Strain Differentiation

After the isolation of DNA from the isolates gel electrophoresis of the DNA was undertaken to check if the isolation was successful. A photograph below was taken of the result

(Photograph 1)

Plate 1 DNA gel electrophoresis for isolates

Lanes:

1-2 Rafingora
3-4 Henderson
5-6 CRI
7-8 Chisumbanje
9-10 Chinhoyi
11 Marker

After running the PCR products photograph was taken and Plate 2 was produced.

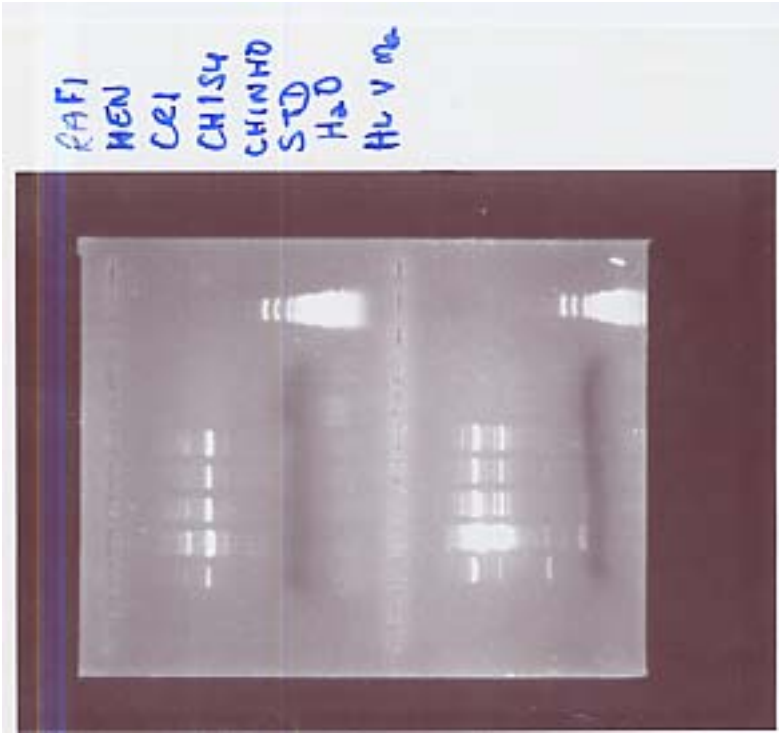

Plate 2 Gel Electrophoresis for PCR products
Notes
First bands (left bands) on the picture are from primer 19/22 and the second bands (right bands) are from primer 42/70
Isolates were loaded in the order first row was left blank, then Rafi-Rafingora, Hen-Henderson, CRI-Cotton Research Institute, Chisu-Chisumbanje, Chinho-Chinhoyi, STD-Standard and H_2O-Water, HLVMA-Marker

The standard fungus failed to produce bands on the gel. Primer 42/70 manage to produce more bands than primer 19/22. Bands produced for each primer after running products of the PCR on gel were scored on a scale of 0-1 with 0 for no band and 1 if a band at a particular length of the gel was produced. The Jaccard coefficient of similarity was used to generate similarity matrix from which dendograms were constructed using Genstat Statistical Package 5 to compare their similarity was done (Appendix 22 and 23). Primer 19/22 produced five bands; Bands 1 and 2 were common in all the isolates except in Rafingora isolate. Band 3 was common in all the isolates. Band 4 was only observed in the Henderson isolate, while Band 5 was

common in the four other isolates but not in the Henderson isolate. Analysis of the banding pattern with the Jaccard revealed closed 80% similarity between the isolates. Isolate from Rafingora and CRI had only 25% similarity, which was very significant. Primer 42/70 managed to produce 11 bands, however a cluster analysis revealed that differences in the isolates with isolate from Henderson and CRI being different from each other. Rafingora and Chisumbanje had a similarity coefficient of 66.7%.

CHAPTER 5

DISCUSSION

All the isolates that were tested were pathogenic on the cotton varieties that were in this study. This was so both in the field and in the green house at Cotton Research Institute. Pathogenesis test of the isolates failed to separate the five isolates from each other based on the results from the study, all the isolates were pathogenic and they were able to incite symptoms. This is in agreement with work by Chinodya (1996) who observed that isolates from three sites in Zimbabwe were virulent on cotton. He only observed the physiological differences on the isolates but could not separate them based on their pathogenic on cotton. Failure to separate the isolates distinctively from each other based on their ability to inflect damage on some varieties or not may be attributed to the narrow genetic background of the varieties that were used in this study. All the materials were derived from ALBAR germplasm hence the lack of clear varietal reaction to the disease. Maybe if germplasm from diverse background were used different reaction from the isolates could have been obtained. Brinkerhoff (1963) advocated the use of differential hosts in the determination of pathogenic variability between the races of Bacterial Blight pathogen, these differential are from diverse genetic background, if we had used such materials our results may have been more meaningful. At Chinhoyi 563-97-12 produced new leaves after the crop was completely defoliated by the disease this phenomenon could not be understood. In the green house, virulence of the isolates may have been enhanced by the way the inoculum was injected into the plant, in which the fungus was directly injected into the plant's transpiration stream. This method although it has been used elsewhere, may have bypassed the plant's defence mechanism thereby overwhelming it hence the succumbing to the disease. Compared to the other isolates and the sites Chinhoyi

isolate was more virulent both in the field and the green house (Table 2 and 4). Low AUDPC on G501 and BC 853 across all the isolates in the green house and in the field reflect that these varieties are more tolerant to Verticillium Wilt and is in agreement with previous findings by Hillocks (1991) who observed high level of wilt tolerance in G501 while BC 853 is recommended for wilt infested fields by Cotton Research Institute in Zimbabwe while SZ 9314 and FQ902 are generally recommended for wilt free land because they are susceptible to the disease. This was confirmed by the results from the study. Cross site analysis revealed significant differences between site infection percentages these differences may be due to differences in the climatic conditions at these sites and also the soils. The other reason for the site differences may have arisen from the differences in the inoculum density at these sites, which were not determined at the start of these experiments. Cross site analysis also revealed significant differences infection percentages and the AUDPC, this may have been due to the influence of the site climatic conditions on the disease expression. It seems some varieties are more resistant at certain sites than the other sites and susceptible to the disease at other sites. Maybe resistance is being influence or suppressed by site conditions.

PCR analysis revealed that the isolates were different from each other at molecular level. However the differences could have been more pronounced if we had tried other markers (only two primer were used instead of 10 primers used elsewhere by Radisek, 2003), maybe the separation of the PCR products could have been better. The differences that were observed between the isolates may confirm the difference we found in the virulence of the isolates both in the field and the green house trial. The molecular difference found with the PCR may form the bases of the physiological

differences between the isolates in Zimbabwe that were observed by Chinodya (1996). This study only managed to show slight differences between the five isolates at molecular level, however correlation between virulence and molecular differences could not be established, this agrees with the work of Radisek *et al*. (2003) who also failed to establish correlation between pathotypes associated with AFLPs groups and level of virulence. Research by Ramsay *et al.* (1996) also confirms the existence of difference on isolates at molecular level but they asserts that difference between isolates is very difficult to pick and a combination of techniques may need to be employed for differences to be picked. Studies elsewhere (Radisek *et al*., 2003) using different molecular techniques have shown high genetic diversity among the species and as well as variation within species indicating pathotypes specialisation, our results although they reveal some differences they could have been meaningful had we used many primer and other molecular techniques which could have picked differences in the isolates (Dr. Garwe, 2005 personal communication) used a combination of various methods.

CHAPTER 6

6.1 CONCLUSIONS

Results from the PCR analysis although they show differences between the isolates, they are not conclusive, further work need to be carried out and there may be need to increase the number of primers that may be able to pick the differences which the two primers we used failed to pick. From our results *Verticillium dahliae* isolates from five locations were able to elicit various reactions on the 5 varieties we used in this study during the pathogenicity tests comparison of banding patterns of the digested DNA established that there maybe molecular differences between the isolates, however this study failed to establish the relationship between virulence and differences at molecular level. Our results indicate that the isolates that were isolated from the fields under the study are virulent on cotton. Chinhoyi isolate was more virulent than other isolates. SZ 9314 and FQ 902 are susceptible to verticillium wilt whilst G501 and BC 853 are tolerant. 563-97-12 is intermediate. Site also influence the disease expression and progress of the disease and a strong interaction between the entries and site was also evident.

6.2 RECOMMENDATIONS

- Further work with the PCR needs to be carried out and there is need to compare the isolates with other molecular methods like the Monoclonal Antibody-based ELISA so that genetic differences between the isolates can be picked.
- Although our results are not conclusive we recommended Cotton Research Institute should use more sites during the screening of elite material, this will

enable them to exposed their material to probably different pathotypes of *Verticillium dahliae* before they release the material for commercial use

- There is need to follow up this project and use other methods like the Vegetative compatibility groups to confirm the existence of different pathotypes of *Verticillium dahliae* attacking cotton in this country as the PCR with the two primers we used failed to pick significant differences between the isolates.
- Lines that are known to be tolerant to certain strains should be sourced outside the country and them used as differential hosts to confirm the existence strains based on their differential reaction to *Verticillium dahliae* isolates

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APPENDICES

Appendix 1: Analysis of variance arc sine at CRI

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	460.5	153.5	0.88	
ENTRY	4	2854.4	713.6	4.07	0.026
Residual	12	2103.4	175.3		
Total	19	5418.3			

l.s.d.	20.40
C.V	27%

Appendix 2: Analysis of variance arc sine Chisumbanje

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	3	109.01	36.34	1.23	
Entry	4	447.07	111.77	3.79	0.032
Residual	12	353.58	29.46		
Total	19	909.66			

l.s.d.	8.36
C.V	10.6%

Appendix 3: Analysis of variance arc sine Henderson

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	3	482.6	160.9	0.81	
entry	4	1243.0	310.7	1.57	0.245
Residual	12	2376.4	198.0		
Total	19	4102.0			

l.s.d.	21.68
C.V	46.8

Appendix 4: Analysis of variance arc sine Chinhoyi

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	3	333.69	111.23	3.02	
Entry	4	1008.47	252.12	6.84	0.004
Residual	12	442.29	36.86		
Total	19	1784.45			

l.s.d.	9.35
C.V	14.6

Appendix 5: Analysis of variance arcsine Rafingora

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	3	378.27	126.09	4.36	
Entry	4	407.84	101.96	3.53	0.040
Residual	12	346.82	28.90		
Total	19	1132.92			

l.s.d.	8.28
C.V	140.9

Appendix 6: Analysis of variance Arcsine across sites

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	750.14	250.05	2.71	
Site	4	30049.25	7512.31	81.50	<.001
ENTRY	4	2408.68	602.17	6.53	<.001
Site.ENTRY	16	3552.06	222.00	2.41	0.006
Residual	72	6636.45	92.17		
Total	99	43396.58			

Appendix 7: Analysis of variance for the area under the curve at CRI

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	25.35	8.45	0.55	
ENTRY	4	136.70	34.17	2.24	0.125
Residual	12	182.90	15.24		
Total	19	344.95			

l.s.d.	6.015
C.V	14.2
s.e.d.	2.761

Appendix 8: Analysis of variance for the area under the curve at Chinhoyi

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	122.15	40.72	1.51	
ENTRY	4	1137.20	284.30	10.54	<.001
Residual	12	323.60	26.97		
Total	19	1582.95			

s.e.d.	3.67
l.s.d.	8.00
C.V	11.7

Appendix 9: Analysis of variance for the area under curve at Chisumbanje

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	8.400	2.800	0.43	
ENTRY	4	69.000	17.250	2.63	0.087
Residual	12	78.600	6.550		
Total	19	156.000			

s.e.d.	1.810
l.s.d.	3.943
C.V	8.8

Appendix 10: Analysis of variance for the area under the curve at Henderson

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	329.8	109.9	1.00	
ENTRY	4	251.3	62.8	0.57	0.687
Residual	12	1312.7	109.4		
Total	19	1893.8			

s.e.d.	7.40
l.s.d.	16.11
C.V	43.4

Appendix 11: Analysis of variance for yield at Chinhoyi

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	0.04703	0.01568	0.93	
ENTRY	4	0.62032	0.15508	9.18	0.001
Residual	12	0.20280	0.01690		
Total	19	0.87015			

s.e.d 0.0919
l.s.d 0.2003
C.V 6.8%

Appendix 12: Analysis of variance for yield at Chisumbanje

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	0.6898	0.2299	1.23	
ENTRY	4	1.5939	0.3985	2.13	0.140
Residual	12	2.2444	0.1870		
Total	19	4.5282			

s.e.d 0.3058
l.s.d 0.6663
C.V 16.3%

Appendix 13: Analysis of variance for yield at CRI

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	0.19631	0.06544	3.47	
ENTRY	4	0.09152	0.02288	1.21	0.355
Residual	12	0.22630	0.01886		
Total	19	0.51412			

s.e.d 0.0971
l.s.d 0.2116
C.V 18.6%

Appendix 14: Analysis of variance for yield at Henderson

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	13.710	4.570	1.35	
ENTRY	4	4.355	1.089	0.32	0.858
Residual	12	40.592	3.383		
Total	19	58.657			

s.e.d 1.301
l.s.d 2.834
C.V. 48.7%

Appendix 15: Analysis of variance for yield at Rafingora

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	7.4309	2.4770	5.58	
ENTRY	4	2.3361	0.5840	1.32	0.319
Residual	12	5.3275	0.4440		
Total	19	15.0945			

s.e.d 0.471
l.s.d 1.027
C.V 61%

Appendix 16: Cross-site analysis of variance for yield at five sites

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	2.4013	0.8004	0.84	
Site	4	120.1891	30.0473	31.69	<.001
ENTRY	4	2.9729	0.7432	0.78	0.539
Site.ENTRY	16	6.0234	0.3765	0.40	0.979
Residual	72	68.2663	0.9481		
Total	99	199.8531			

Appendix 16: Analysis of variance Arcsine

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	3	750.14	250.05	2.71	
Site	4	30049.25	7512.31	81.50	<.001
ENTRY	4	2408.68	602.17	6.53	<.001
Site.ENTRY	16	3552.06	222.00	2.41	0.006
Residual	72	6636.45	92.17		
Total	99	43396.58			

s.e.d.	6.789
l.s.d.	13.533
C.V	27.3

Appendix 17: Analysis of Variance for area under the curve for five varieties inoculated with five isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	1.947	0.973	1.79	
Main	4	176.887	44.222	81.26	<.001
Residual	8	4.353	0.544	0.36	
Subplot	4	137.487	34.372	22.47	<.001
Main.Subplot	16	53.613	3.351	2.19	0.023
Residual	40	61.200	1.530		
Total	74	435.487			

Appendix 18: Malt Extract Broth

Malt Extract	20g
Glucose	20g
Peptone	2g
Distilled Water	1l

Appendix 19: Derandomised Raw Data for yield across sites

Site	ENTRY	REP	t/ha
Chinho	563-97-12	1	2.07
Chinho	563-97-12	2	2.00
Chinho	563-97-12	3	1.99
Chinho	563-97-12	4	2.22
Chinho	BC853	1	1.80
Chinho	BC853	2	1.96
Chinho	BC853	3	1.81
Chinho	BC853	4	1.82
Chinho	FQ 902	1	1.63
Chinho	FQ 902	2	1.65

Chinho	FQ 902	3	1.63
Chinho	FQ 902	4	2.01
Chinho	G501	1	2.09
Chinho	G501	2	2.15
Chinho	G501	3	2.41
Chinho	G501	4	2.06
Chinho	SZ 9314	1	1.74
Chinho	SZ 9314	2	1.67
Chinho	SZ 9314	3	1.77
Chinho	SZ 9314	4	1.87
Chisu	563-97-12	1	3.56
Chisu	563-97-12	2	2.75
Chisu	563-97-12	3	2.79
Chisu	563-97-12	4	2.82
Chisu	BC853	1	3.00
Chisu	BC853	2	2.24
Chisu	BC853	3	2.90
Chisu	BC853	4	3.00
Chisu	FQ 902	1	1.79
Chisu	FQ 902	2	2.89
Chisu	FQ 902	3	2.17
Chisu	FQ 902	4	1.90
Chisu	G501	1	3.12
Chisu	G501	2	2.77
Chisu	G501	3	2.90
Chisu	G501	4	2.56
Chisu	SZ 9314	1	3.23
Chisu	SZ 9314	2	2.79
Chisu	SZ 9314	3	2.03
Chisu	SZ 9314	4	1.94
CRI	563-97-12	1	0.73
CRI	563-97-12	2	0.78
CRI	563-97-12	3	0.78
CRI	563-97-12	4	0.53
CRI	BC853	1	0.55
CRI	BC853	2	0.68
CRI	BC853	3	0.87
CRI	BC853	4	0.55
CRI	FQ 902	1	0.62
CRI	FQ 902	2	0.86
CRI	FQ 902	3	0.57
CRI	FQ 902	4	0.85
CRI	G501	1	0.67
CRI	G501	2	0.96
CRI	G501	3	1.15
CRI	G501	4	0.68
CRI	SZ 9314	1	0.53
CRI	SZ 9314	2	0.93
CRI	SZ 9314	3	0.80
CRI	SZ 9314	4	0.71

Hinder	563-97-12	1	4.86
Hinder	563-97-12	2	2.96
Hinder	563-97-12	3	3.59
Hinder	563-97-12	4	6.86
Hinder	BC853	1	0.37
Hinder	BC853	2	5.18
Hinder	BC853	3	6.04
Hinder	BC853	4	2.57
Hinder	FQ 902	1	2.39
Hinder	FQ 902	2	4.07
Hinder	FQ 902	3	6.79
Hinder	FQ 902	4	2.28
Hinder	G501	1	4.59
Hinder	G501	2	2.42
Hinder	G501	3	6.10
Hinder	G501	4	1.94
Hinder	SZ 9314	1	2.69
Hinder	SZ 9314	2	3.92
Hinder	SZ 9314	3	3.19
Hinder	SZ 9314	4	2.79
Rafing	563-97-12	1	3.45
Rafing	563-97-12	2	0.85
Rafing	563-97-12	3	0.15
Rafing	563-97-12	4	0.84
Rafing	BC853	1	2.62
Rafing	BC853	2	1.69
Rafing	BC853	3	1.39
Rafing	BC853	4	0.36
Rafing	FQ 902	1	0.90
Rafing	FQ 902	2	0.29
Rafing	FQ 902	3	0.23
Rafing	FQ 902	4	1.36
Rafing	G501	1	1.47
Rafing	G501	2	0.35
Rafing	G501	3	0.35
Rafing	G501	4	0.56
Rafing	SZ 9314	1	2.27
Rafing	SZ 9314	2	1.06
Rafing	SZ 9314	3	1.42
Rafing	SZ 9314	4	0.27

Appendix 20: Raw data Green House trial

Rep	Main	Subplot	week1	week2	week3	week4	AUDPC
1	CHINHO	563-97-12	2	3	4	5	10.5
2	CHINHO	563-97-12	2	3	4	5	10.5
3	CHINHO	563-97-12	2	3	3	4	9.0
1	CHINHO	BC853	2	3	3	4	9.5
2	CHINHO	BC853	1	2	2	3	6.0

3	CHINHO	BC853	1	2	3	4	7.5
1	CHINHO	FQ902	2	3	4	5	10.5
2	CHINHO	FQ902	2	4	5	6	13.0
3	CHINHO	FQ902	3	4	5	6	13.5
1	CHINHO	G501	2	2	3	4	8.0
2	CHINHO	G501	2	3	3	4	9.0
3	CHINHO	G501	2	3	4	4	10.0
1	CHINHO	SZ9314	2	3	4	5	10.5
2	CHINHO	SZ9314	2	4	5	6	13.0
3	CHINHO	SZ9314	3	4	5	6	13.5
1	CHISU	563-97-12	1	2	3	4	7.5
2	CHISU	563-97-12	2	2	3	4	8.0
3	CHISU	563-97-12	2	3	4	5	10.5
1	CHISU	BC853	1	2	2	3	7.0
2	CHISU	BC853	1	2	2	2	5.5
3	CHISU	BC853	1	2	2	3	7.0
1	CHISU	FQ902	1	2	3	3	7.0
2	CHISU	FQ902	2	3	3	3	8.5
3	CHISU	FQ902	2	3	3	4	9.0
1	CHISU	G501	1	2	2	3	65.5
2	CHISU	G501	1	2	2	2	5.5
3	CHISU	G501	1	2	2	2	5.5
1	CHISU	SZ9314	2	3	3	4	9.0
2	CHISU	SZ9314	2	2	3	4	8.0
3	CHISU	SZ9314	1	2	3	3	7.0
1	CRI	563-97-12	2	3	4	5	10.5
2	CRI	563-97-12	3	4	5	5	12.5
3	CRI	563-97-12	2	3	4	4	10.0
1	CRI	BC853	1	2	3	3	7.0
2	CRI	BC853	1	1	2	3	5.0
3	CRI	BC853	2	3	3	3	8.5
1	CRI	FQ902	2	3	4	5	10.5
2	CRI	FQ902	2	3	4	5	10.5
3	CRI	FQ902	2	3	4	5	10.5
1	CRI	G501	1	2	3	3	7.0
2	CRI	G501	1	2	3	3	7.0
3	CRI	G501	1	2	2	3	6.0
1	CRI	SZ9314	3	3	4	5	11.0
2	CRI	SZ9314	2	3	4	5	10.5
3	CRI	SZ9314	3	3	4	5	11.0
1	HENDER	563-97-12	1	2	2	3	6.0
2	HENDER	563-97-12	1	2	2	3	6.0
3	HENDER	563-97-12	1	1	2	2	4.5
1	HENDER	BC853	1	2	2	2	3.5
2	HENDER	BC853	1	1	2	3	5.0
3	HENDER	BC853	1	2	2	2	5.0
1	HENDER	FQ902	2	2	3	4	8.0
2	HENDER	FQ902	1	2	3	3	7.0
3	HENDER	FQ902	1	2	3	3	7.0
1	HENDER	G501	1	2	2	3	6.0

2	HENDER	G501	1	2	3	3	7.0
3	HENDER	G501	1	2	2	2	3.5
1	HENDER	SZ9314	1	3	3	4	8.5
2	HENDER	SZ9314	1	2	3	4	7.5
3	HENDER	SZ9314	2	2	3	4	8.0
1	RAFING	563-97-12	1	2	2	2	3.5
2	RAFING	563-97-12	1	1	2	3	5.0
3	RAFING	563-97-12	1	2	2	3	6.0
1	RAFING	BC853	1	1	2	2	4.5
2	RAFING	BC853	1	2	3	3	7.0
3	RAFING	BC853	1	1	2	2	4.5
1	RAFING	FQ902	1	2	3	4	7.5
2	RAFING	FQ902	2	2	3	3	7.5
3	RAFING	FQ902	2	3	3	3	8.5
1	RAFING	G501	2	2	3	3	8.5
2	RAFING	G501	2	2	2	3	8.5
3	RAFING	G501	1	2	2	2	5.5
1	RAFING	SZ9314	2	2	3	3	8.5
2	RAFING	SZ9314	2	2	3	3	8.5
3	RAFING	SZ9314	2	3	4	4	10.0

Appendix 21: Raw data for infection percentages on five sites

Site	REP	ENTRY	% IN	Arc sine
Chinhoyi	1	563-97-12	54.5	47.58
Chinhoyi	2	563-97-12	25.5	30.33
Chinhoyi	3	563-97-12	29.1	32.65
Chinhoyi	4	563-97-12	25.2	30.13
Chinhoyi	1	BC853	49.5	44.71
Chinhoyi	2	BC853	48.5	44.14
Chinhoyi	3	BC853	42.9	40.92
Chinhoyi	4	BC853	57	49.02
Chinhoyi	1	FQ 902	68.3	55.73
Chinhoyi	2	FQ 902	42.5	40.69
Chinhoyi	3	FQ 902	68.7	55.98
Chinhoyi	4	FQ 902	28.7	32.39
Chinhoyi	1	G 501	34	35.67
Chinhoyi	2	G 501	32	34.45
Chinhoyi	3	G 501	26.7	31.11
Chinhoyi	4	G 501	17	24.35
Chinhoyi	1	SZ 9314	67.3	55.12
Chinhoyi	2	SZ 9314	62	51.94
Chinhoyi	3	SZ 9314	55.4	48.10
Chinhoyi	4	SZ 9314	51.9	46.09
chisumbanje	1	563-97-12	46.8	43.17
chisumbanje	2	563-97-12	54.9	47.81
chisumbanje	3	563-97-12	53.1	46.78
chisumbanje	4	563-97-12	64.8	53.61
chisumbanje	1	BC853	63	52.54
chisumbanje	2	BC853	64.5	53.43
chisumbanje	3	BC853	56.1	48.50

chisumbanje	4	BC853	49.4	44.66
chisumbanje	1	FQ 902	78.8	62.58
chisumbanje	2	FQ 902	48.6	44.20
chisumbanje	3	FQ 902	71.2	57.54
chisumbanje	4	FQ 902	73.4	58.95
chisumbanje	1	G 501	46.9	43.22
chisumbanje	2	G 501	47.3	43.45
chisumbanje	3	G 501	49.4	44.66
chisumbanje	4	G 501	57.9	49.55
chisumbanje	1	SZ 9314	68.3	55.73
chisumbanje	2	SZ 9314	56.7	48.85
chisumbanje	3	SZ 9314	79.2	62.87
chisumbanje	4	SZ 9314	79.5	63.08
CRI	1	563-97-12	67.7	55.37
CRI	1	BC853	50	45.00
CRI	1	FQ 902	73.4	58.95
CRI	1	G 501	32.1	34.51
CRI	1	SZ 9314	58.8	50.07
CRI	2	563-97-12	34.9	36.21
CRI	2	BC853	57.5	49.31
CRI	2	FQ 902	81	64.16
CRI	2	G 501	25	30.00
CRI	2	SZ 9314	27.2	31.44
CRI	3	563-97-12	74.7	59.80
CRI	3	BC853	46.4	42.94
CRI	3	FQ 902	100	90.00
CRI	3	G 501	17.1	24.43
CRI	3	SZ 9314	25.3	30.20
CRI	4	563-97-12	97.2	80.37
CRI	4	BC853	71.2	57.54
CRI	4	FQ 902	57.7	49.43
CRI	4	G 501	38	38.06
CRI	4	SZ 9314	64.6	53.49
HEND	1	563-97-12	50	45.00
HEND	1	BC853	33.3	35.24
HEND	1	FQ 902	57.1	49.08
HEND	1	G 501	7.7	16.11
HEND	1	SZ 9314	50	45.00
HEND	2	563-97-12	38.5	38.35
HEND	2	BC853	16.7	24.12
HEND	2	FQ 902	54.5	47.58
HEND	2	G 501	0	0.00
HEND	2	SZ 9314	18.2	25.25
HEND	3	563-97-12	35.7	36.69
HEND	3	BC853	6.3	14.54
HEND	3	FQ 902	12.5	20.70
HEND	3	G 501	7.7	16.11
HEND	3	SZ 9314	38.5	38.35
HEND	4	563-97-12	37.5	37.76
HEND	4	BC853	36.4	37.11

HEND	4	FQ 902	0	0.00
HEND	4	G 501	33.3	35.24
HEND	4	SZ 9314	37.5	37.76
Rafingora	1	563-97-12	0	0.00
Rafingora	2	563-97-12	0	0.00
Rafingora	3	563-97-12	0	0.00
Rafingora	4	563-97-12	0	0.00
Rafingora	1	BC853	23.3	28.86
Rafingora	2	BC853	5.2	13.18
Rafingora	3	BC853	1.6	7.27
Rafingora	4	BC853	0	0.00
Rafingora	1	FQ 902	5.5	13.56
Rafingora	2	FQ 902	0	0.00
Rafingora	3	FQ 902	0	0.00
Rafingora	4	FQ 902	0	0.00
Rafingora	1	G 501	5.4	13.44
Rafingora	2	G 501	0	0.00
Rafingora	3	G 501	0	0.00
Rafingora	4	G 501	0	0.00
Rafingora	1	SZ 9314	0	0.00
Rafingora	2	SZ 9314	0	0.00
Rafingora	3	SZ 9314	0	0.00
Rafingora	4	SZ 9314	0	0.00

Appendix 22: Nearest neighbour cluster analysis for bands produced by Primer 19/22****

**** Dendrogram ****
** Levels 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0

1 .....
5 ..)
4 ..))
3 ..).....)
2 .....).....).....

Key:
1: Rafingora
2: Henderson
3: CRI
4: Chisumbanje
5: Chinhoyi

**** Neighbours table derived from Primer 19/22 ****
1 3 25.0
2 3 80.0
3 4 100.0
4 3 100.0

5 3 100.0

**** Mean similarities between and within groups ****
**** Similarity matrix: Primer 19/22 ****

** Between and within groups similarity matrix **

1 ----
2 23.8 90.0

1 2

Appendix 23: Nearest neighbour cluster analysis for bands produced by 42/70 primer on isolates

**** Dendrogram ****
** Levels 90.0 80.0 70.0

1 .....
4 .....)
5 ..))
3 ..)..).....)
2 .....)..)........

Key:
1: Rafingora
2: Henderson
3: CRI
4: Chisumbanje
5: Chinhoyi

**** Neighbours table derived from Primer 42/70 ****
1 4 66.7
2 3 70.0
3 5 85.7
4 5 83.3
5 3 85.7

**** Mean similarities between and within groups ****
**** Similarity matrix: Primer 42/706 ****

** Between and within groups similarity matrix **

1 ----
2 57.9 80.2
3 36.4 60.0 ----

1 2 3