

**DEVELOPMENT OF DISEASE RESISTANT FENUGREEK FOR WESTERN  
CANADA**

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## **DEDICATION**

This thesis is dedicated to my parents.

## ABSTRACT

Cercospora leaf spot (CLS) caused by *Cercospora traversiana* is an important phytopathological problem of fenugreek (*Trigonella foenum graecum*), a multiuse legume crop. Knowledge about the inheritance of genes controlling CLS resistance is essential when selecting suitable breeding approaches while information about epidemiological factors affecting the disease can help develop new control strategies. Our greenhouse and field experiments showed CLS resistance in fenugreek (L3717 and PI138687) to be governed by a single dominant gene which is moderately heritable (46% narrow sense heritability). This indicates a relatively simple pathway for transfer of genes to adapted fenugreek cultivars. Rapid screening techniques (detached leaf assay and whole plant assay) were developed to identify the degree of resistance to *C. traversiana* in fenugreek genotypes. Several epidemiological factors such as temperature, physical injury (wounding), level of host resistance, plant age and inoculum concentration were found influencing CLS severity in controlled environment conditions.

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## TABLE OF CONTENTS

<b>Contents</b>	<b>Page</b>
ABSTRACT .....	iv
ACKNOWLEDGEMENT .....	v
TABLE OF CONTENTS .....	vi
LIST OF TABLES .....	x
LIST OF FIGURES .....	xii
LIST OF ABBREVIATIONS .....	xiii
 <b>CHAPTER – 1 INTRODUCTION .....</b>	 1
 <b>CHAPTER – 2 LITERATURE REVIEW .....</b>	 6
2.1. Fenugreek Introduction: .....	6
2.1.1 Taxonomy: .....	6
2.1.2. Origin, history, and distribution: .....	7
2.1.3. Plant description: .....	8
2.1.4. Cytogenetics: .....	9
2.1.5. Economic Uses: .....	9
2.2. Breeding approaches for Fenugreek improvement: .....	11
2.2.1. Selection: .....	11
2.2.2. Hybridization: .....	12
2.2.3. Mutagenesis: .....	13
2.2.4. Tissue culture:.....	15
2.2.5. Modern breeding techniques: .....	16
2.3. Traits of interest for fenugreek:.....	17
2.4. Improvement of disease resistance in fenugreek:.....	18
2.4.1. Important diseases of fenugreek in western Canada: .....	18
2.4.2. Cercospora leaf spot: .....	18
2.4.2.1. Causal agent of CLS: .....	19
2.4.2.2 Microscopic description:.....	21
2.4.2.3 Symptoms of CLS:.....	21
2.4.3. Epidemiology of Cercospora leaf spot: .....	23
2.4.3.1. Nature of pathogen:.....	24
2.4.3.2. Host characteristics: .....	25
2.4.3.3. Environmental factors:.....	26
2.5. CLS management: .....	28

2.6. Genetics of Disease resistance: .....	30
2.7. Inheritance of CLS resistance: .....	32
2.8. Heritability of CLS resistance: .....	33

**CHAPTER – 3 HYBRIDIZATION BETWEEN RESISTANT AND SUSCEPTIBLE FENUGREEK ACCESSIONS AND EVALUATION OF CERCOSPORA LEAF SPOT RESISTANCE IN SEGREGATING GENERATIONS.....**

3.1. Introduction: .....	35
3.2. Materials and Methods: .....	39
3.2.1 Development and Identification of resistant genotypes: .....	39
3.2.1.1. Choice of parents and experimental design: .....	39
3.2.1.2. Artificial hybridization:.....	40
3.2.1.3. Advancement of generations: .....	41
3.2.1.4. Identification of resistant genotypes: .....	42
3.2.1.5. Inoculum preparation: .....	45
3.2.1.6. Artificial inoculation and disease assessment: .....	45
3.2.2. Inheritance of CLS resistance: .....	46
3.2.3. Data Analysis:.....	49
3.3. Results: .....	50
3.3.1. Artificial hybridization: .....	50
3.3.2. Identification of top performing family:.....	52
3.3.3. Selection of high-yielding and resistant genotypes from the field: .....	52
3.3.4. Correlation between different morphological traits of fenugreek: .....	56
3.3.5. Generation mean analysis from greenhouse experiment for CLS scores:.....	58
3.3.6. Genetics of resistance to <i>C. traversiana</i> in fenugreek:.....	60
3.3.7. Variance components and heritability estimates: .....	64
3.4. Discussion: .....	66

**CHAPTER-4 NOVEL TECHNIQUES FOR SCREENING CERCOSPORA LEAF SPOT RESISTANT FENUGREEK GENOTYPES.....**

4.1. Introduction: .....	72
4.2. Material and methods .....	76
4.2.1. Preparation of plant material .....	76
4.2.1.1. Whole plant assay: .....	76
4.2.1.2. Detached leaf assay:.....	76

4.2.2. Culture maintenance and inoculum preparation: .....	77
4.2.3. Inoculum application: .....	77
4.2.3.1. Whole plant assay: .....	77
4.2.3.2. Detached leaf assay:.....	78
4.2.4. Disease assessment: .....	78
4.2.4.1. Whole plant assay: .....	78
4.2.4.2. Detached leaf assay:.....	79
4.2.5. Data analysis:.....	79
4.3. Results: .....	80
4.3.1. Whole plant assay: .....	80
4.3.1.1. Days to first disease appearance: .....	80
4.3.1.2. Disease score (AUDPC): .....	81
4.3.1.3. Lesion diameter and number of lesions: .....	81
4.3.1.4. Correlation between components of CLS resistance in whole plant assay: .....	82
4.3.2. Detached leaf assay: .....	86
4.3.2.1 Effect of temperature on disease severity: .....	86
4.3.2.2. Effect of wounding on disease severity: .....	87
4.3.2.3. Difference among genotypes for disease severity: .....	87
4.3.3. Correlation between WPA and DLA: .....	90
4.4. Discussion: .....	93

<b>CHAPTER – 5 EFFECTS OF HOST SUSCEPTIBILITY, PLANT AGE AND INOCULUM CONCENTRATION ON CERCOSPORA LEAF SPOT SEVERITY IN FENUGREEK.....</b>	<b>98</b>
5.1. Introduction:.....	98
5.2. Material and Methods: .....	101
5.2.1. Host plants: .....	102
5.2.2. Experimental design: .....	102
5.2.3. Inoculum preparation and inoculation: .....	103
5.2.4. Disease assessment: .....	103
5.2.5. Data analysis:.....	105
5.3. Results: .....	106
5.3.1. Area under disease progress curve (AUDPC): .....	108
5.3.2. First disease appearance (Incubation Period): .....	110

5.3.3. Defoliation: .....	112
5.3.4. Pod Infection:.....	114
5.3.5. Seed yield loss: .....	116
5.3.6. Forage Yield loss: .....	119
5.4. Discussion: .....	121
<b>CHAPTER-6 SUMMARY AND FUTURE DIRECTIONS .....</b>	<b>128</b>
6.1. Summary: .....	128
6.2. Future directions:.....	129
<b>REFERENCES: .....</b>	<b>132</b>
<b>APPENDICES .....</b>	<b>152</b>

## LIST OF TABLES

<b>Table 3.1:</b> List of top five and bottom five fenugreek F <sub>2</sub> families along with 3 parental genotypes ranked based on their respective seed and forage yield.....	54
<b>Table 3.2:</b> List of the top 20 genotypes ranked based on their biomass (seed + forage) yield. Resistant category for each genotype was determined after treating their progenies (F <sub>2:3</sub> ) with suspension inoculum of <i>Cercospora traverisana</i> .....	55
<b>Table 3.3:</b> Pearson correlation coefficient between various morphological parameters of fenugreek genotypes developed through artificial hybridization grown at LRDC field.....	57
<b>Table 3.4a:</b> Results of analysis of variance (ANOVA) for Cercospora leaf spot (CLS) score of different generations of fenugreek grown in controlled environment conditions at LRDC.....	59
<b>Table 3.4b:</b> Results of Cercospora leaf spot (CLS) mean disease score and standard error of the mean for six generations of the 2 different cross combinations.....	59
<b>Table 3.5:</b> A monogenic dominant model to explain segregation pattern of CLS resistance in fenugreek. Each individual plant in different generations were inoculated with <i>C. traversiana</i> in two controlled environment experiments conducted in a greenhouse at the Lethbridge Research and Development Center. Frequency of plants with various CLS scores within different generations were subjected to $\chi^2$ - test.....	62
<b>Table 3.6:</b> Frequency of F <sub>2:3</sub> families of fenugreek which showed two different contrasting categories (homozygous resistant and heterozygous resistant) while treated with inoculum of <i>C. traverisana</i> .....	63
<b>Table 3.7:</b> Genetic variance components and heritability estimates for resistance to cercospora leaf spot in fenugreek.....	65
<b>Table 4.1:</b> Mean values for different components of disease resistance after treating individual plants with conidial suspension ( $2 \times 10^5$ conidia/ml) of <i>Cercospora traversiana</i> .....	83
<b>Table 4.2:</b> Correlation analysis of different parameters of resistance in relation to Cercospora leaf spot of fenugreek while inoculating whole plants.....	85
<b>Table 4.3a:</b> Analysis of variance for detached leaves of 46 genotypes and their state (wounding and un-wounding) in terms of disease score and first disease appearance. ....	88

<b>Table 4.3b:</b> Mean disease score and first visible symptoms appearance (days after inoculation) of wounded and un-wounded detached leaves irrespective of genotypes.....	88
<b>Table 4.3c:</b> Categorisation of 46 different genotypes into resistant and susceptible based on their average disease score obtained after treating detached leaves irrespective of state of leaves (wounding and un-wounding) .....	89
<b>Table 5.1:</b> Analysis of variance (ANOVA) conducted for various components of CLS resistance.....	107
<b>Table 5.2:</b> AUDPC values of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations. ....	105
<b>Table 5.3:</b> Incubation period (number of days) of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.....	109
<b>Table 5.4:</b> Defoliation (%) for CLS affected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations. ....	111
<b>Table 5.5:</b> Pod infection (%) of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations. ....	113
<b>Table 5.6:</b> Seed yield loss (%) for fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.....	117
<b>Table 5.7:</b> Forage yield loss (%) for fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.....	120

## LIST OF FIGURES

<b>Figure 2.1:</b> Fenugreek plant.....	6
<b>Figure 2.2:</b> Possible disease cycle of <i>Cercospora traversiana</i> on fenugreek.....	20
<b>Figure 2.3:</b> Symptoms of Cercospora leaf spot Caused by <i>Cercospora traversiana</i> on fenugreek: (A) leaf lesions; (B) coalescence of lesions; (C) infected pod; (D) difference between CLS infected and healthy seeds.....	22
<b>Figure 2.4:</b> Generalised Disease triangle showing possible interactions between factors that can help cause a disease epidemic.....	23
<b>Figure 3.1:</b> Mean air temperature and precipitation from May 2017 to September 2018 as compared to 30-year average in LRDC field while growing F <sub>2</sub> generations. Source: <a href="http://ablethr2/weather/weathermain.aspx?Language=0">http://ablethr2/weather/weathermain.aspx?Language=0</a> .....	44
<b>Figure 3.2:</b> Percentage of crossing success; (A) = Crossing success within a day, (B) = Crossing success within a year. Bars with different subscript are significantly different (P ≤ 0.05). .....	51
<b>Figure 3.3:</b> Frequency distribution of Cercospora leaf spot (CLS) scores observed in individual plants of 6 different generations within two different experiments conducted in greenhouse at Lethbridge Research and Development Center.....	61
<b>Figure 4.1:</b> Changes in Cercospora leaf spot lesion diameter and number of lesions over time in fenugreek leaves. Error bars represent standard error of mean and means with different superscripts are significantly different (P < 0.05) from each other; a, b, c are for lesion diameter and A, B, C are for number of lesions.....	84
<b>Figure 4.2:</b> Correlation of mean disease score between detached leaf assay (unwounded leaves kept at 25/14°C) and whole plant assay conducted in controlled environment at Lethbridge Research and Development Center.....	91
<b>Figure 4.3:</b> Some glimpse of experiment: <b>A</b> -Maintaining humidity by keeping plants along with pods in a tray containing water and covered by transparent lid. <b>B</b> -Difference between resistant and susceptible plants in WPA 30 days after inoculation. <b>C</b> - Detached leaves in a 6cm petri plate <b>D</b> -Difference between resistant and susceptible leaves. <b>E</b> - Asymptomatic leaf samples after 45 days kept at 20°C. <b>F</b> - Disease assessment chart as percent of leaf area infected .....	92
<b>Figure 5.1:</b> Relationship of percent seed yield loss of fenugreek to percent pod infection due to Cercospora leaf spot for resistant and susceptible genotypes.....	118
<b>Figure 5.2:</b> Non-linear (cubic) relationship between AUDPC values with inoculum concentration [ $\log_{10}$ (conidia/ml)] for resistant and susceptible genotypes 20, 40 and 60 days after sowing (DAS).....	127

## LIST OF ABBREVIATIONS

- 2, 4-D = 2,4-Dichlorophenoxyacetic acid  
AAFC = Agriculture and Agri-Food Canada  
AFLP = Amplified Fragment Length Polymorphism  
ANOVA = Analysis of variance  
AUPDC = Area under disease progress curve  
BC<sub>1</sub>(R) = Backcross with resistant parent  
BC<sub>1</sub>(S) = Backcross with susceptible parent  
CABI = Center for Agriculture and Biosciences International  
CLS = Cercospora leaf spot  
DAI = Days after inoculation  
DAS = Days after sowing  
dd = Distilled deionised  
DF = Degrees of freedom  
DES = Diethyl sulphate  
DLA = Detached leaf assay  
DMS = Dimethyl sulfate  
DNA = Deoxyribonucleic acid  
DS = Disease score  
EMS = Ethyl methane sulphonate  
F<sub>1</sub> = First filial generation  
F<sub>2</sub> = Second filial generation  
FDA = First disease appearance  
GA = Gibberellic acid  
GLS = Gray leaf spot  
IAA = Indole-3-acetic acid  
IPA = Indole-3-pyruvic acid  
ISSR = Inter simple sequence repeat  
LRDC = Lethbridge Research and Development Center  
LSD = Least significant difference  
LSMEAN = Least-squares mean  
MMS = Methyl methane sulfonate  
MS = Murashige and Skoog  
NAA = 1-Naphthaleneacetic acid  
NS = Non-significant  
PDA = Potato dextrose agar  
QTL = Quantitative trait locus  
*r* = Correlation coefficient  
*r*<sup>2</sup> = Coefficient of determination  
RAPD = Random Amplified Polymorphic DNA  
RCBD = Randomized Complete Block Design  
RFLP = Restriction Fragment Length Polymorphism  
SE = Standard error  
SS = Sum of Squares

UV = Ultra-violet  
WPA = Whole plant assay

## **CHAPTER – 1**

### **INTRODUCTION**

Forage fenugreek, a newly introduced crop to Canada, is being developed at Lethbridge Research and Development Center (LRDC). This new crop has many important features that are expected to benefit Canada's agri-food industry in general and the cattle industry in particular. This annual bloat free legume crop produces high-quality forages in all growth stages and contains precursors of natural growth promoting hormones such as diosgenin and tigogenin unlike other forage legumes (Acharya et al. 2008). Fenugreek's seed and leaves are used as a spice to enhance the taste of various dishes particularly in south Asia and the Mediterranean region. Being endowed with a number of medicinal properties, fenugreek is used in the treatment of many diseases since ancient times. It's use in curing diabetes (Al-Habbori and Raman 1998; Babu et al. 2010), high cholesterol (Stark and Madar 1993), ulcer (Helmy 2011), cancer (Alsemari et al. 2014), abdominal disorders (Moradikor et al. 2013), inflammation (Chauhan et al. 2010), arthritis (Pundarikakshudu et al. 2016), wounds (Muhammed and Salih 2012) and many more diseases has been reported. The seeds of fenugreek can be used in the production of gums, essential oils, natural dyes, cosmetics and flavoring agents (Petropoulos 2002). Furthermore, Petropoulos (2002), in his book has emphasized the nitrogen-fixing nature of fenugreek and use of this crop as a green manure (a fertilizer consisting of growing plants that are ploughed back into the soil) which can help replenish the soil in degraded areas whereas insect repellent properties of this crop help itself as well as other crops by keeping away harmful insects in field and storage facilities. This may save a considerable amount of money of farmers by reducing the cost of chemical fertilizers and insecticides.

Being a drought tolerant crop, it can be grown in semi-arid regions like western Canada with minimal irrigation (Acharya et al. 2008). All these features make fenugreek an attractive option for farmers, seed companies, the dairy and beef industries and pharmaceutical industries in Canada.

A number of diseases constrain the production of fenugreek. Leaf spot, caused by *Cercospora traversiana*, is one of the major problems associated with fenugreek cultivation as it can cause considerable economic loss (Leppik 1959; Prasad et al. 2014; Zimmer 1984). This fungus affects mainly shoot parts causing blight and damping off (Mohamed and Ghoneem 2002) ultimately reducing seed and biomass yield. In addition to that, this seed borne pathogen imparts a dark color to the seeds rendering them unacceptable to consumers (Ryley 1989). Although *Cercospora* leaf spot (CLS) is native to the south Asia region, it is found to be equally problematic in other regions mainly Australia, Europe and South America (Prasad et al. 2014; Ryley 1989). This disease was observed to cause up to 80% yield loss in Manitoba and other regions of the Canadian prairies (Zimmer 1984). Assessment of loss by this disease on an economic basis has not been done yet in Canada. Acharya et al. (2010) have reported that farmers in other parts of the world along with Canada are using different fungicides to control this disease with various rates of success. However, these chemicals may have a negative impact on humans as well as beneficial organisms in the surrounding area and may lead to ecological problems while increasing the cost of production. Development of disease resistant cultivars to control the pathogen is considered a good alternative to combat the

disease as it is also environmentally friendly, durable and cost effective (Trivoli et al. 2006).

Statistics show that the acreage of cultivation of fenugreek is increasing day by day in Canada (Ministry of Agriculture, Saskatchewan 2014; Slinkard et al. 2006) and other countries where it has been introduced recently. This expansion in the cultivated area may increase the area contaminated with the pathogen and cause losses to farmers. The widely adapted cultivar in Canada; AC Amber and recently introduced high-yielding forage cultivar Tristar, though performing well, have been categorized as susceptible to CLS infection in the screening tests done at LRDC by Prasad et al. (2014). Widespread use of fenugreek requires that we solve these production problems through a proper understanding of the agronomical aspects of growing the crop, develop genotypes with disease resistance and select germplasm that can adapt well particularly to the western Canadian region. Keeping the above in mind, the present study was undertaken to develop high yielding CLS resistant genotypes which can act as a good preventive measure and can instill a sense of security in fenugreek growers. This study started with crossing the disease resistant lines L3717 and PI138687 (Prasad et al. 2014) with Tristar, a high yielding cultivar developed at LRDC for western Canada. The new genotypes were screened against the pathogen followed by selection and evaluation of suitable genotypes under greenhouse and field conditions. A concurrent experiment was also set-up to identify the inheritance pattern and heritability of genes responsible for CLS resistance in fenugreek. This information, we believe, will be helpful for future researchers who want to breed crops for CLS resistance.

Artificial hybridization, controlled mating between different parents, is considered one of the best methods to generate genetic variability in breeding populations (Fehr 1987). However, the small floral structure, lack of sufficient knowledge on anthesis behavior and highly self-pollinated nature of the flowers makes fenugreek difficult crop to intercross artificially (Petropoulos 2002). Although there are reports of improvement of fenugreek through artificial hybridization as a breeding tool (Cornish et al. 1983; Saleh 1996), detailed information on artificial hybridization in fenugreek is scarce. Our intention through this research is not only to generate high yielding resistant genotypes, but also to add information on an efficient technique of artificial hybridization of fenugreek which may help researchers and students who aspire to do research on improvement of fenugreek in the future.

Generalised information for *Cercospora* spp suggests its growth is highly favored in warm and humid climatic situations (Cooperman and Jenkins 1986; Ghosh et al. 2012; Khan and Khan 2009; Munkvold 2005) leading to maximum crop loss. Higher inoculum concentration, longer leaf wetness duration, earlier growth stages and physiological damage to plant parts were found to exacerbate CLS (Bradley and Ames 2010; Pundhir and Mukhopadhyay 1987; Weiland and Koch 2004; Wnidels et al. 1998; Wolf and Verreet 2005). Complexities may arise due to positive interaction between biotic (host, pathogen) and abiotic (environmental) factors leading to disease epidemics. Therefore, knowledge about epidemiological factors affecting disease establishment, their development and dispersal is of prime importance. Although, one of the most devastating diseases of fenugreek (Acharya et al. 2010; Malhotra 2010; Prasad et al. 2014), limited

knowledge about the environment in which *C. traversiana* causes infections has hindered progress in managing CLS. Even, the life cycle of *C. traversiana* is poorly understood. At present, there are no recommendations for the control of CLS in fenugreek in western Canada.

Therefore, this research project aims to develop and evaluate new germplasms of fenugreek with resistance to Cercospora leaf-spot and their adaptation to western Canada growing conditions and to identify the epidemiological factors affecting CLS severity through field and greenhouse trials.

The specific research objectives are to:

- Study floral morphology of fenugreek and develop methods for successful artificial hybridization of fenugreek.
- Create genetic variability in fenugreek so as to select high yielding *Cercospora* leaf spot resistant genotypes and study genetics of CLS resistance in fenugreek.
- Develop effective methods of screening fenugreek plants for disease resistance in controlled environmental conditions.
- Add to the present state of knowledge on epidemiology and morphology of *Cercospora traversiana*.
- Identify the effect of host susceptibility, plant age and inoculum concentration on CLS severity through controlled environment experiments.

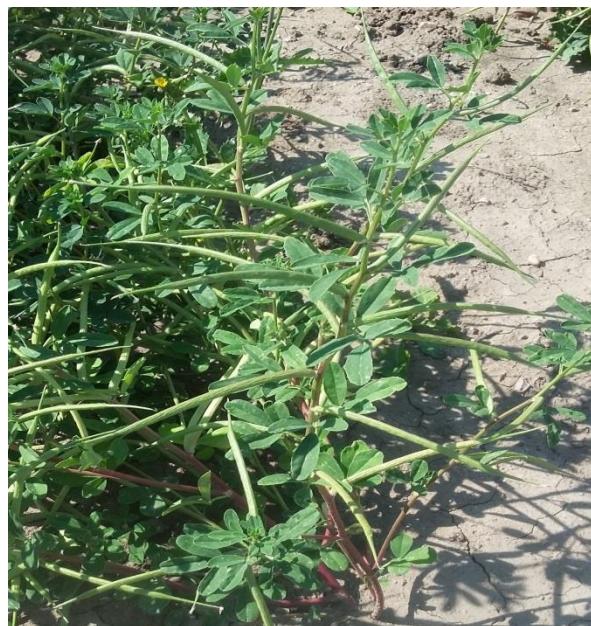
## CHAPTER – 2

### LITERATURE REVIEW

#### **2.1. Fenugreek Introduction:**

##### **2.1.1 Taxonomy:**

Domain	:	Eukarya
Kingdom	:	Plantae
Division	:	Magnoliophyta (or Anthophyta)
Class	:	<i>Magnoliopsida</i>
Order	:	<i>Fabales</i> (or <i>Leguminales</i> )
Family	:	<i>Fabaceae</i>
Sub-family	:	<i>Trifolieae</i>
Genus	:	<i>Trigonella</i>
Sub-genus	:	<i>Foenum-graecum</i>
Species	:	<i>Trigonella foenum-graecum</i> (L.)



**Figure 2.1:** Fenugreek plant

### **2.1.2. Origin, history, and distribution:**

The origin of fenugreek (*Trigonella foenum-graecum*) is debatable (Sinskaya 1961).

Divergent opinions have been expressed by different authors about the origin of fenugreek. Vavilov (1926, 1951) suggested that fenugreek originated in the Mediterranean region, while De Candolle (1964) and Fazli and Hardman (1968) proposed an Asian origin for the crop and Dangi et al. (2004) suggested that *T. foenum-graecum* is native to Turkey.

Fenugreek is used for different purposes since ancient times. The species name of fenugreek, *foenum-graecum*, came from "Greek hay" which suggests the use of this crop as forage (Acharya et al. 2008, Petropoulos 2002). This crop is one of the oldest known medicinal herbs found to be used in Chinese traditional medicine and Indian Ayurvedic medicine (Petropoulos 2002). Recorded history also suggests that this crop was predominantly used as a spice crop to enhance the taste of different European and south Asian dishes. 2000-1700 B.C. old carbonized fenugreek seeds were found in the Punjab state of India, suggesting its use in ancient times (Saraswat 1984). Ebers Papyrus, a famous 1500 B.C. old medicinal document found in Egypt, also has enlisted benefits of the fenugreek plant (Betty 2008).

Fenugreek, either in a cultivated or wild form, is widely distributed throughout the world. Most countries have their local names for this crop which indicates its wide distribution. It is presently cultivated in all habitable continents of the world (Acharya et al. 2008); though some continents have only started growing this crop a few decades back. India is the leading fenugreek producer which supplies almost 70-80% of the total world

requirement (Edison 1995). Commercial cultivation of fenugreek in Canada started at around 1990. So far, five cultivars of fenugreek have been released for different uses in Canada (Thomas et al. 2011).

According to Petropoulos (2002), a temperate climate with moderate to low rainfall and annual mean temperature of 7.8-27.5°C is best suited for the cultivation of fenugreek. This crop also likes a well-drained loamy soil with a slightly alkaline pH. Experience in western Canada suggests that a prolonged period of cool and wet conditions causes the plants to suffer from leaf diseases and consequently failure of plants to mature for seed harvest.

### **2.1.3. Plant description:**

Fenugreek is an annual dicot belonging to the legume family with a lifespan of about four months (Acharya et al. 2007). After germination of seeds following the epigeal method, the vegetative phase of the plant starts. Growth rate of fenugreek is slow at the beginning of the growing season (McCormick et al. 2006). Fenugreek has simple trifoliate leaves with erect, slender, hollow stems which may reach up to 1m in height. It has a taproot system with nodules on the primary and secondary roots, which grow in symbiotic association with *Rhizobium meliloti* and can fix up to 42.4kg/acre of nitrogen into the soil (Campbell and Reid 1982). Most cultivars of fenugreek grown in western Canada have an indeterminate growth habit, which means vegetative growth continues even after the plant is in the reproductive phase.

Flowering in fenugreek, in general, starts after 35-40 days of sowing. The flowers are mostly found in pairs which are initially yellowish and look white when they mature (Petropoulos 2002). They have typically papilionaceous flowers with 5 fused sepals, 5

petals (1 standard, 2 wings, and 2 keels), 10 stamens, and a pistil. Two keels are fused together and cover all stamens and the pistil throughout the entire life of the flower preventing cross pollination in fenugreek (Petropoulos 2002). Pods are about 15 cm long containing 10-20 seeds which are hard, smooth, dull yellow to brownish yellow in color (Altuntas et al. 2005; Moradi kor and Moradi 2013).

#### **2.1.4. Cytogenetics:**

According to Darlington and Wylie (1945), many species of fenugreek along with *Trigonella foenum-graecum* are diploids with  $2n=16$  chromosomes. They also reported that some other species of the genus *Trigonella* have a diploid set of 18, 22 and 28 chromosomes. Furthermore, *T. hamosa* from Egypt have 16 and 44 chromosomes; *T. geminiflora* from Iran and Asia Minor, and *T. grandiflora* from Turkey have 44 chromosomes; *T. polycerata* collected around the Mediterranean region and Asia was reported to have 28, 30 and 32 chromosomes, while *T. ornithopodioides* was reported to possess 18 chromosomes in its genome (Petropoulos 2002). Ahmad et al. (1999) have observed the presence of  $\beta$ -chromosomes in many fenugreek accessions.

#### **2.1.5. Economic Uses:**

Fenugreek seeds and fresh or dried leaves have been widely used as a spice for enhancing the taste of various foods in different parts of the world primarily south Asia, Africa and countries of the Mediterranean region (Acharya et al. 2008). Green succulent leaves are also used as a green vegetable. Its use as a bread mix in Africa, a condiment in the Arabic region, a cheese flavor in Switzerland, a soup seasoning and tea decoction in China, for beverage making in Sudan and Egypt, are highlighted (Petropoulos 2002). In terms of

nutrients, fenugreek is rich in protein, oils and several minerals such as Mg, Ca, Fe, Zn, Mn, Co, Ba, Cu and Br (Duke 1981).

Use of fenugreek as a medicinal herb has been recognized since very early times. Its use in Chinese traditional and Indian Ayurvedic medication has been documented in several historical records and publications. Also supported with modern day medicinal research, fenugreek is identified as having anti-diabetic (Al-Habbori and Raman 1998; Babu et al. 2010), anti-ulcer (Helmy 2011), anti-cancer (Alsemari et al. 2014), anti-inflammation (Chauhan et al. 2010), anti-allergy (Bae et al. 2012), anti-microbial (Sharma et al. 2016), anti-helminthic (Swarnakar et al. 2014), cholesterol reducing (Stark and Madar 1993) and wound-healing (Muhammed and Salih 2012) properties. Furthermore, it can be used as a galactagogue to promote milk production in weaning mothers, as a tonic to gargle for a sore throat and as a stimulant of appetite and the digestion process (Petropoulos 2002). Several other important medicinal features of this crop have been reported. Thus, there is increasing interest of pharmacological companies towards this crop.

Several studies conducted in Canada and other parts of the world have already shown fenugreek as a new potential annual forage crop with a multitude of attractive features. This crop is similar to alfalfa, a dominant forage crop worldwide, regarding nutrition, yield, and quality of forage, and is observed to provide similar rumen conditions as alfalfa (Acharya et al. 2008, Mir et al. 1998). Unlike alfalfa, fenugreek is bloat-free and can provide quality forage in all growth stages (Acharya et al. 2008). Fenugreek has also been reported to increase milk flow from lactating cattle (Edison 1995). Presence of diosgenin, a precursor of the different steroidal compounds, in seeds and forage of

fenugreek can help weight gain in cattle (Acharya et al. 2008; Mir et al. 1998) thus can reduce the use of artificial growth promoting hormones in growing cattle.

The seeds of fenugreek can also be used in the production of gums, essential oils, natural dyes, cosmetics and as a flavoring agent. Pharmaceutical and nutraceutical industries are interested in it due to the presence of high-value compounds such as diosgenin, galactomannan, sapogenin and isoleucine which are known to have huge medicinal importance (Acharya et al. 2008). The peculiar smell from the volatile compounds present in fenugreek can be used in making artificial maple syrup, flavoring tobacco and several foods such as bread, cheese, tea and pizza (Slinkard et al. 2006; Smith 1982).

## **2.2. Breeding approaches for Fenugreek improvement:**

Researchers working on the improvement of fenugreek have mainly concentrated on increasing production of seeds and biomass, increasing the level of biochemicals particularly of pharmacological importance and tolerance to different biotic and abiotic stresses (Petropoulos 2002). Various approaches for the improvement of fenugreek have been adapted to date.

### **2.2.1. Selection:**

Selection is an important method of plant breeding which constitutes selecting top performers for an area and propagating them for commercial production (Petropoulos 2002). Identifying plants with a particular superior character is essential for crop improvement, so selection forms the basis of every crop improvement program. Furthermore, selection is required even after the creation or an assemblage of genetic variation (Zandi et al. 2015). Several cultivars of fenugreek have been developed

employing this method (Saleh 1996). Out of 23 developed varieties of fenugreek in India, 20 were through the selection method (Malhotra, 2010). Petropoulos (2002) in his book ‘Fenugreek’ has reported that presence of double pods is an indication of higher diosgenin level in seeds, which is supported by Ahmed et al. (1989), who developed a variety of fenugreek by passively selecting plants for double pods. Selection of fenugreek is basically done for; a) improvement of morphological characters such as high seed and forage yield, early maturity, etc. and, b) improved level of chemical constituents with nutraceutical importance. Continuous selection of fenugreek plants with superior traits lead in the development of cultivars RH 2701, RH3128 and RH2699 with higher diosgenin level, higher seed yield, and higher protein content respectively (Petropoulos 2002, 1973). ‘Tristar,’ the first North American forage cultivar of fenugreek developed at Lethbridge Research and Development Center, was also developed by selecting vigorous plants from an introduced fenugreek line L3314 with good biomass yield among 55 entries (Acharya et al. 2007). Prasad et al. (2014) have selected two lines L3717 and PI 138686 as Cercospora leaf spot resistant genotypes from 53 world accessions by screening plants against *Cercospora traversiana*.

### **2.2.2. Hybridization:**

Artificial hybridization, controlled mating between different parents, is considered one of the best methods for generating genetic variability in breeding populations (Fehr 1987). However, small floral structure, lack of sufficient knowledge on anthesis behavior and highly self-pollinated nature of the flowers make fenugreek one of the difficult crop to cross pollinate (Petropoulos 2002). Information about artificial hybridization of fenugreek is scarce. However, there are some reports of improvement of fenugreek

through artificial hybridization as a breeding tool (Chaudhary and Singh 2000; Cornish et al. 1983; Edison 1995; Petropoulos 2002; Saleh 1996). Cornish et al. (1983) employed artificial hybridization as a method to develop fenugreek with high diosgenin content. Chaudhary and Singh (2001) in an experiment to determine the inheritance pattern of growth habit obtained only four percent success while crossing indeterminate and determinate fenugreks. Artificial hybridization can be helped by regularizing and understanding the meiotic behavior of parental plants. It is possible to increase vigor, seed fertility and resistance to different diseases and pests such as powdery mildew, fusarium wilt, root rot, downy mildew and thrips through interspecific hybridization of fenugreek (Malhotra 2010).

### **2.2.3. Mutagenesis:**

Artificially induced mutations are a valuable tool in creating genetic variability especially when an important trait is missing in the breeding material of a crop (Acharya et al. 2007). Among various methods of mutagenesis, irradiation and chemical treatments are most common. Most significant sources of radiation primarily used by biologists are X-rays,  $\gamma$ -rays,  $\alpha$ -particle,  $\beta$ - particle, neutrons and protons where as they use methyl methane sulphonate (MMS), ethyl methane sulphonate (EMS), diethyl sulphate (DES), dimethyl sulphate (DMS) ethyleneimine, ethyl nitroso urea, ethyl nitroso urethane and methyl nitroso urea as chemical mutagens for crop improvement (The Manual of Mutation Breeding 1977). Mutation breeding has been successfully applied for improvement of a wide range of plants which include safflower (Fernandez and Munoz 1987; Sahu and Kumar 1997), arabidopsis (Henikoff and Comai 2003), wheat (Millet and

Feldman 1994; Williams et al. 1992), rice (Min et al. 1989), barley (Zhu et al. 2003), banana (Novak et al. 1990) and many more.

In fenugreek, use of both irradiation and chemicals to induce mutations has been attempted (Basu et al. 2008; Petropoulos 2002). Increasing the level of diosgenin and other useful chemicals, increasing the seed and biomass yield and shortening the maturity date were the prime objectives of the researchers while using mutation breeding in fenugreek. Petropoulos (2002) has mentioned the use of chronic  $\gamma$ -rays ( $\text{Co}^{60}$ ) in the open field for irradiation. In addition to that, dry seeds of fenugreek were treated with acute  $\gamma$ -rays which resulted in delaying of flowering and decrease in height and seed yield. From the same study, he also suggested that the dose of radiation should be low to produce useful fenugreek mutants. In the case of chemical mutations, EMS is the most preferred mutagen in fenugreek because it induces localized DNA change (point mutations) rather than a drastic alteration in the plant genome (Acharya et al. 2007; Henikoff and Comai 2003). Basu et al. (2008) used EMS with the intention of producing some early maturing fenugreek mutants with a determinate growth habit, higher seed yield, and seed quality. They obtained some early maturing mutants but with lower seed yield which, according to them, was due to the selection criteria for determinant types in each generation. Acharya et al. (2006) in research conducted at AAFC, Lethbridge, were able to produce fenugreek with high diosgenin content and increased biomass using EMS as a mutagen. A similar result was also obtained by Petropoulos (2002). Use of chemical mutagens other than EMS is also documented. Sodium azide (Prabha et al. 2010), methyl methanesulphonate (Laxmi and Dutta 1987; Laxmi et al. 1980), dimethyl sulfate and

diethyl sulfate (Jain and Agarwal 1987) were used for altering the morphological characters of *Trigonella* species.

#### **2.2.4. Tissue culture:**

Different approaches such as anther and pollen culture, ovule culture, in-vitro selection, cell and tissue culture, somaclonal variation and genetic transformation can also be used to produce new, high yielding fenugreek types with improved biochemical constituents. These techniques strengthen the breeding program of fenugreek by offering new research tools and avenues to rapidly develop variations within fenugreek. Owing to the lower success rate in hand pollination, somatic hybridization has proved to have high potential in the breeding of fenugreek even in distantly related species with a more economical investment (Malhotra 2010).

*In-vitro* culture has been successfully used in the production of secondary metabolites which can be used as drugs, flavor and fragrances, dye and pigments, pesticides, and food additives (Ahmed et al. 2013; Al-Qudah et al. 2011; Hussain et al. 2012; Karuppusamy 2009). Oncina et al. (2000) found a different level of diosgenin in callus from different parts of fenugreek with leaf callus exceeding the level over the stem and root callus. A similar result was obtained by Ciura et al. (2015) suggesting that leaves can be used as a place for the accumulation of saponins. In a root hair culture experiment, Merkli et al. (1997) obtained the fastest callus growth in McCown's woody plant (WP) medium supplemented with 3% sucrose whereas, the highest diosgenin content was obtained in half-strength WP medium with 1% sucrose. The increment in diosgenin level was obtained from callus when they were treated with UV radiation and methyl methanesulphonate (MMS) (Jain and Agarwal, 1994).

Choice of a suitable medium supplemented with an appropriate type and concentration of plant hormones determines the success of any tissue culture experiment. Ciura et al. (2015) found the highest level of diosgenin in the 21<sup>st</sup> and 27<sup>th</sup> day on Murasighe and Skoog (MS) medium supplemented with 3% sucrose. A similar result was obtained by Ortuno et al. (1998). Suspension cultures of fenugreek grown on media supplemented with various concentrations of cholesterol produced higher sapogenin contents than those grown on medium without cholesterol (Khanna et al. 1975). Trisonthi et al. (1980) demonstrated that use of mevalonic acid, in suspension culture grown on Miller or MS medium, promotes the synthesis of steroidal sapogenins in fenugreek tissue. Oncina et al. (2000) reported that MS medium supplemented with 15% (v/v) coconut milk and 3×10-6M NAA was the most suitable medium for callus growth. Radwan and Kokate (1980) studied the role of different plant growth regulators on the induction of callus and found that 10 mg/l 2, 4-D, IAA, IPA and NAA significantly increased the trigonelline content. In contrast to that, treatment with 1 mg/l GA, and 2 mg/l kinetin did not show a significant effect.

#### **2.2.5. Modern breeding techniques:**

Combining different genes, from species belonging to a different genera, family or kingdom, for developing new plant varieties and hybrids through recombinant DNA technology have been practiced in many crops (Azevedo and Araujo 2003). However, there are only a few reports on the use of this technique for fenugreek improvement. Merkli et al. (1997) used *Agrobacterium rhizogenes* strain A4 for the biosynthesis of diosgenin in fenugreek. This is the first report of the use of genetic transformation in fenugreek (Aasim et al. 2014). Using A281 oncogenic strain of *Agrobacterium*

*tumefaciens*, Khawar et al. (2004) tried to induce tumors on root, cotyledon and hypocotyl explants and concluded that the plants are highly susceptible to transformation. There are some other reports on genetic transformation in fenugreek (Raheleh et al. 2011; Reid et al. 2003).

Molecular marker techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), ISSR (inter simple sequence repeats) and AFLP (amplified fragment length polymorphism) are also being used commonly in fenugreek for diversity analysis and various genetic studies (Dangi et al. 2011; Haliem and Al-Huqail 2013; Harish et al. 2011; Kakani et al. 2011; Kumar et al. 2012; Sundaram and Purwar 2011).

### **2.3. Traits of interest for fenugreek:**

Fenugreek is a crop of multiple uses. Considerable emphasis has been placed on breeding fenugreek to improve seed and forage yield. Seed yield is important where this crop is primarily used for spices and raw material for nutraceutical industries whereas forage yield (total biomass) is essential when your objective is to develop a cultivar for a cattle feed. Four Canadian cultivars ‘AC Amber’, ‘CDC Quattro’, ‘CDC Canagreen’ and ‘CDC Canafen’ were developed mainly for use as a spice and for obtaining nutraceutical extracts from seeds. AC Tristar has a high forage yield on the other hand and was developed primarily for use as a livestock forage (Thomas et al. 2011). Research was also conducted to increase the level of diosgenin in seeds and forages; i.e., for use in steroidial industries (Cornish et al. 1983). Other traits of interest in fenugreek include: adaptation to different climatic conditions, large and clean seeds, early maturing, decreased pod shattering, resistance to diseases and insects, drought, salt and other stress

tolerance (Malhotra 2010). Breeding objectives should be determined considering the farmer's knowledge and preferences to be included in a cultivar which subsequently leads to easier and wider adoption of a cultivar (Muhinyuwa et al. 2016).

#### **2.4. Improvement of disease resistance in fenugreek:**

##### **2.4.1. Important diseases of fenugreek in western Canada:**

Fenugreek is affected by several diseases. Although this crop was initially considered to be less affected by diseases, a spectrum of diseases has been reported in different countries (Malhotra 2010). An array of fungal pathogens from the genus *Cercospora*, *Ascochyta*, *Erysiphe*, *Peronospora*, *Fusarium*, *Rhizoctonia*, *Alternaria* are found to affect fenugreek. Similarly, yield reductions due to several bacteria, viruses, and root knot nematodes have also been reported (Acharya et al. 2010; Malhotra 2010). *Cercospora* leaf spot (CLS) has been found to be problematic when fenugreek was grown in western Canada (Acharya et al. 2010).

##### **2.4.2. Cercospora leaf spot:**

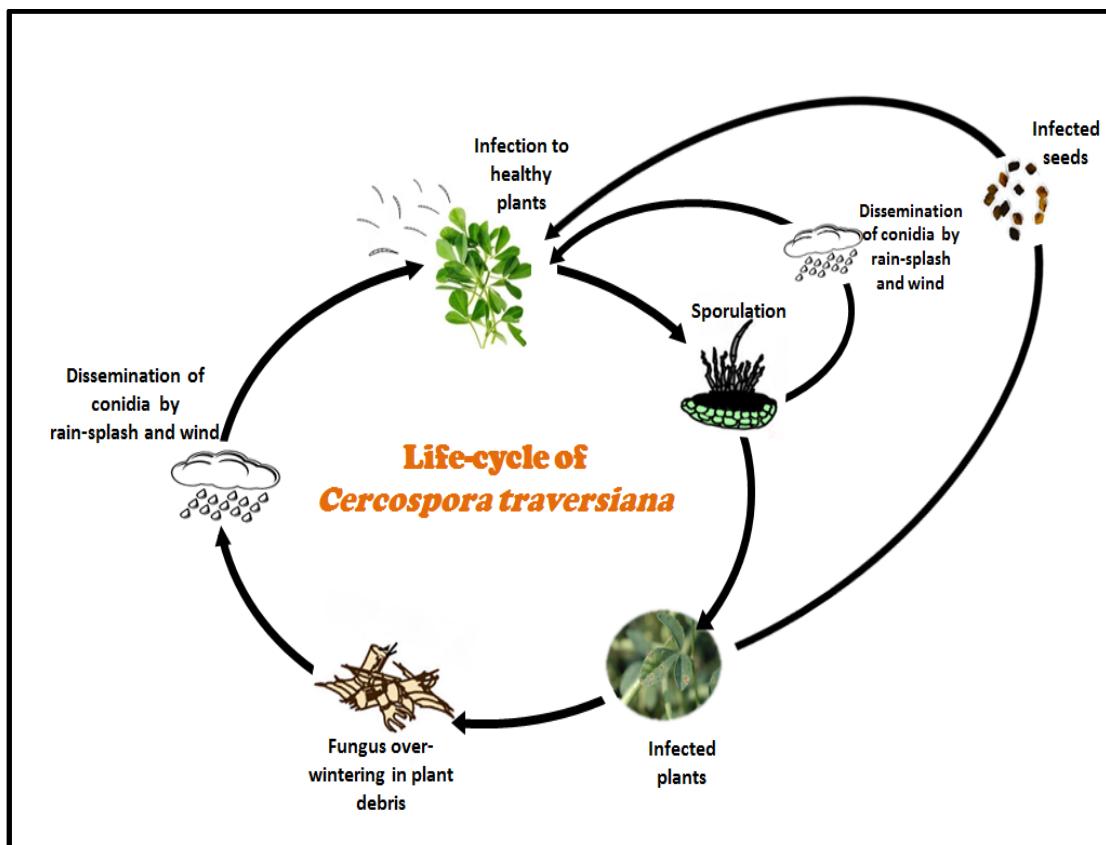
The genus *Cercospora* has been recorded as a highly destructive group of pathogens causing leaf spot on a diversity of crop species worldwide. CLS in groundnut (Alderman and Beute 1987), sugar beet (Jacobsen et al. 2004a; Weiland and Koch 2004), maize (Caldwell et al. 1982; Dagne et al. 2003), asparagus (Cooperman and Jenkins 1986), soybean (Mian et al. 2009; Mengistu et al. 2012), mungbean (Mishra et al. 1988; Thakur et al. 1977a, 1977b), faba bean (Kimber and Paull 2011), tobacco (Fajola and Alasoadura 1970), coffee (Silva et al. 2016), mulberry(Ghosh et al. 2012), cowpea (Fery et al. 1976; Fery and Dukes 1977; Castro et al. 2003), celery (Strandberg and White 1978), rose

(Davis 1938), spinach (Hasan et al. 2016), carrot (Westerveld et al. 2008), rice (Mani et al. 2017), eggplant (Srivastava and Nelson 2012) and sesame (Enikuomehin 2005) are highlighted due to severe production loss. In fenugreek, CLS is caused by *Cercospora traversiana* which is one of the most devastating fungal pathogens accounting for major economic loss in fenugreek around the world (Acharya et al. 2010; Petropoulos 2002; Ryley 1989). When environmental conditions are conducive, this disease has been reported to cause over 80% yield losses in susceptible cultivars of fenugreek (Zimmer 1984). Affected leaves were defoliated in severe conditions and plants are left with only a few leaves at the apical region (Prasad et al. 2014). Photosynthesis is drastically reduced consequently leading to a low seed and forage yield. Not only affecting seed and forage yield, *Cercospora* imparts a black color to infected seeds rendering them unsuitable for the market (Ryley 1989). Though economically important, reviews on this disease have not been carried out intensively. Most of the literature has been in response to the first reported occurrence of the disease (Bobev et al. 1999; Ryley 1989; Voros and Nagy 1972; Zimmer 1984). A screening test to identify CLS resistant genotypes of fenugreek among all collected world accessions was carried out in Canada by Prasad et al. (2014). They identified two genotypes L3717 and PI138687 as being resistant to CLS.

#### **2.4.2.1. Causal agent of CLS:**

The causal agent of CLS in fenugreek is *Cercospora traversiana*, fungi imperfecti belonging to the order Capnodiales. This seed borne pathogen is considered to have originated in southern Asia (Leppik 1959), and transport of infected seeds could be the reason behind its occurrence all around the globe (Leppik 1959; Prasad et al. 2014). *C. traversiana* is the only species of *Cercospora* infecting fenugreek (Ryley 1989). As other

*Cercospora* species, *C. traversiana* is thought to survive between crop seasons as conidiophores or dormant mycelia (stromata) in infected fenugreek debris (Acharya et al. 2010), and its method of transmission is predominately by infected seeds, blowing air and splashing rain. Dispersal of the disease inoculum with such divergent media makes this disease difficult to control. Information regarding the pattern of inoculum dispersal and factors influencing its dissemination is still lacking. In addition, the mechanism of survival of the pathogen during the extreme cold temperatures often encountered in Canada is yet to be studied.



**Figure 2.2:** Possible disease cycle of *Cercospora traversiana* on fenugreek.

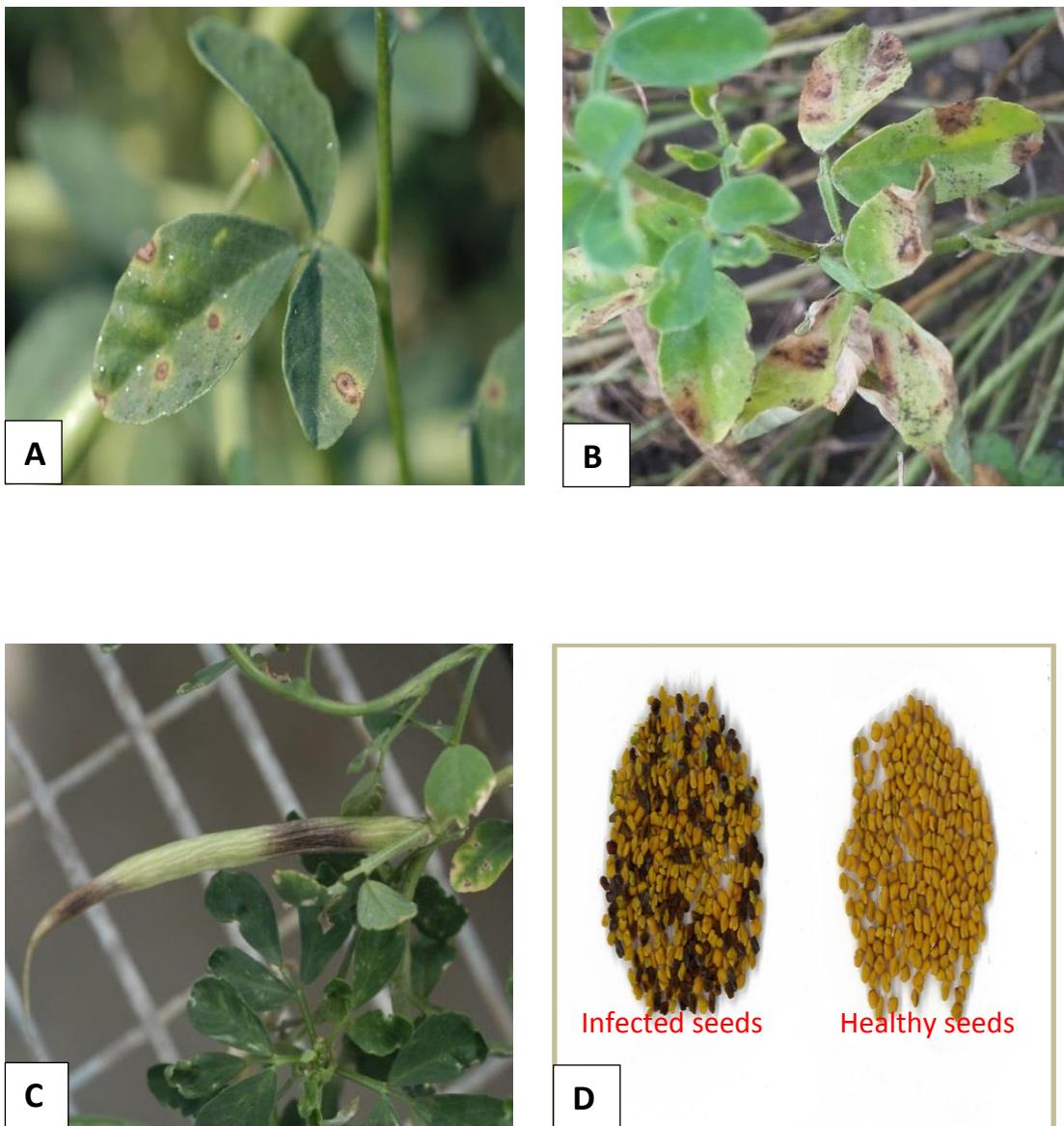
#### **2.4.2.2 Microscopic description:**

Ryley (1989) found variation in size of conidia, septum number and conidiophore length of *C. traversiana* which he assumed were due to variation in the substrate used. Colonies on PDA are circular with a cottony white growth on the upper side whereas it has an olivaceous black appearance from the underside. Conidiophores are arranged in minute bundles (fascicle) of 3 to 12 conidiophores which are 17.6 to 28.8  $\mu\text{m}$  long and 1.78 to 3.01  $\mu\text{m}$  wide (Prasad et al. 2014). These conidiophores are darker towards the base, paler above, unbranched, sparingly septate, uniform but slightly bent (Ryley 1989). Conidia are translucent, multicellular, solitary, acicular, slightly curved, with a truncate base and rounded apex, and 2.3 to 2.8  $\mu\text{m}$  long, 1.2 to 1.9  $\mu\text{m}$  wide (Prasad et al. 2014; Ryley 1989).

#### **2.4.2.3 Symptoms of CLS:**

Cercospora leaf spot is primarily considered a foliar disease; however, this may affect stem and pods as well. Visible symptoms initiate with the appearance of circular sunken spots on the lower leaves of the plant. Sporulation within the lesion begins and leads to secondary inoculum production and infection. This allows disease to progress towards other parts if suitable environment condition prevailed. Lesions first may appear 1-2 mm in diameter but expand rapidly and coalesce with adjacent lesions. Further advancement of disease leads to necrosis of the affected area which is surrounded by a characteristic

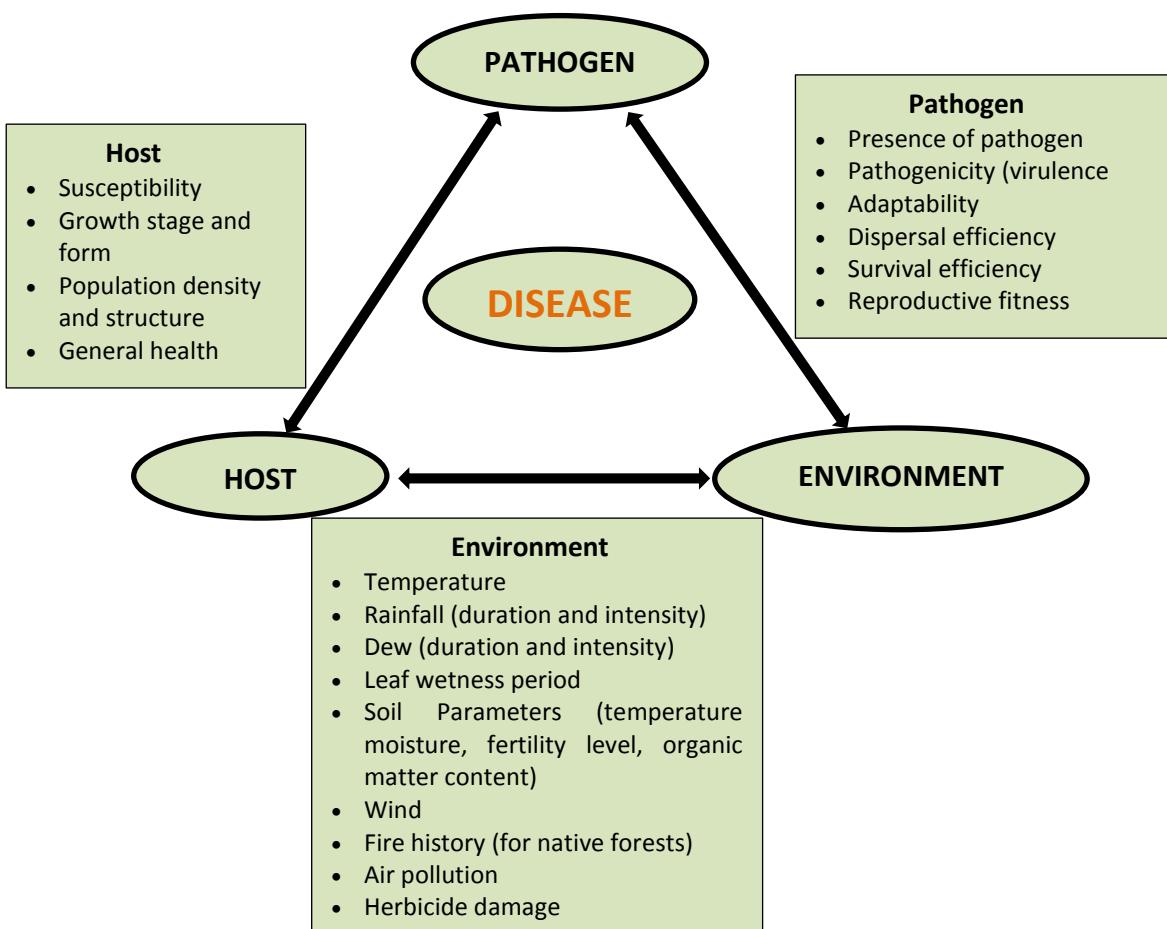
yellow halo (Prasad et al. 2014). Severe infection leads to extensive defoliation of leaves leaving no or few leaves on the apical region. Infected secondary branches dry and pods turn blackish in color which finally shrink and twist (Prasad et al. 2014). Seeds look darker than normal uninfected seeds (Ryley 1989).



**Figure 2.3:** Symptoms of Cercospora leaf spot Caused by *Cercospora traversiana* on fenugreek: (A) leaf lesions; (B) coalescence of lesions; (C) infected pod; (D) difference between CLS infected and healthy seeds.

### 2.4.3. Epidemiology of Cercospora leaf spot:

Different factors acting solely or in combination play an important role in onset of disease epidemics (Agrios 1997). Under favorable environmental conditions, virulent pathogen attacking susceptible plants may lead to epidemic levels. This can be well explained with the help of the disease triangle.



**Figure 2.4:** Generalised Disease triangle showing possible interactions between factors that can help cause a disease epidemic. **Source:** Keane and Kerr (1997).

#### **2.4.3.1. Nature of pathogen:**

For disease establishment, a minimum number of pathogen propagules are required even under favorable conditions (Gaumann 1946). However, after establishment, a number of factors such as virulence and aggressiveness, ability to adapt with changing environment and host, mode of spread and survival efficiency of the pathogen determine the level of disease severity within an area (Keane and Kerr 1997).

Genes in sexually reproducing pathogens undergo recombination in a regular fashion thus they are more diversified than those which reproduce asexually (McDonald and Linde 2002; Milgroom and Peever 2003; Bolton et al. 2012). Also, there is a high risk of an outbreak of new fungal races and development of fungicide resistance in sexually reproducing pathogens (McDonald and Linde 2002). Because sexual reproduction is not well understood in *Cercospora*, several asexual processes such as vegetative compatibility, parasexual recombination, and mutation, though occurring less frequently, may have acted solely or in combination to generate genetic diversity in *Cercospora* (Bolton et al. 2012).

Propagule dispersion also affects the disease incidence rate within an area. Soil-borne pathogens are considered to be localized within an area as compared to air-borne pathogens which can travel a long distance and start a new infection cycle, allowing epidemics to develop rapidly over a broad area (Keane and Kerr 1997). *Cercospora* is an air-borne pathogen which can also transmit through splashing rain. In addition to that, formation of resting spores in plant residues, weeds, and infected seeds help to perpetuate the disease cycle over many seasons (Vaghefi et al. 2017) thereby making this disease difficult to control.

#### **2.4.3.2. Host characteristics:**

Level of resistance or susceptibility of the host plant has a huge impact on disease development (Mundt 2014). Disease development is hindered if the pathogen lands on resistant plants, therefore, reducing the risk of disease havoc. In extreme cases, such resistant genotypes though infected, force the disease to develop slowly and limit economic impacts of the disease to an acceptable level (Gaurilcikiene et al. 2006; Mani et al. 2017). Monoculture of susceptible cultivars within a cropping system can make the situation worse. Therefore, increasing host diversity by mixing cultivars is recommended for field crops when disease resistance is not available. This diversity may help build up partial resistance against pathogens (Gigot et al. 2012; Raboin et al. 2012).

Growth stage of the plants also has a huge influence on disease development. Generally, plants in earlier growth stages are more susceptible to disease than in later stages reflecting an improvement in the plant's ability to control infection and colonization over time also known as developmental resistance (Lazarovits et al. 1980; Salzman et al. 1998). Furthermore, disease severity seems to be influenced by cultivar characteristics (plant morphology). Stem canker susceptibility in soybean was found to be increased in genotypes with thin stems due to a lodging problem (Freitas 2002). Infection of *Fusarium* head blight in wheat under field conditions was severe in dwarf genotypes as compared to tall ones (Mesterházy 1995). In this case, easier transfer of inoculum from soil to upper parts of the plants by splashing rain and closely placed leaves due to shorter internodes was the cause behind the greater severity. Vertically oriented leaves prevent the formation of moisture films on the leaf surface and, inhibit the growth of pathogens reliant on water (Russell 1975). A similar defense mechanism acts if a cuticle is present

in the leaves (Freeman and Beattie 2008). However, there are reports suggesting the presence of a cuticle on leaves favors disease development through fungal cutinolytic activity. (Gilbert et al. 1996; Hansjakob et al. 2010; Hegde and Kolattukudy 1997; Podila et al. 1993; Ringelmann et al. 2009).

#### **2.4.3.3. Environmental factors:**

Environmental factors primarily temperature and relative humidity play a significant role in the development of *Cercospora* leaf spot (Wolf and Verreet 2005). Actual prediction of disease severity is difficult because the weather may fluctuate every year leading to severe crop loss if no preventive measures are taken. The literature regarding optimal environmental conditions required for proliferation of CLS in fenugreek is scanty. However, general principles may be drawn from research on other species of *Cercospora* affecting several other crops.

Optimal temperature is required for the pathogen to maintain disease pressure within a crop. Temperature has a great influence on conidial germination, time required for germination and germ tube elongation (Agrios 2005), consequently affecting the latent period (time from infection to first spore production) and disease severity (Jacobsen et al. 2004b). Temperature also governs the physiological condition of host and expression of disease symptoms (Terefe et al. 2015). Paul and Munkvold (2005) observed that maximum spore production and the rate of lesion expansion of CLS in maize caused by *C. zeae-maydis* was highest at 25 and 30°C. Sporulation of *C. arachidicola* in peanut leaves was highest at 24 and 28°C and poorest at 16 and 32°C (Alderman and Beute 1987). Carisse et al. (1993) also stated that conidia of *C. carotae* are produced more readily at 28°C; however, they observed no sporulation at 16 and 32°C. In asparagus, the

optimum temperature for maximum sporulation of *C. asparagi* is 24 to 28°C (Cooperman and Jenkins 1986). Therefore, it is assumed that the most suitable temperature for CLS development is around 25-30°C.

A prolonged period of high humidity and leaf wetness accelerates development of the disease. Field results from Bangladesh show that mulberry trees were profoundly affected by *C. moricola* when the temperature was 25-30°C accompanied by a relative humidity higher than 80% (Ghosh et al. 2012). In a study on *C. beticola* causing CLS in sugar beet, Khan and Khan (2009) observed a steady increment in the conidial count when the temperature increased from 10°C with relative humidity always higher than 87%. Several other studies also show that relative humidity and leaf wetness duration has a positive relation with the disease progression (Windels et al. 1998; Wallin and Loonan 1971; Khan et al. 2005; Wolf and Verreet 2005). These reports suggest that warm and wet conditions are favorable for Cercospora leaf diseases in many crops and so it is suspected that these conditions are also suitable for CLS development and progress in fenugreek.

Wind, through dispersal of air-borne conidia, may lead to primary infection, secondary infection and proliferation of CLS (Khan and Khan 2009). Fire can decrease disease potential, especially soil and residue borne pathogens (Cox et al. 2004), thus may be applicable in controlling *Cercospora* species. Direct effects of other factors such as soil temperature, soil water level, soil organic matter, soil fertility, herbicide damage and air pollution on CLS have not been studied or reported.

## **2.5. CLS management:**

Methods of control of *Cercospora* species in other crops primarily include use of resistant cultivars, improved cultural practices, and fungicidal treatment. This is no different for CLS in fenugreek. Use of resistant cultivars is preferred over other approaches since this reduces the use of expensive chemicals and is effective irrespective of environmental conditions (Kaiser et al. 2010; Tivoli et al. 2006). Even moderately resistant cultivars hinder rapid disease progression, thus delay and reduce the frequency of fungicide application (Mani et al. 2017). However, commercial cultivars resistant to CLS in fenugreek have not been reported so far. Two fenugreek genotypes L3717 and PI138687 are identified as resistant to CLS in Canada but are low yielding (Prasad et al. 2014). Hybridization of these genotypes can be done with high yielding and adapted cultivars to obtain CLS resistant and high yielding fenugreek genotypes.

The control of this disease all around the world is reliant on spraying multiple fungicides such as Chlorothalonil, Mancozeb, Maneb, Bordeaux mixture and Benomyl (Acharya et al. 2010). However, there are no fungicides registered in Canada for the control of this disease. CLS being a seed-borne disease, selection of healthy seeds and treatment of seeds before sowing is also suggested for effective control (Khare et al. 198; Leppik 1960). Suitable timing of fungicide application in plants against CLS is right after the disease forecast and before the appearance of symptoms in the field (Harveson 2013). Delay in the first application of fungicide may lead to ineffective control. Alternate spraying of fungicide or fungicides from the same class of chemicals in every cropping season will be rewarding in controlling CLS (Khan and Smith 2005). However, chemical treatments are costly and possess health risks. In addition to that, gradually increasing

resistance by pathogen against several fungicides is arising as a serious threat to farmers (Allen 1983; Nakawuka 1997).

Cultural methods represent an efficient and eco-friendly practice to suppress pest populations to a threshold level. This may include practices such as crop rotation, intercropping, residue management, alteration in sowing and harvesting date, proper plant densities, wider spacing and fertilization (Ogle and Dale 1997). Longer rotations of about four years with other non-host crops are suggested to reduce the severity of leaf spot diseases (Kemerait et al. 2017). This provides enough time for crop debris to degrade, which in turn eliminates any inoculum in host tissues to survive. Leaf spot threat in peanut increases when planting is delayed (late May to June). This is because of warmer temperature in later seasons which favors disease growth and spread (Kemerait et al. 2017). Plant density within an area also influences disease severity. Crowded plants increase humidity and maintain a uniform temperature around them, which favors further disease development. In addition to that, closely located plants help to ease the transfer of inocula from one plant to another (Ogle and Dale 1997). However, sometime a wider spacing of plants may be detrimental leading to higher disease incidence, especially when disease is dispersed by splashing rain. (Mmbaga et al. 1979; Pataky and Lim 1981). Contrasting results have been reported for the effect of fertilization in leaf spot disease severity (McCoy 1973; Huber 1980; Vos and Frinking 1997; Caldwell and Ward 2002; Caldwell et al. 2002; Westerveld et al. 2008) and no explanation regarding the cause was provided. Studies on the effect of cultural methods on the incidence, severity and economic impact of *C. traversiana* on fenugreek are almost non-existent.

## **2.6. Genetics of Disease resistance:**

Breeding for disease resistance is one of the most important phases of a crop improvement program. Every year 9 to 15% of yield loss is reported to be due to disease (Cramer 1967). The consequences are not only limited to yield reductions but also to reduced harvest quality (food processing) and safety (harmful toxins), which are generally underestimated (Savary et al. 2012). Chemicals have been used to control diseases for a long time, but cost and environmental issues associated with them have reduced their credibility in present days (Kibria et al. 2010; Komarek et al. 2010). Host plant resistance is the simplest and most effective way to manage different problematic phyto-pathogens which not only ensures protection against diseases but also saves time, energy and money by restricting the use of costly equipment and chemicals (Mundt 2014). Identification of a proper source of disease resistant genes in germplasm from a gene pool consisting of wild, close relatives and cultivated genotypes is the first step in the breeding of disease resistant genotypes, and this can be achieved through suitable screening techniques (Malhotra 2010). If adopted cultivars are found susceptible to a disease, then a gene of interest can be transferred from a resistant genotype or created through gene modification to obtain variation within a population. For this, traditional breeding or modern techniques of molecular biology can be used. Such resistant cultivars act as a low cost and reliable insurance for farmers (Petropoulos 2002).

Disease resistance is most commonly found to be inherited in a Mendelian fashion which is also known as qualitative disease resistance (Fehr 1987). Controlled by one or two major genes, such resistance can be readily transferred from one genotype to another (Fehr 1987). Presence or absence of a resistant gene in a plant within a

population can be identified by closely observing the individual plants as they segregate into different contrasting categories of resistance. Therefore, a resistant cultivar can be developed through visualization techniques without the use of molecular markers which require sophisticated tools with high investments. Though easier to transfer and screen, such resistance is not retained (Poland et al. 2008) as the plants become vulnerable to new races of pathogen eventually. Furthermore, some reports of elimination of resistance genes while exposing the pathogen population to an increased level of selection pressure has also been observed (Poland et al. 2008). Consequently, breeders should dedicate more efforts in finding new resistance genes to replace those that have been compromised by pathogens. The outbreak of a new strain of wheat powdery mildew caused by *Blumeria graminis f.sp tritici* can be considered the best example of qualitative resistance overcome by a pathogen (Hsam et al. 2002; Marone et al. 2013). Quantitative disease resistance, on the other hand, is controlled by multiple minor genes with small effects and may provide partial resistance against pathogens (Fehr 1987). Inheritance of such genes is complex. Quantitative resistance allows disease development in plants to some extent (below the threshold level) but offers the stability of the resistant genes over an extended period of time (Leach et al. 2001). Gene pyramiding — combining genes— is a useful approach for development of quantitative disease resistance. Best example suggesting the importance of gene pyramiding is: control of wheat stem rust, a ubiquitous fungal disease of wheat, which has been maintained for over five decades (Mundt 2014). Identification of the best combination of genes for increasing durability is nevertheless an easy task. Gene × Environment and Gene × Gene interactions play a significant role in the phenotypic expression of quantitative resistance

genes thus, breeders need to employ extensive field testing under various environments before recommending any cultivar to farmers (Mundt 2014).

## **2.7. Inheritance of CLS resistance:**

Knowledge of the inheritance of a character of interest is crucial for the development of a suitable breeding methodology (Mishra et al. 1988). Diversity of inheritance mechanism has been identified for CLS disease resistance in various crops. Several studies suggest that a single dominant gene is responsible for CLS resistance in cowpea (Fery et al. 1976; Fery and Dukes 1977; Castro et al. 2003). A similar observation was obtained in faba bean (Kimber and Paull 2011) as well as mung bean (Thakur et al. 1977a, b). However, Mishra et al. (1988) reported a single recessive gene controlling CLS resistance in mung bean. Smith and Campbell (1996) concluded that at least four minor genes control resistance to *C. beticola* in sugar beet (*Beta vulgaris*), thus developing resistance while maintaining yield is difficult. This suggestion was supported by a QTL study of 204 segregating sugar beet plants which identified 5 QTLs with mostly additive and some negative dominance effects contributing 7 to 18% of the phenotypic variation. However, 37% of the phenotypic variation was unexplained (Nilsson et al. 1999). Maroof et al. (1996) found that resistance to gray leaf spot (GLS) in maize caused by *Cercospora zea maydis* is controlled by a small number of QTLs or oligogenes. Coates and White (1998), in support, discovered more than one gene with a dominant effect being responsible for resistance to GLS in maize. However, contrasting results were also observed explaining the preponderance of additive gene action in controlling resistance to GLS in maize (Huff et al. 1988; Crowley et al. 2002; Wegary et al. 2003).

In the case of fenugreek, a study of the inheritance of diseases other than powdery mildew has not been reported. An experiment conducted by Raje et al. (2003) concluded that inheritance pattern for powdery mildew resistance was monogenic recessive. However, Avtar (2004) found an additive-dominance model suitable to explain the variability among the majority of crosses undertaken by him. All these examples show a generalization of inheritance pattern and selection procedure for a crop may be erroneous sometimes, therefore, it would be prudent to set experiments to identify the nature and number of genes controlling disease resistance within a genotype.

## **2.8. Heritability of CLS resistance:**

Broad and narrow sense heritability of traits are taken into account while considering the improvement of traits. Heritability in a broad sense measures the fraction of phenotypic variance that is due to the genetic differences among individuals in a population while, heritability in a narrow sense measures the proportion of phenotypic variance that is due to genetic effects which are capable of being passed from one generation to the next (Falconer 1989; Fehr 1987). Heritability value can range from 0 (no genetic contribution) to 1 (all differences attributed to genetic effects), and it is not necessarily a constant value. Nature of genotypes, their population structure, environment to which those genotypes are subjected and methodology applied can affect the heritability of the trait under study (Dabholkar 1992; Fehr 1987; Weiland & Koch 2004). The knowledge about heritability helps breeders to decide a suitable breeding approach (Dabholkar 1992). Estimation of heritability for CLS resistance in fenugreek has not been reported so far. Although heritability of CLS resistant genes in several other crops has been determined, and the results are contradictory. A high broad sense heritability value of 0.85 was

obtained for resistance to gray leaf spot disease caused by *Cercospora zae-maydis* in maize (Zhang et al. 2012). The estimate of heritability by Gordon et al. (2006) in gray leaf spot in maize, however, was in the range of 0.46 to 0.81. Smith and Gaskill (1970) reported broad sense heritability of CLS resistance in sugar beet F<sub>2</sub> generation ranges from 0.6 to 0.71. Jogloy et al. (1987) while conducting experiments in the greenhouse of leaf spot on peanut reported moderate (0.49-0.68) broad-sense heritability for lesion size and sporulation but found narrow-sense heritabilities to be extremely low (<0.03). Iroume and Knauf (1987) obtained heritabilities of 0.16 to 0.26 for necrotic area and defoliation from segregating populations of peanut in the field. Narrow-sense heritability calculated from the parent-offspring regression method was 0.18-0.74 for leaf spot resistance in peanuts (Anderson et al. 1991).

For a breeding program to be successful requires a sound understanding of variability present in the breeding material for the trait of interest (Hugar and Savithramma 2015; Padmaja 2015). Furthermore, knowledge about the inheritance pattern, heritability, and association of targeted traits with others helps to confirm the breeding approaches (Zongo et al. 2017). Heritability estimates can be used to predict genetic gain from selection (Falconer and Mackay 1996). Early generation selection will be rewarding when additive genetic effects are substantial and targeted traits are highly heritable (Kearsey and Pooni 1996) thus facilitating a thorough assessment of the breeding population using a relatively small sample size (Slater et al. 2014).

## **CHAPTER – 3**

### **HYBRIDIZATION BETWEEN RESISTANT AND SUSCEPTIBLE FENUGREEK ACCESSIONS AND EVALUATION OF CERCOSPORA LEAF SPOT RESISTANCE IN SEGREGATING GENERATIONS.**

#### **3.1. Introduction:**

Fenugreek (*Trigonella foenum-graecum*) is an annual herbaceous legume plant belonging to the family Fabaceae and subfamily trifoliae. This crop is predominately grown as a spice and medicinal crop all around the world. India, however, is the leading producer which fulfills about 75% of the world fenugreek requirement (Pavlista and Santra 2016).

Introduced a few decades back in Canada, fenugreek is gaining popularity as a viable forage crop alternative and source of pharmaceuticals as well as nutraceuticals.

Fenugreek, unlike alfalfa, is bloat free and is comparable to alfalfa in terms of dry matter yield and nutritive value (Acharya et al. 2008; Mir et al. 1998). Presence of diosgenin, a precursor of natural growth promoting hormone, in its forage and seed helps to reduce the dependency of cattle farm owners towards synthetic hormones. Considerable interest towards fenugreek in western Canada is arising because of its easier incorporation into short-term crop rotations, superior drought tolerance, nitrogen fixation and insect repellent properties. In addition to that, several agronomic studies carried out to identify edaphic, climatic and cultural requirements have proved its suitability to western Canada growing conditions (Acharya et al. 2008; Moyer et al. 2003).

Cercospora leaf spot caused by *Cercospora traversiana* is one of the problematic diseases of fenugreek which is arising as a threat to farmers on every continent. (Leppik 1959; Malhotra 2010; Prasad et al. 2014; Ryley 1989; Zimmer 1984). Already reported in Canada in Morden, Manitoba and other regions of the prairies a few decades back, this

disease was found to reduce yield up to 80% (Zimmer 1984). *Cercospora* not only affects leaves and causes defoliation; it can affect stems, pods, and seeds (Prasad et al. 2014). Severely infected plants have only a few leaves in the apical region (Prasad et al. 2014) therefore reducing the photosynthetic area drastically. Infected seeds look darker than normal which is considered unmarketable (Ryley 1989). Being transmitted through diverse modes such as blowing wind, splashing rain, weeds, plant debris and seeds, *Cercospora* is regarded as one of the most challenging diseases to control (Vaghefi et al. 2017).

Control of CLS in fenugreek so far is heavily reliant on several fungicides such as Chlorothalonil, Mancozeb, Maneb, Bordeaux mixture and Benomyl in different parts of the world (Acharya et al. 2010); but, there is no registered fungicides recommended for the control of CLS in fenugreek within Canada. Though effective to some extent, these fungicides have raised some serious concerns related to environmental quality, sustainable food security and pesticide tolerance (Piquerez et al. 2014). Thus, alternative disease management techniques primarily cultural manipulation and plant disease resistance are seen as important these days as they already have proven to be effective, eco-friendly and economically feasible (Katan 2010; Mundt 2014; Narayanasamy 2013). Cultural practices such as crop rotation, intercropping, residue management, alteration in sowing and harvesting date, proper plant densities, wider spacing, rouging and fertilization help in controlling disease through reduction of initial inoculum level in the field (Ogle and Dale 1997). However, no such research has been undertaken to identify suitable crop management practices for controlling CLS in fenugreek. Growing plants resistant to CLS not only hinders the development of disease

through internal (resistance genes) and external (leaf orientations, thick cuticle etc.) mechanisms (Agrios 2005) but also prevents the rapid spread of disease within an area if grown in as a cultivar mixture (Gigot et al. 2012; Raboin et al. 2012). Virtually, all cultivars of fenugreek currently grown in western Canada are susceptible to CLS, although two fenugreek introductions (Line 3717 and PI138687) have been identified with lower susceptibility against CLS (Prasad et al. 2014). Incorporation of resistant genes from those genotypes into adapted cultivars to develop germplasms with CLS resistance and acceptable agronomic traits could be an effective strategy for controlling this rapidly expanding disease.

There is no formal report on the inheritance pattern and heritability of CLS in fenugreek. In other crop species, the genetics of resistance to *Cercospora* leaf spot has been described as either qualitative or quantitative. A single dominant gene was found to be responsible for CLS resistance in cowpea (Fery et al. 1976; Fery and Dukes 1977; Castro et al. 2003), mung bean (Chankaew et al. 2014; Thakur et al. 1977b), faba bean (Kimber and Paull 2011) and soybean (Pace et al. 1993). In contrast, resistance to CLS in sugarbeet caused by *Cercospora beticola* and gray leaf spot in maize caused by *Cercospora zea maydis* has been attributed to multiple genes (Coates and White 1998; Maroof et al. 1996; Nilsson et al. 1999; Setiawan et al. 2000; Smith and Campbell 1996). Crowley et al. 2002 and Wegary et al. 2003 identified a preponderance of additive genes associated with resistance to gray leaf spot in corn. Knowledge about the number of genes controlling a trait and their heritable behavior helps breeders to determine a suitable approach before starting any breeding project (Fehr 1987). Heritability estimate, however, does not adhere to a single value as it depends on several

parameters such as the nature of parental genotype, sample size, environment to which the segregating population is subjected and method of heritability calculation (Falconer and Mackay 1996; Fehr 1987). Genes controlling CLS resistance in various crops are reported to have a varying degree of heritability. Broad-sense heritability values are moderate to high in maize (0.46 to 0.81), sugar beet (0.6 to 0.71) and peanut (0.49 to 0.68) genotypes identified by Gordon et al. (2006), Smith and Gaskill (1970) and Jogloy et al. (1987) respectively, suggesting a low to moderate environmental effect on CLS expression and high transferability of characters from parents to offspring. In contrast, there are also reports of low heritability for CLS within the same crops (Iroume and Knauft 1987; Anderson et al. 1991). Such low heritability of genes can make selection difficult due to the masking effect of the environment over the genotypic effect (Singh 1993). Setting up experiments to discern the number of genes involved and their inheritance mechanisms in relation to resistance for *Cercospora traversiana* in fenugreek would provide more insight to breeders and thus allow better planning for breeding fenugreek populations.

The main objectives of this study were: i) to obtain homozygous CLS resistant lines which can be used in future breeding programs, and ii) to determine the mode of inheritance and heritability estimates of CLS resistance in fenugreek based on CLS ratings of the segregating population through field and controlled environment experiments. Using this process, we were able to describe a suitable technique for crossing fenugreek flowers.

### **3.2. Materials and Methods:**

#### **3.2.1 Development and Identification of resistant genotypes:**

##### **3.2.1.1. Choice of parents and experimental design:**

Identification of the source of resistance to plant pathogenic disease always constitutes the first step in research for developing a disease resistance genotype (Leppik 1970). A study conducted at the Lethbridge Research and Development Center (LRDC) to identify resistant fenugreek genotypes through screening world accessions of fenugreek against *C. traversiana* (Prasad et al. 2014), found two plant introductions L3717 (India) and PI 138687 (Iran) resistant to Cercospora leaf spot. However, those two genotypes were poor performers in terms of seed and biomass yield both in greenhouse and field conditions. Therefore, for the development of high yielding Cercospora leaf spot resistant genotypes, those two genotypes were crossed with Tristar, a high yielding cultivar developed at LRDC primarily for forage production (Acharya et al. 2007). These parental genotypes were grown in the greenhouse in 4-inch pots to facilitate easy handling of plants during hybridization and provide a favorable environment. A soil mixture composed of Cornell mix (Basu et al. 2008), sand and fine soil in equal proportions was used for fenugreek growing. The greenhouse temperature was maintained at 22°C and 14°C during day and night time respectively. Daylight time was maintained at 16 hours.

For crossing flowers of three genotypes, an experiment was designed to determine the efficiency of crossing success within different times of a day. Four blocks (replication) each consisting of ten pots was considered for the crossing trial. Within each block there were three pots (containing single plants) each of L3717 and PI138687 and four pots of Tristar initially. Later, while performing backcrosses, varying numbers of F1 plants were

also included in each block. Time of a day when the crossings were performed (treatment) was divided into four sections: 8am-10am, 10am-12pm, 12pm-2pm and 2pm-4 pm. Date and time of crossings were noted in the tags which were later used for data analysis. Data analysis was also done by dividing the whole experiment into two sections viz. summer (July 15- September 15) and fall (September 16- November 15).

### **3.2.1.2. Artificial hybridization:**

Hybridization is the process of combining the desirable traits from two or more non-identical parents into a single plant. It involves first the removal/killing anthers (emasculuation) of the selected female flower and then the transfer of pollen grains (pollination) from the different parent in the same day or after some days depending upon the receptive nature of stigma of female flower. Artificial hybridization of self-pollinated legumes is complicated compared to cross-pollinated crops because of the flower structure; male and female parts of the flower are organized in a cleistogamous nature (Petropoulos 2002). In the case of fenugreek, crossings were done manually with the help of fine tweezers. Flowers of the female parent were emasculated after 2 to 3 days of flower bud initiation by opening the keel petal and removing all ten anthers carefully preventing mechanical damage to the pistil. At this stage, the style is taller than the filaments, and anther bags are intact which prevents the pollen from falling on the stigmatic surface. After emasculation (removal of anthers), the flowers are considered female. The stigma of the fenugreek flower matures and become receptive earlier than the anthers do (Petropoulos 2002). This allowed us to perform pollination of the emasculated flowers on the same day. For this purpose, white and fully open flowers were selected and the busted anthers along with pollen dust of the male parent were

removed and pollination was done using a fine tweezer. In some cases, multiple pollinations were done to ensure fertilization. The pollinated flowers were then tagged with the date and time of the cross and name of the female × male parent. Initially, crossings were performed at normal greenhouse conditions without maintaining the humidity but no success was obtained. Thus, arrangements were made to maintain humidity by placing the plants in a temporary humidity chamber which consisted of a tray containing thin film of water, and covered with a transparent plastic lid. Plants with crossed flowers were kept in humidity chambers for three days and were transferred in a normal greenhouse conditions. Crossed pods were harvested when the seeds looked mature and they were allowed to dry before the seeds were separated from the pods. Seeds along with their tags were stored in a cool and dry place.

### **3.2.1.3. Advancement of generations:**

The F<sub>1</sub> seeds obtained from artificial hybridization were grown in the greenhouse with similar conditions as mentioned above in section 3.2.1.1. Three seeds per hybrid line were sown following a Randomized Complete Block Design (RCBD). Plants were regularly irrigated and sprayed with insecticides- Kontos (Bayer CropScience Canada Inc.) and Avid (Syngenta Canada Inc.) alternately to prevent damage caused by western flower thrips (*Frankliniella occidentalis* Pergande) in the greenhouse. Seeds from each F<sub>1</sub> hybrid pod were collected separately, levelled and stored in cold storage at 4°C.

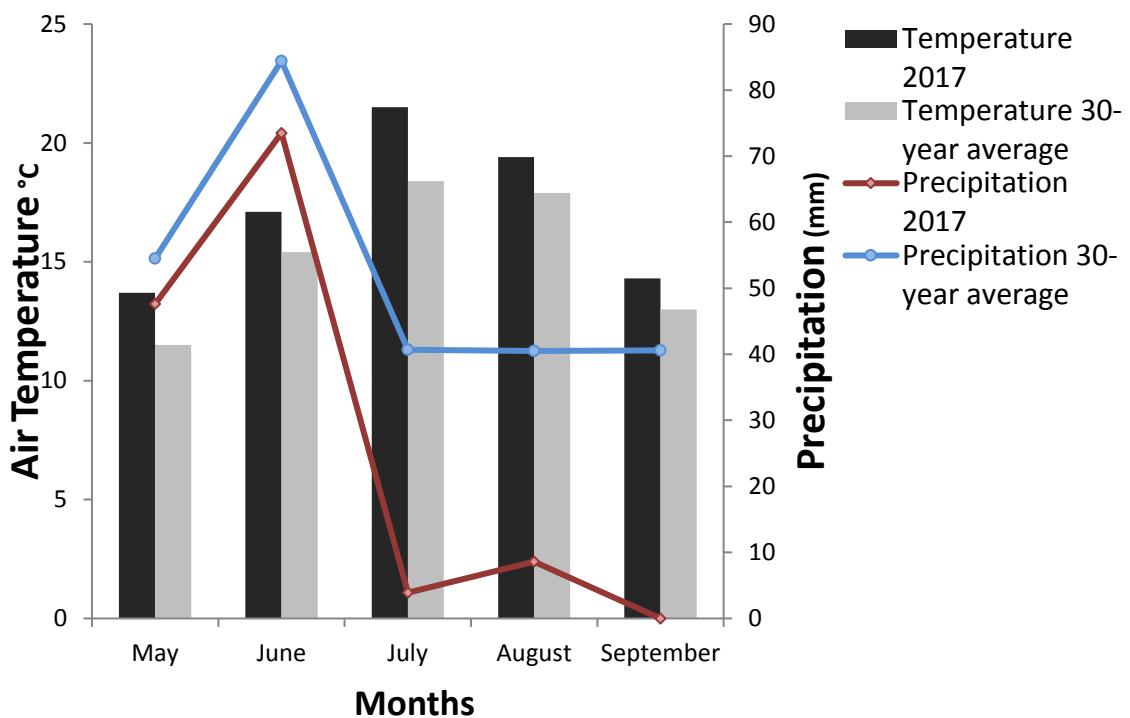
Forty-three different F<sub>2</sub> populations along with three parents were grown in the LRDC field following a three-times replicated RCBD. Prior to seeding, the plots were cultivated to kill existing weeds; Edge (Dow AgroScience Canada Inc.) was applied at a rate of 8.9

kg/ac and worked into the soil in two directions. Seeds were sown 2.5cm deep into the soil and 5cm apart with the help of small plot seeder. Each genotype was grown in a row of 2 meters and there was 1meter distance between rows. 5 rows (each 20cm apart) of F80 (fenugreek line) seeds were grown all around the research plot to minimize the border effect. A sprinkler irrigation system was arranged for the uniform distribution of water within the field. Weeding was done twice manually with the help of a hand hoe. 10 random plants from each generation were selected and tagged 30 days after germination and data were collected on flowering time, plant height, seed yield, biomass yield, number of branches, number of pods and maturity date. Harvesting of those selected plants was done manually after pods turned yellow and they were air dried for 72 hours. Threshing was done with the help of a small vogel plot thresher (ALMACO).

#### **3.2.1.4. Identification of resistant genotypes:**

The F<sub>2</sub> population is always heterogeneous consisting of both homozygous and heterozygous seeds. Natural infection was expected in the field therefore, no artificial inoculation was done. However, weather of summer 2017 was hotter and drier than long-term averages (Figure: 3.1), and so disease did not develop on the field grown plants even though the frequency of irrigation was increased above normal to increase humidity within the plot. Furthermore, CLS being a seed-borne disease, treatment of whole plants was not possible as our objective was not only to identify resistant plants but also to obtain healthy seeds and use it for further generation analysis. Thus, we planned an alternative method, detached trifoliolate treatment, to identify CLS resistant fenugreek. This method had already shown a good correlation with field screenings and allowed us to avoid treatment of whole plants (Twizeyimana et al. 2007). Seventy vigorous and

healthily growing plants were selected and tagged during the early flowering stage. Some of the plants were even selected on the basis of peculiar characteristics depicted by them such as early flowering, profuse branching, and high number of pods and double pods. Newly developed apical trifoliate leaves were collected from those selected plants and three parental genotypes, stored in a cooler containing ice and then taken to the lab for detached trifoliate assessment. The leaves then were washed twice with distilled water to make them free of any physical impurities and were kept in plastic petri-plates containing moist filter paper. There were three replications of leaves for each selected plant. Leaves from separate ninety randomly selected plants were also included in detached trifoliate treatment for the correlation study.



**Figure 3.1:** Mean air temperature and precipitation from May 2017 to September 2018 as compared to 30-year average in LRDC field while growing F<sub>2</sub> generations.

Source: <http://ablethr2/weather/weathermain.aspx?Language=0>

### **3.2.1.5. Inoculum preparation:**

Isolate 1M1 318080, the only available isolate initially obtained from the Centre for Agriculture and Biosciences International (CABI), was chosen to represent the population of *C. traversiana* in western Canada and used for lab and greenhouse experiments. A portion of frozen culture was transferred into three Potato Dextrose Agar (PDA) plates using a sterilized scalpel to obtain a maximum number of mycelia and conidia, to be used for further increment of inoculum. The plates were then incubated at room temperature ( $20 \pm 2^{\circ}\text{C}$ ) for 28 days in a dark room. Mass inoculum was prepared by transferring those growing cultures into new petri plates containing PDA with a sterilized spatula which was used to spread the culture to the whole plate (making circular streaks) by placing them on a turntable. Again, the plates were incubated at room temperature ( $20 \pm 2^{\circ}\text{C}$ ) for 28 days in a dark room. A suspension of the culture was prepared by adding 10 ml of distilled deionized (dd) water and 1 drop of tween-20 (wetting agent) into the media plates, scratching the culture with the help of a scalpel, and staining it through a double layer of cheesecloth. A required amount of dd water was added and the concentration of the suspension was adjusted to  $1 \times 10^6$  conidia/ml by counting the spores using a haemocytometer.

### **3.2.1.6. Artificial inoculation and disease assessment:**

The spore suspension was then sprayed in each individual petri-plate containing detached leaves until the suspension produced runoff from the leaves. Petri-plates were then covered with lids and kept in a tray containing moist paper. The trays were also covered with a transparent lid and placed in a greenhouse maintained at  $22/14^{\circ}\text{C}$  day-night temperature and 16-hour photoperiod. A cover was placed above the transparent lid of

the tray to prevent direct sunlight on the leaves. Water was regularly sprayed in the trays to keep the paper moist; subsequently maintaining humidity around the treated leaves. Disease scoring was done 25 days after inoculation and disease severity of each genotype was measured by a visual estimate of percentage of leaf area affected. Trifoliates with less than 30% leaf area affected by the *C. traversiana* in each of the replicates were considered resistant. For the confirmation of CLS within the leaves, conidia of *C. traversiana* were removed from the lesions, placed in a temporary slides and watched under the compound microscope.

### **3.2.2. Inheritance of CLS resistance:**

Three parents viz. Tristar, L3717 and PI138687, two F1 populations, two F2 populations and two backcross populations derived from a cross of resistant and susceptible parents were grown in the greenhouse. Seeds were sown in root trainers ( $5 \times 9$  cells fixed in a box of size  $30 \times 20$  cm) containing soil mixture composed of Cornell mix (Basu et al. 2008), sand and fine soil in equal proportions. Greenhouse temperature during the day and night was maintained at  $22^{\circ}\text{C}$  and  $14^{\circ}\text{C}$  respectively. Photoperiod was 16 hours per day. The experiment was laid out in a RCBD design with 4 replications. Number of plants varied in each generation as per seed availability (Table 3.5).

Inoculation was done when plants were 30 days old (delayed because of thrips and powdery mildew). Inoculum of *C. traversiana* (Isolate 1M1 318080) was prepared as per the method described in section 3.2.1.5; however, in this experiment concentration of the suspension was  $1 \times 10^5$  conidia/ml. Spraying was done uniformly with the help of a hand-held sprayer in mist form until runoff was observed from every plant. Pots were then placed in a tray containing a thin layer of water and covered with a transparent lid. This

helped in maintaining high humidity around the plants and thus helped in easier disease development. Lids were removed after 5 days and plants were subjected to natural greenhouse conditions. Plants were sprayed with tap water twice a day until disease assessment was done.

Disease Assessment was conducted 28 days after inoculation based on a 0-9 disease scoring chart where; 0 = no infection; 1 = 1 to 5% leaves infected; 2 = 6 to 10% leaves infected, 3 = 11 to 20% leaves infected; 4 = 21 to 30% leaves infected; 5 = 31 to 40% leaves infected; 6 = 41 to 50% leaves infected; 7 = 51 to 60% leaves infected; 8 = 61 to 80% leaves infected and 9 = 81 to 100% leaves infected. After scoring the disease, number of resistant (with CLS score 0 to 4) and susceptible (with CLS score 5 to 9) plants were counted which were later used to identify the inheritance pattern of CLS resistance.

Thirty-two top performing (according to field performances) F<sub>2:3</sub> families from the ones which showed a resistant reaction against disease during detached trifoliolate treatment were also grown in the greenhouse in order to identify homozygous lines. A similar growing and epiphytotic condition was maintained and disease was scored based on the same disease scoring chart mentioned above.

The whole experiment mentioned above was repeated once, however the number of plants in the second experiment were different as per seed availability in different generations.

In order to determine variance components: environmental variance, genotypic variance, additive variance and dominance variance, and heritability of two different crosses Tristar × Line 3717 and Tristar × PI138687, the method as described by Kearsey and Pooni (1996) was followed as detailed below:

$$\text{Environmental variance } (\sigma^2 e) = \frac{[SS(P_1) + SS(P_2) + SS(F_1)]}{[DF(P_1) + DF(P_2) + DF(F_1)]}$$

$$\text{Phenotypic Variance } (\sigma^2 p) = [\sigma^2 F_2]$$

$$\text{Genotypic variance } (\sigma^2 g) = \sigma^2 F_2 - \sigma^2 e$$

$$\text{Additive variance in } [\sigma^2 A] = (2 \sigma^2 F_2) - [\sigma^2 BC_1 P_1 + \sigma^2 BC_1 P_2]$$

$$\text{Dominance variance in } [\sigma^2 D] = \sigma^2 BC_1 P_1 + \sigma^2 BC_1 P_2 - \sigma^2 (F_2) - \sigma^2 e$$

$$\text{Broad-sense heritability } (h^2 b) = [\sigma^2 g / \sigma^2 p] \times 100$$

$$\text{Narrow-sense heritability } (h^2 n) = [\sigma^2 A / \sigma^2 p] \times 100$$

Where,

$SS(P_1)$  = Sum of squares of susceptible parent,

$SS(P_2)$  = Sum of squares of resistant parent,

$SS(F_1)$  = Sum of squares of first filial generation,

$DF(P_1)$  = Degrees of freedom of susceptible parent,

$DF(P_2)$  = Degrees of freedom of resistant parent,

$DF(F_1)$  = Degrees of freedom of first filial generation,

$\sigma^2 F_2$  = Variance of  $F_2$  generation,

$\sigma^2 BC_1 P_1$  and  $\sigma^2 BC_1 P_2$  = Variance of backcross to female and male parents respectively.

The heritability was categorized as low (0-30%), moderate (30- 60%) and high (60% and above) as given by Robinson et al. (1949).

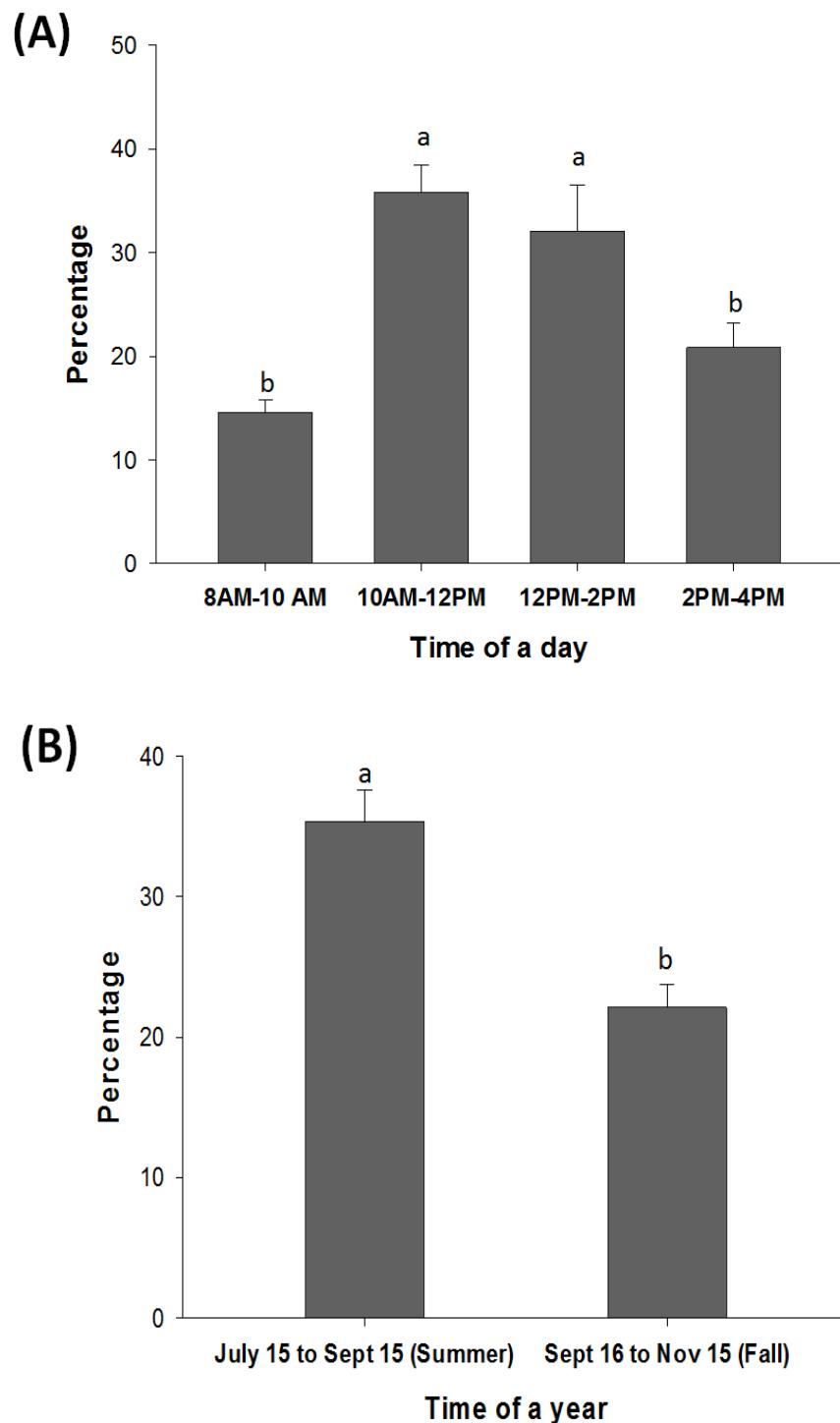
### **3.2.3. Data Analysis:**

Disease score data were analyzed using PROC MIXED (SAS Institute, Inc., 2010) with experiment and generation as a fixed factor and replication as a random factor. Treatment mean comparisons were made using Tukey's Honestly Significant Difference test and the probability level was set at a 5% level. Field data for different morphological traits from the  $F_2$  generation were analyzed on Microsoft excel 2010 (Microsoft Corporation) to calculate means, range and standard errors. Linear relationships (Pearson Correlation coefficient) between different morphological traits of fenugreek were tested using CORR procedure in SAS.

### **3.3. Results:**

#### **3.3.1. Artificial hybridization:**

Initially, when performed without maintaining the humidity around the floral region, none of the crossings out of 76 total crosses were successful. However, we were able to harvest 104 pods from a total number of 364 flower buds pollinated during the overall period of crossing when there was humidity maintained. This represents an average crossing success of 28.57% among the different parental combinations. Successful crosses were identified after comparing the F<sub>1</sub> seeds with the parental ones because three parents had contrasting shape, size and color of seeds. When there was any doubt, F<sub>1</sub> plants were compared with the parents for different parameters such as leaf shape, size, plant height, growing nature, flowering time and several other visible morphological differences. Comparison of efficiency of successful crosses vs different time within a day is presented in figure 3.2A. Hand crossings done earlier (8 am to 10 am) and later (2 pm to 4 pm) in the day were less successful (14.61% and 20.86%, respectively) and were significantly different from 10 am to 12 pm and 12pm to 2 pm (35.88% and 32.09%, respectively). The same experiment was divided into two different periods within a year (figure 3.2B). Crossing success (35.40%) was significantly higher in the period from July 15 to September 15 (summer) as compared to crossing success (22.13%) during the period from September 16 to November 15 (fall). Some of the pods aborted after few days of development and they were considered unsuccessful crosses. Number of seeds in crossed pods varied from 2 to 21 with an average of 10.54 seeds per pod.



**Figure 3.2:** Percentage of crossing success; (A) = Crossing success within a day, (B) = Crossing success within a year. Bars with different subscript are significantly different ( $p \leq 0.05$ ).

### **3.3.2. Identification of top performing family:**

Several parameters such as flowering time, plant height, number of branches, number of pods, maturity time, seed yield and forage yield were measured from 10 plants of each family to identify the top performing family (Appendix-2) to be used in a future fenugreek improvement program. High variability for those parameters was observed within and between the F<sub>2</sub> families grown under field conditions.

Results for the top 5 and bottom 5 families (based on seed and forage yield) along with three parental genotypes are presented in table 3.1. Average seed yield was highest in family RT-11 followed by RT-13, PiT-4, RT-9 and PiT-1 respectively. Only two genotypes RT-13 and PiT-4 from the top five seed yielding families were common in the top five forage yielding families. The other three among the top five forage yielding families were PiT-7, PiT-10 and RT-12. The adapted (Check) cultivar Tristar ranked fourth and first in terms of seed yield and forage yield respectively. RT-22, RT-23 and TR-2 were common in both the lowest five seed and forage yielding categories.

### **3.3.3. Selection of high-yielding and resistant genotypes from the field:**

The detached trifoliolate treatment used for differentiating genotypes for resistance to CLS in fenugreek was found to be effective in differentiating genotypes. The suspension concentration of  $1 \times 10^6$  conidia/ml generated sufficient symptoms within the leaves to make identification of resistant genotypes easier. Out of 160 (70 + 90) selected individual plants from the field, leaves from 150 plants were treated and 104 plants showed resistant reaction with each trifoliolate treated being affected by less than 30% of the total leaf area while 46 of them showed susceptibility to CLS. Trifoliolate leaves from the resistant

parents L3717 and PI138687 were affected 25% and 30% respectively. The susceptible cultivar Tristar was affected the most with 75% diseased leaf area. The frequency of resistant (104) and susceptible (46) plants obtained through detached trifoliolate inoculation were subjected to goodness of fit in a chi-square test. The observed ratio fit into the expected ratio 3:1 ( $\chi^2 = 2.567$ , P = 0.109) suggested a single dominant gene was responsible for controlling CLS resistance in fenugreek. Out of 70 initially tagged vigorous plants, 44 of them showed resistant reaction thus they were traced back in the field. Seed and forage yield were recorded after harvesting them and the seeds were stored for future use. The 20 top performing genotypes among those harvested ones based on total biomass (seed + forage) yield are presented in the table 3.2. PiT-7-5 had the highest forage yield (72.97gm) while RT-11-4 was the one with the highest seed yield (52.41gm). Both genotypes showed a homozygous nature in terms of CLS resistance while treated with pathogen.

**Table 3.1:** List of top five and bottom five fenugreek F<sub>2</sub> families along with 3 parental genotypes ranked based on their respective seed and forage yield.

Rank	Family	Seed yield ± SE (gm)	Rank	Family	Forage yield ± SE (gm)
1	RT-11	27.74 ± 3.76	2	PiT-7	31.69 ± 2.60
2	RT-13	27.49 ± 3.93	3	PiT-10	31.60 ± 2.83
3	PiT-4	27.17 ± 4.90	4	RT-12	31.52 ± 3.26
5	RT-9	26.93 ± 2.86	5	RT-13	30.65 ± 3.04
6	PiT-1	26.49 ± 3.12	6	PiT-4	30.52 ± 2.54
42	RT-10	18.47 ± 3.07	42	TPi-3	21.57 ± 3.89
43	TR-2	17.38 ± 2.91	43	TPi-3	21.19 ± 4.23
44	RT-23	17.58 ± 2.84	44	RT-22	20.75 ± 4.95
45	RT-22	16.38 ± 2.63	45	TR-2	19.87 ± 2.16
46	RT-19	16.13 ± 2.58	46	RT-23	17.96 ± 2.05
4	Tristar	26.95 ± 1.86	1	Tristar	34.44 ± 1.78
40	L3717	19.65 ± 0.87.	29	L3717	24.74 ± 3.00.
37	PI138687	20.36 ± 1.18	22	PI138687	26.30 ± 1.76

**Table 3.2:** List of the top 20 genotypes ranked based on their biomass (seed + forage) yield. Resistant category for each genotype was determined after treating their progenies ( $F_{2:3}$ ) with suspension inoculum of *Cercospora traverisana*.

Rank	Genotype	Seed yield (gm)	Forage yield (gm)	Resistant Category
1	PiT-7-5	50.14	72.97	<b>Homozygous Resistant</b>
2	PiT-7-7	50.62	72.08	Heterozygous resistant
3	RT-11-4	52.41	65.16	<b>Homozygous resistant</b>
4	RT-11-7	49.18	67.52	Heterozygous resistant
5	PiT-2-4	45.56	70.48	<b>Homozygous resistant</b>
6	RT-7-5	44.12	71.18	Heterozygous resistant
7	PiT-1-6	43.14	71.12	Heterozygous resistant
8	RT-22-9	43.1	71.10	Heterozygous resistant
9	RT-20-10	43.92	69.71	Heterozygous resistant
10	PiT-12-2	42.14	71.17	Heterozygous resistant
11	RT-11-6	43.74	69.18	Heterozygous resistant
12	RT-9-5	42.86	68.81	<b>Homozygous resistant</b>
13	RT-19-15	44.86	62.98	Heterozygous resistant
14	RT-22-10	35.7	70.71	<b>Homozygous resistant</b>
15	RT-17-6	40.62	65.63	Heterozygous resistant
16	TR-2-5	36.45	69.34	Heterozygous resistant
17	RT-2-6	47.49	57.25	Heterozygous resistant
18	RT-4-8	43.97	60.02	Heterozygous resistant
19	TR-4-3	40.57	62.83	Heterozygous resistant
20	RT-13-4	41.4	61.87	Heterozygous resistant

### **3.3.4. Correlation between different morphological traits of fenugreek:**

Data for various morphological traits: flowering time, maturity time, seed yield, forage yield, number of branches, number of pods and number of double pods, were gathered for the randomly selected fenugreek plants from the F<sub>2</sub> generation. Pearson correlation coefficient (r) values representing association among the traits are presented in table 3.3. There was no negative correlation between the morphological traits measured in this study. Results demonstrate that seed yield and number of pods are very strongly correlated ( $r = 0.878$ ,  $p < 0.001$ ) which indicates that number of pods can be used as an indicator for seed yield. Seed yield was also found very strongly correlated with forage yield, moderately correlated with number of branches and number of pods, and weakly correlated with flowering time and maturity time. Forage yield was significantly correlated with flowering time ( $r = 0.381$ ), number of branches ( $r = 0.51$ ), number of pods ( $r = 0.757$ ) and number of double pods ( $r = 0.389$ ). Number of branches and number of pods within a plant were found to positively respond to flowering time and maturity time. In a similar manner, number of double pods seemed to be increase with an increase in the number of branches ( $r = 0.427$ ) and number of pods ( $r = 0.665$ ) in a fenugreek plant.

The correlation study (table 3.3) also shows a negative and very weak (non-significant) correlation between level of susceptibility and different other morphological traits. A very low correlation between seed and forage yield with level of susceptibility indicates that deployment of CLS resistance into fenugreek genotypes may not impose a large yield penalty. However, several generation studies with a large number of samples need to be carried out to support this result.

**Table 3.3:** Pearson correlation coefficient between various morphological parameters of fenugreek genotypes developed through artificial hybridization grown at LRDC field.

Parameters	Flowering time	Maturity time	Seed yield	Forage yield	# branches	# pods	# double pods	Level of CLS Susceptibility
<b>Flowering time</b>	1							
<b>Maturity time</b>	0.474***	1						
<b>Seed yield</b>	0.223*	0.255*	1					
<b>Forage yield</b>	0.318**	0.206 <sup>NS</sup>	0.836***	1				
<b># branches</b>	0.459***	0.247*	0.510***	0.570***	1			
<b># pods</b>	0.288**	0.273*	0.878***	0.757***	0.647***	1		
<b># double pods</b>	0.147 <sup>NS</sup>	0.132 <sup>NS</sup>	0.588***	0.389***	0.427***	0.665***	1	
<b>Level of CLS Susceptibility</b>	-0.072 <sup>NS</sup>	-0.110 <sup>NS</sup>	-0.098 <sup>NS</sup>	-0.084 <sup>NS</sup>	-0.035 <sup>NS</sup>	-0.070 <sup>NS</sup>	-0.078 <sup>NS</sup>	1

NS, \*, \*\* and \*\*\* denote non-significant at  $P < 0.05$ , significant at:  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  respectively.

### **3.3.5. Generation mean analysis from greenhouse experiment for CLS scores:**

There was no significant difference ( $P = 0.9398$ ) between two experiments conducted under greenhouse conditions. However, the mean disease score of various generations of both cross combinations Tristar  $\times$  L3717/ L3717 $\times$  Tristar and Tristar  $\times$  PI138687/ PI138687  $\times$  Tristar differed significantly ( $P < 0.0001$ ). In experiment-1, the mean disease score (7.49) for the susceptible parent ‘Tristar’ was the highest among all the generations treated. The other two parental lines L3717 and PI138687 consistently displayed a resistant reaction with lower mean disease scores of 3.44 and 3.88 respectively. No plants of L3717 and PI138687 were immune to CLS.  $F_1$  generations of both cross combinations showed a resistant reaction with a mean disease score close to their respective resistant parents. The mean disease score increased in the  $F_2$  generation in both cross combinations which may be due to presence of susceptible plants within the segregating population. A backcross with the resistant parent (L3717) showed a lower disease severity closer to the resistant parent while a backcross with the susceptible parent (Tristar) showed an increase in disease severity (table 3.4b). There was no distinct difference within the generation between experiment-1 and experiment-2 in terms of mean disease score (table 3.4b). In general, the grand mean disease score result in both experiments indicated that the number of resistant plants is higher than susceptible plants within the population of both the cross combinations.

**Table 3.4a:** Results of analysis of variance (ANOVA) for Cercospora leaf spot (CLS) score of different generations of fenugreek grown in controlled environment conditions at LRDC.

Source	Tristar × L3717			Tristar × PI138687		
	DF	F value	p-value	DF	F value	p-value
<b>Generation</b>	5	90.62	<.0001	3	99.54	<.0001
<b>Experiment</b>	1	0.47	0.4945	1	0.01	0.9398
<b>Generation*Experiment</b>	5	1.94	0.0848	3	2.56	0.0542

**Table 3.4b:** Results of Cercospora leaf spot (CLS) mean disease score and standard error of the mean for six generations of the 2 different cross combinations.

Generations	Mean Disease score ± SEM			
	Tristar × L3717		Tristar × PI138687	
	Experiment-1	Experiment-2	Experiment-1	Experiment-2
<b>Parent (S)</b>	7.49 ± 0.17 <sup>a</sup>	7.25 ± 0.29 <sup>a</sup>	7.49 ± 0.17 <sup>a</sup>	7.25 ± 0.29 <sup>a</sup>
<b>Parent (R)</b>	3.44 ± 0.17 <sup>d</sup>	3.17 ± 0.27 <sup>c</sup>	3.88 ± 0.17 <sup>c</sup>	3.81 ± 0.27 <sup>c</sup>
<b>F<sub>1</sub></b>	3.52 ± 0.14 <sup>d</sup>	3.65 ± 0.23 <sup>c</sup>	3.98 ± 0.18 <sup>c</sup>	3.68 ± 0.30 <sup>c</sup>
<b>F<sub>2</sub></b>	4.44 ± 0.08 <sup>c</sup>	5.00 ± 0.14 <sup>b</sup>	4.40 ± 0.08 <sup>b</sup>	5.05 ± 0.23 <sup>b</sup>
<b>BC<sub>1</sub>(R)</b>	3.56 ± 0.12 <sup>d</sup>	3.87 ± 0.26 <sup>c</sup>	-	-
<b>BC<sub>1</sub>(S)</b>	5.24 ± 0.11 <sup>b</sup>	5.21 ± 0.25 <sup>b</sup>	-	-
<b>Grand mean</b>	4.614	4.522	4.94	4.95
<b>LSMean (p&lt;0.05)</b>	0.37009	0.67552	0.42185	0.7636

S= Susceptible parent (Tristar), R = Resistant parent (L3717/PI138687),

F<sub>1</sub> = First filial generation,

F<sub>2</sub> = Second filial generation,

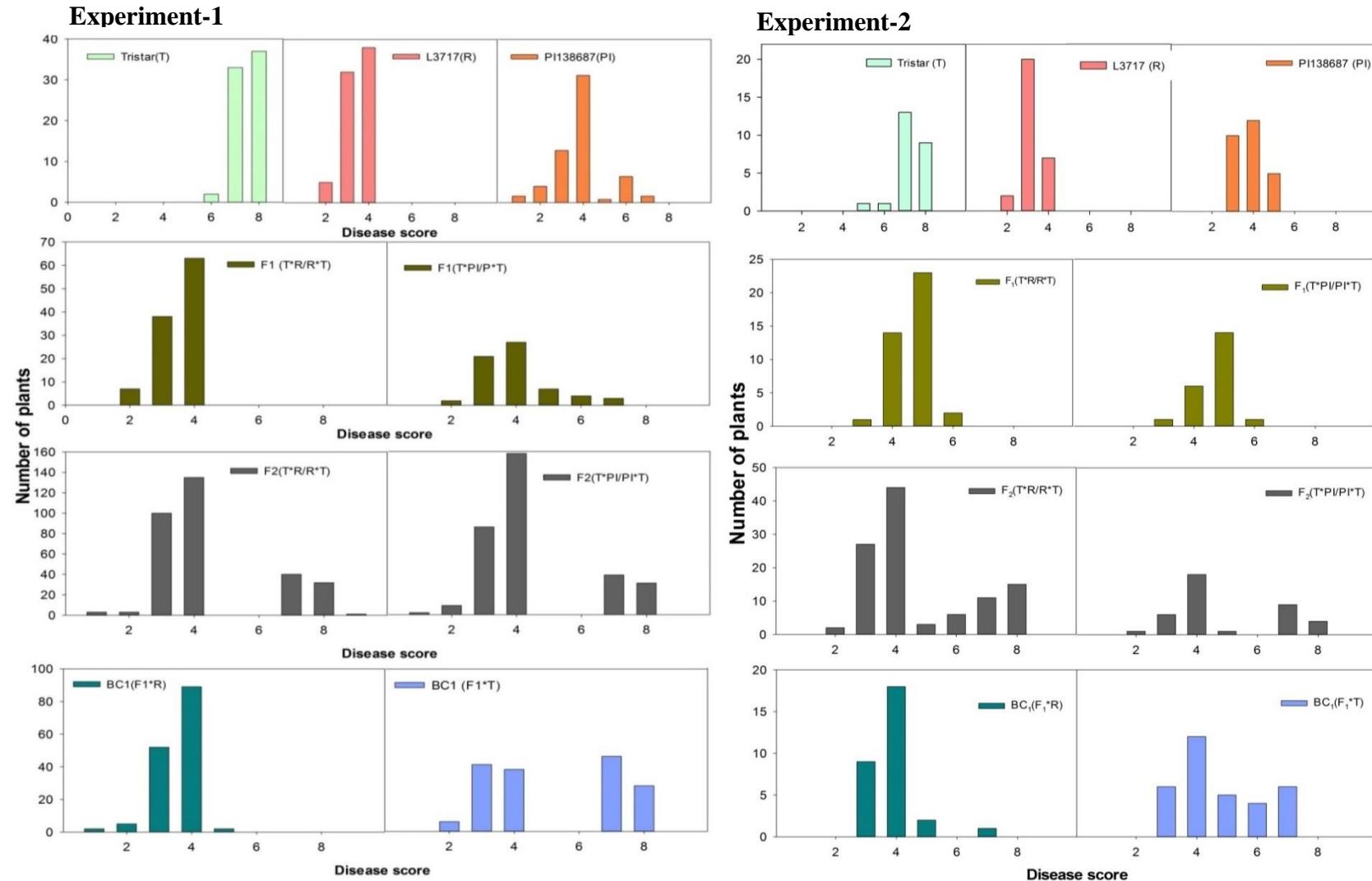
BC<sub>1</sub> (R) = Backcross with resistant parent, BC<sub>1</sub> (S) = Backcross with susceptible parent.

LSMean = Least Significant Mean,

SEM = Standard Error of Mean

### **3.3.6. Genetics of resistance to *C. traversiana* in fenugreek:**

Fenugreek plants treated with conidial suspension of  $1 \times 10^5$  conidia/ml in a controlled environment were able to produce sufficient symptoms in order to differentiate their reaction against CLS. The disease reaction depicted by individual plants in various generations was scored and the frequency of resistant and susceptible plants was used to identify the number and nature of genes involved in CLS resistance. There was considerable variability within and between the generations of fenugreek treated. Every plant from Line 3717 showed a resistance reaction but a few plants in PI138687 were susceptible which may have arisen due to mixing of seeds while storage. All Tristar were susceptible to CLS. In the F<sub>1</sub> generation, all plants from Tristar × L3717 cross combination were categorised resistant to CLS. In contrast, some F<sub>1</sub> plants from Tristar × PI138687 cross combination depicted susceptible disease reaction which may be due to contamination of parental seeds. In the F<sub>2</sub> generation, the observed ratio of resistant and susceptible plants when subjected to a goodness of fit test using a chi-square test fit into an expected ratio of 3:1 [ $\chi^2 = 0.514$ , P = 0.473 (Tristar × L3717) and  $\chi^2 = 2.077$ , P = 0.149 (Tristar × PI138687)] in both cross combinations. Repeated experiment also fit into the expected 3:1 ratio [ $\chi^2 = 3.160$ , P = 0.075 (Tristar × L3717) and  $\chi^2 = 2.47$ , P = 0.116 (Tristar × PI138687)] although probability values were less than the first experiment which may be due to lower number of plants used in the second experiment. F<sub>2:3</sub> families, in a similar manner, fit into the expected ratio of 1 homozygous resistant: 2 heterozygous resistant ( $\chi^2 = 1.35$ , P < 0.245) in both the experiments undertaken. Segregation results from the F<sub>2</sub> population, F<sub>2:3</sub> families and detached trifoliate treatment mentioned in section 3.3.3 suggest that a single dominant gene is responsible for resistance to CLS in fenugreek (table 3.5 and 3.6).



**Figure 3.3:** Frequency distribution of Cercospora leaf spot (CLS) scores observed in individual plants of 6 different generations within two different experiments conducted in greenhouse at Lethbridge Research and Development Center.

**Table 3.5:** A monogenic dominant model to explain segregation pattern of CLS resistance in fenugreek. Each individual plant in different generations were inoculated with *C. traversiana* in two controlled environment experiments conducted in a greenhouse at the Lethbridge Research and Development Center. Frequency of plants with various CLS scores within different generations were subjected to  $\chi^2$ - test.

Generation	Total # of plants	Observed Ratio		Expected ratio	$\chi^2$ Value	P Value	Total # of plants	Observed Ratio		Expected ratio	$\chi^2$ Value	P Value
		Resistant	Susceptible					Resistant	Susceptible			
<b>EXPERIMENT- 1</b>							<b>EXPERIMENT- 2</b>					
Tristar(T) <sup>a</sup>	72	0	72	-	-	-	24	0	24	-	-	-
L3717(R) <sup>b</sup>	75	75	0	-	-	-	29	29	0	-	-	-
PI138687(PI) <sup>b</sup>	73	62	11	-	-	-	27	22	5	-	-	-
<b>F<sub>1</sub> (T×R/R×T)</b>	108	108	0	-	-	-	40	38	2	-	-	-
<b>F<sub>1</sub>(T×PI/PI×T)</b>	64	50	14	-	-	-	22	21	1	-	-	-
<b>F<sub>2</sub>(T×R/R×T)</b>	314	241	73	3:1	0.514	0.473	108	73	35	3:1	3.160	0.075
<b>F<sub>2</sub>(T×PI/PI×T)</b>	325	255	70	3:1	2.077	0.149	39	25	14	3:1	2.47	0.116
<b>F<sub>2</sub> (field)</b>	150	104	46	3:1	2.567	0.109	-	-	-	-	-	-
<b>BC<sub>1</sub>(F<sub>1</sub>×R)</b>	150	148	2	-	-	-	30	27	3	-	-	-
<b>BC<sub>1</sub> (F<sub>1</sub>×T)</b>	159	85	74	1:1	0.761	0.383	33	18	15	1:1	0.273	0.601

a= Susceptible parent b= Resistant parents

**Table 3.6:** Frequency of F<sub>2:3</sub> families of fenugreek which showed two different contrasting categories (homozygous resistant and heterozygous resistant) while treated with inoculum of *C. traverisana*.

Generation	Total number of Family accessed	Observed Ratio		Expected ratio	$\chi^2$ Value	P Value
		Homozygous Resistant	Heterozygous Resistant			
<b>Experiment -1</b>						
F <sub>2:3</sub> Family	30	7	23	1:2	1.35	0.245
<b>Experiment -2</b>						
F <sub>2:3</sub> Family	30	7	23	1:2	1.35	0.245

### **3.3.7. Variance components and heritability estimates:**

The results demonstrating different variance components and heritability estimates are presented in table 3.7. Broad sense heritability estimates from first experiment for CLS score were 88.4% and 67.2% respectively for Tristar × L3717/ L3717 × Tristar and Tristar × PI138687/PI138687 × Tristar cross combinations. From the second experiment, broad sense heritability estimates for same cross combinations were obtained 88.3% and 81.2% respectively. Narrow sense heritability estimates for cross combination Tristar × L3717/ L3717 × Tristar were 47.7% and 66.1% respectively for experiment-1 and experiment-2.

**Table 3.7:** Genetic variance components and heritability estimates for resistance to Cercospora leaf spot in fenugreek

Variance components and Heritability estimates	Tristar × L3717		Tristar × PI138687	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
<b>Environmental variance (<math>V_E</math>)</b>	0.363	0.397	0.994	0.505
<b>Phenotypic variance (<math>V_P</math>)</b>	3.116	3.383	3.031	2.682
<b>Genotypic variance (<math>V_G</math>)</b>	2.753	2.986	2.037	2.177
<b>Additive variance (<math>V_A</math>)</b>	1.487	2.235	-	-
<b>Dominance variance (<math>V_D</math>)</b>	1.266	0.751	-	-
<b>Narrow sense heritability (<math>h^2_n</math> %)</b>	47.7	66.1	-	-
<b>Broad sense heritability (<math>h^2_b</math> %)</b>	88.4	88.3	67.2	81.2
<b>Grand mean (<math>\bar{x}</math>)</b>	4.614	4.522	4.94	4.95

### **3.4. Discussion:**

Producing intraspecific hybrids through artificial hybridization between different accessions of fenugreek was demanding due to the small sized flower and stigma in fenugreek being deeply located fully covered by ten stamens and two fused keel petals. Mastering the crossing techniques is required along with proper physical facilities such as magnifying lens and sharply pointed forceps. Factors, especially relative humidity and light played a significant role while performing artificial hybridization. Not a single cross (out of 76 crosses) was successful when high humidity was not maintained around the floral region; i.e., drying of floral parts was visible (Fehr 1987). Although able to accommodate a few plants, keeping plants in artificial humidity chambers was helpful and overall crossing success increased to 28.57% among different parental combinations. Success rate was almost double (33.99%) when crosses were done from 10 a.m. to 2 p.m. compared to 17.74% if done outside this time period. A study carried out in Brazil on artificial hybridization of soybean also identified 10 am to noon as the best time for pollination (Matsuo et al. 2015). Similarly, success rate of crosses made during the summer time was significantly higher than those done in the fall. Greenhouses during the fall were supplemented with tungsten light as natural light was insufficient. Thus, we presume that difference in crossing success in both cases (within a day and within a year) was due to variation in natural light intensity and quality (Fehr 1987). This kind of difference was also noticed by Cornish et al. (1983) while conducting artificial hybridization in fenugreek where crossing success was lower in the greenhouse under artificial light provided by fluorescent and tungsten lamps. Experiments in diverse crops suggested that insufficient light negatively affect flowering and fruit set which may be due to abnormalities in various reproductive parts or variations produced in enzymatic

activity within the flower (Karapanos et al. 2008). Since all other factors within the greenhouses were similar during emasculation and pollination, the differences in success rate could be attributed to differences in natural lighting. It is also interesting to note that our overall crossing success for fenugreek was almost 7 times higher than the previously reported study by Choudhary (2003).

Symptoms in susceptible plants started to show up about 9 days after inoculation while in resistant plants, it took around 12 days for the first visible disease symptoms to appear. Symptoms were characterized by formation of wet spots which later turned into black lesions fully covered with fungal growth and surrounded by yellow halos (Prasad et al. 2014). No such contrasting visual differences in symptoms between the two resistant parental genotypes used (i.e. L3717 and PI138687) were observed. In general, resistant plants had relatively small-sized lesions compared to susceptible ones and early necrosis of invaded cells was visible in them which could be a defense mechanism of plants to cease fungal growth and eventual disease spread. Rapid coalescing of lesions in susceptible plants under favorable conditions led to invasion of whole leaves and later defoliation. Yellowing of the lower leaves was prominent, especially in susceptible plants even when no lesions were present on them. Microscopic observation of *Cercospora traversiana* structures from the infected leaves showed long, slender, slightly curved, solitary, translucent and multicellular conidia—which was no different from observations made by Prasad et al. 2014.

Variability in the F<sub>2</sub> generation in terms of different parameters was obtained through artificial hybridization between resistant and susceptible genotypes used in this experiment (Appendix -2). This variability can be exploited to select the most promising

genotypes; high productivity and CLS resistance in our case. Individual performances (table 3.2) of some individuals were even better than the check (Tristar), depicting their potential to be released as a new cultivar. However, it is too early to judge those genotypes; selection of superior genotypes based on available agronomic traits requires series of multi-location trials over several generations. Along with being resistant to CLS, another important feature of L3717 which was unnoticed in previous studies was its early maturing nature. It took around 98 days to mature under field condition which was almost 24 days less time to mature compared to Tristar. This can facilitate selection of early maturing and CLS resistant fenugreek genotypes from a single experiment and thus can be exploited to develop new fenugreek cultivars primarily for seed purposes. Such cultivars, if developed, can attain complete seed set and maturity especially in western Canadian conditions where only around 100 frost free days are available (Basu et al. 2008; Thomas et al. 2011).

The current study provides valuable information on the genetics of resistance to CLS in fenugreek. The crosses between resistant and susceptible parents in our research produced no (or negligible) progeny with an intermediate disease score indicating the presence of qualitative gene resistance. To bolster, chi-square test performed for various generations in green house plants and detached leaf samples of the F<sub>2</sub> generation from the field suggested CLS resistance in fenugreek introductions L3717 and PI138687 is governed by a single dominant gene. This result is in general agreement with what was reported earlier for CLS resistance by several scientists in various crops such as cowpea (Fery et al. 1976; Fery and Dukes 1977; Castro et al. 2003), mung bean (Chankaew et al. 2014; Thakur et al. 1977, faba bean (Kimber and Paull 2011) and soybean (Pace et al.

1993). However, drawing general conclusions for CLS resistance in fenugreek requires identification of a large number of resistant sources and testing them against divergent pathogen isolates/races (Liu and Rimmer 1991). Currently, a single strain of *Cercospora traversiana* has been identified all over the world (Ryley 1989; Prasad et al. 2014) thus the qualitative gene resistance, we identified, may be sufficient to check the pathogen population within the field. However, generation and outbreak of new strains of pathogen cannot be neglected in the future, which may demand identification of new sources of resistance, possibly pyramiding of identified genes within a genotype (Mundt 2014). This can act as a long term sustainable solution to the CLS problem in fenugreek. Although, two genotypes (L3717 and PI38687) have already been identified as potential sources of CLS resistant genes, additional study is required to determine whether those genes are the same and are located within the same chromosome.

Both crosses in the present study showed high broad-sense heritability (67% to 88%) indicating the fraction of phenotypic variance that is due to genetic differences among individuals in a population. Narrow-sense heritability, indicating the proportion of phenotypic variance that is due to genetic effects which are capable of being passed from one generation to the next however, was moderate (47.7%) for Tristar × L3717/L3717 × Tristar cross combination. The results are in close agreement with prior studies by Zhang et al. (2012) and Gordon et al. (2006) which also indicated high broad sense heritability up to 85% in gray leaf spot in maize caused by *Cercospora zeae-maydis*. In contrast, CLS resistance in sugar beet (60 to 71%) and peanut (49 to 68%) has been identified to have moderate broad sense heritability (Jogloy et al. 1987; Smith and Gaskill 1970). Anderson et al. 1991 identified narrow-sense heritability values ranging from 18% to

74% for CLS resistance in peanut genotypes. The high broad sense heritability value in our experiment indicated existence of high genetic variability within the genotypes and a low level of environmental influence on CLS expression. Furthermore, the moderate narrow sense heritability for disease score suggests CLS resistant traits are under additive gene effects, and therefore selection in early generations for them will be effective (Ali and Khan 2007; Ayele 2011). Some scientists also have proclaimed that heritability higher than 30% allows genetic gain if selection is made in early generations, such as the F<sub>3</sub> generation (Brogan et al. 2003; Santos et al. 2017). A notable discrepancy of results between our two experiments for the heritability is not unexpected because heritability estimation is highly dependent on several factors such as environment, genetic background of parents, sample size and methods used to calculate heritability (Falconer and Mackay 1996; Griffiths 2000). In this study, the second experiment had a lower number of plants due to limited availability of seeds which may have played a role in generating the varied heritability results.

To the best of our knowledge, this is the first report of genetic analysis of resistance to *Cercospora traversiana* in fenugreek. The results observed from this study were primarily for artificial hybridization, correlation between morphological traits and genetics of CLS resistance. These are of practical relevance and are valuable for the successful implementation of fenugreek breeding programs which in Canada are at an early stage. CLS resistance being governed by a single dominant gene with moderate narrow sense heritability facilitates easier selection of progeny. Identification of phenotypic markers as well as molecular markers which are closely related to CLS resistance can help in the selection process. Homozygous CLS resistant F<sub>3</sub> seeds

identified from this experiment can be further tested through a series of field and greenhouse screenings to confirm their resistance and check their superiority in agronomic traits as well. Variation obtained through artificial hybridization in this experiment can be exploited to enhance the genetic base of fenugreek within which selection can be practiced in western Canada.

## CHAPTER-4

### NOVEL TECHNIQUES FOR SCREENING CERCOSPORA LEAF SPOT RESISTANT FENUGREEK GENOTYPES.

#### 4.1. Introduction:

Cercospora leaf spot (CLS) on fenugreek, caused by *Cercospora traversiana*, is an important constraint to use of fenugreek as a crop. This fungal disease is prevalent all around the globe and was found to severely infect fenugreek even in countries such as Hungary (Voros and Nagy 1972), Bulgaria (Bobev et al. 1999), Australia (Ryley 1989) and Canada (Zimmer 1984) where the crop was newly introduced and not grown intensively. Yield loss attributed to CLS in susceptible fenugreek cultivars has been noticed up to 80% when environmental conditions are conducive to disease development (Zimmer 1984). Besides reducing yield, the disease also has an adverse effect on seed quality—affecting marketability (Ryley 1989) and forage quality which renders it unsuitable as animal feed. Though, considered one of the major constraints of fenugreek production a critical study of this disease has not been done in the past.

*Cercospora traversiana* is a seed borne fungus which falls under the division Ascomycota (Agrios 1997). Researchers believe that *C. traversiana* is the only species of the genus *Cercospora* infecting fenugreek (Cook 1978; Ryley 1989; Voros and Nagy 1972). The center of origin for this pathogen is southern Asia and occurrence of this pathogen in other countries likely is due to transport of infected seeds (Leppik 1959, 1960). *Cercospora* have unbranched, sparingly septate, uniform but slightly bent conidiophores which are 17.6 to 28.8  $\mu\text{m}$  long and 1.78 to 3.01  $\mu\text{m}$  wide while conidia are translucent, multicellular, solitary, acicular, slightly curved, with a truncate base and rounded apex, 2.3 to 2.8  $\mu\text{m}$  long and 1.2 to 1.9  $\mu\text{m}$  wide (Prasad et al. 2014; Ryley

1989). Spread of these conidia, located at different living plant parts and in decaying debris, can happen through splashing rain and wind (Acharya et al. 2010). The initial symptom of CLS infection is marked by circular sunken lesions with 1-2mm chlorotic halos. These lesions coalesce as the infection progresses and leads to necrosis. Severe infection is marked by defoliation along with infected stems and pods (Prasad et al. 2014).

Disease resistance is a sustainable solution to plant disease control due to economic and environmental factors (Kibria et al. 2010; Komarek et al. 2010). Among several methods used to access the level of resistance among plants or genotypes, field screening through natural or artificial inoculation is the best preferred as it matches the situation that a producer confronts while growing crops. However, field screening demands significant time, area and a workforce to maintain the number of plants required for an experiment (Gurung et al. 2015; Twizeyimana et al. 2007a). In addition to that, weather conditions in the field may not always be favourable for disease propagation (Twizeyimana et al. 2007b), which eventually may lead to failure of the overall experiment. Whole plant assay (WPA) is an alternative screening method where every plant is kept in a controlled environment which is very conducive to disease. This method helps in scaling up the number of plants screened (Evans et al. 1999) while reducing the space and time needed to access the disease (Dong et al. 2017; Twizeyimana et al. 2007a). Environment around the plants can be maintained as required and the plant gets only the specified inocula thus increasing the reliability of study (Blechert and Debener 2005; Dong et al. 2017). Several previous works have been done to identify a suitable model for whole plant assay of cultivated crops against diversified pathogens. Screening of fenugreek through whole

plant assay in a controlled environment to identify resistance was carried out in Lethbridge, Canada by Prasad et al. (2014).

Use of various living plant parts such as leaves, stems, roots and fruit have already been practiced differentiating genotypes for disease resistance. A detached leaf assay (DLA) can facilitate rapid screening of a large number of genotypes without the use of a large space and amount of inoculum (Felsenstein et al. 1998). It allows screening of a single plant against different races of the pathogen at a time. Moreover, this method can be utilized best while screening a segregating population (Dong et al. 2017; Vail and Vandenberg 2010) where each plant has unique genetics; therefore, seeds from those plants can be saved for future breeding purposes. DLA has already been employed to differentiate the level of resistance in several plants ranging from herbaceous crops to large tree species with a high success rate (Browne and Cooke 2004; Hansen et al. 2005; Huang et al. 2016; Jackson et al. 2008). However, use of this method to screen fenugreek genotypes for *Cercospora* leaf spot has not been reported so far.

Temperature plays a significant role in pathogen survival, dissemination, disease appearance and its severity (Agrios 2005; Jacobsen et al. 2004b; Terefe et al. 2015). Pathogens have their own ideal temperature regime where disease development is maximum. The effect of temperature on the ability of *Cercospora* to cause disease in diverse crops has been examined by numerous researchers (Alderman and Beute 1987; Carisse et al. 1993; Cooperman and Jenkins 1986; Khan et al. 2009; Paul and Munkvold 2005). After close scrutiny of those experiments, the optimum temperature for maximum disease severity of CLS was found to be around 25°C. At that temperature, conidia germination and mycelial growth occur at full potential and thus depict maximum symptoms (Cooperman and Jenkins 1986; Paul and Munkvold 2005). Either human or

natural factors can cause physical damage or wounding in plants; leading to direct economic loss. It is supposed that physical damage facilitates easier entry of pathogen into the plants eventually increasing proneness of plants to severe disease (Savatin et al. 2014). Fenugreek grown under field conditions are not always free from damage which may arise due to natural causes such as hailstones, wind, insects, frost and snowfall, and mechanical damage while intercropping. Physical damage accompanied by favorable temperature around plants may lead to maximum possible damage. Thus, this study is essential not only to develop suitable methods for screening germplasm and studying disease ecology and morphology but also to infer effective management strategies to minimize economic consequences to farmers.

Our objectives of this experiment are to:

- Develop rapid screening methods to evaluate host resistance needed to accelerate the progress in developing disease resistance in cultivars of fenugreek.
- Add to the present state of knowledge on epidemiology by critically observing morphological symptoms of CLS.
- Identify effects of temperature and wounding on CLS severity in fenugreek.

## **4.2. Material and methods**

### **4.2.1. Preparation of plant material**

#### **4.2.1.1. Whole plant assay:**

The experiment was laid out using a RCBD design with four replications. Single seed from three parents viz. Tristar (susceptible), L3717 (resistant) and PI138687 (resistant), and 43 F1s developed by artificially crossing of those three parents was sown in each pot (10cm×10cm×12 cm) filled with a non-sterile 30, 30, 30 soil mixture. This soil mix was composed of fine sand, soil and Cornell mix (Basu et al. 2008) in equal proportions. Re-sowing was followed, if no germination was observed. The pots were kept in the greenhouse at the Lethbridge Research and Development Center (LRDC). Plants were exposed to temperature of 25°C during daytime and 14°C during night-time. A photoperiod of 16 hours was maintained throughout the experiment. Plants were irrigated regularly and sprayed with the insecticides- Kontos (Bayer CropScience Canada Inc.) and Avid (Syngenta Canada Inc.) alternately to prevent plant damage by western flower thrips (*Frankliniella occidentalis* Pergande) in the greenhouse.

#### **4.2.1.2. Detached leaf assay:**

Leaves for this experiment were obtained from the genotypes used for whole plant assay. This experiment was conducted in a split plot design with four replications using two different temperatures regimes (day/night) 20/12°C and 25/14°C as main plot factors. The combination of genotypes and leaf state (wounding and non-wounding) formed sub-plots, which were arranged randomly in a RCBD design. Leaves, recently formed but fully open, were excised from the apical region of the branches and kept in a moist plastic bag until they were taken to the lab for the assay. These leaves were then washed

gently with distilled water twice to make them free of physical impurities. Wounds were made by puncturing leaflets on both sides of the midribs with the help of forceps. Four leaves from different genotypes were kept randomly in each petri plate of 6 cm diameter (figure 4.3.C) which was lined with moist filter paper. Inoculation was done right after placing them in petri plates.

#### **4.2.2. Culture maintenance and inoculum preparation:**

A frozen vial of live pure culture of *Cercospora traversiana* was used for sub culturing (initially obtained from CABI, United Kingdom). The microorganism was then streaked on potato-dextrose agar (PDA) plates. Those plates were then incubated at room temperature ( $20 \pm 2^{\circ}\text{C}$ ) in darkness for 28 days to promote sporulation. A conidial suspension for inoculation was prepared after full sporulation within the media plate. The cultures were washed with distilled deionised (dd) water and passed through a double layer of cheese cloth to form a suspension. The resulting conidial suspension was diluted to a final concentration of  $1 \times 10^7$  conidia /ml as determined with a haemocytometer to be used for the detached leaf assay. A separate suspension of concentration  $2 \times 10^5$  conidia/ml was prepared for whole plant assay. One drop of tween 20 was added per 100 ml of suspension.

#### **4.2.3. Inoculum application:**

##### **4.2.3.1. Whole plant assay:**

30 day old plants from the greenhouse were inoculated with a hand sprayer using a fine mist until runoff from the leaves was observed. The pots were then placed in a tray containing a moist paper towel and covered with a transparent lid (figure 4.3.A). Lids were removed after 7 days and trays were placed in normal greenhouse conditions.

Temperature (day/night) and photoperiod were maintained to 25/14°C and 16 hours respectively throughout the experiment.

#### **4.2.3.2. Detached leaf assay:**

The leaves (adaxial side) were inoculated with a hand sprayer using a fine mist until runoff from the leaves was observed. The Petri dishes were then covered with lids and kept in a tray containing a moistened paper towel. This helped in maintaining the required humidity within the system by preventing moisture loss from the petri dishes. Trays containing samples were then kept in two different greenhouses with the temperature (day/night) 20/12°C and 25/14°C and maintained at a 16-hour photoperiod. Arrangements were made to prevent direct sunlight on the samples.

#### **4.2.4. Disease assessment:**

##### **4.2.4.1. Whole plant assay:**

Disease for WPA was visually scored three times viz. at 10, 20 and 30 days after inoculation based on a scale of 0-5, where: 0 = 0-5% of total leaves infected (highly resistant); 1 = 6-15% of total leaves infected (Resistant); 2 = 16-30% of total leaves infected (moderately resistant); 3 = 31-50% of total leaves infected (moderately susceptible); 4= 51-80% of total leaves infected (susceptible); 5 = 81-100% of total leaves infected (highly susceptible). Area under disease progress curve (AUDPC) for each genotype was calculated using the following formula;

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left\{ \frac{Y_i + Y_{i+1}}{2} \right\} \times (T_{i+1} - T_i)$$

Where,

$Y_i$  = disease severity on the  $i^{\text{th}}$  date,

$T_i$  = time on which  $Y_i$  was recorded and

$n$  = number of times observations were taken.

AUDPC was also computed for number of lesions and lesion diameter in the same manner. Lesions from ten randomly selected leaves were counted to determine average number of lesions per leaf. To obtain lesion diameter, ten lesions from those randomly selected leaves were measured and averaged.

#### **4.2.4.2. Detached leaf assay:**

Four weeks after inoculation, the symptoms were visually scored on the same scale of 0-5 as mentioned in WPA; however, this time percentage of individual leaf infected was assessed instead of percentage total leaves infected (figure 4.3.F).

#### **4.2.5. Data analysis:**

Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test with probability level set at 5% ( $p<0.05$ ) was performed on the data for each factor obtained for the 46 genotypes using PROC MIXED—SAS statistical software package (SAS Institute, Cary, NC, USA). Correlation coefficient between different components of disease resistance and different graphs were generated using Sigma Plot (Systat Software, San Jose, CA).

### **4.3. Results:**

#### **4.3.1. Whole plant assay:**

Under the above-mentioned conditions of the experiment, the propagules (conidia) from the suspension induced typical symptoms of leaf spot even in resistant genotypes, though the severity was low in them. Symptoms were initiated with the formation of sunken lesions after 7 to 8 days which later enlarged to form circular brown or black spots. The gradual outward progression of a lesion led to the formation of concentric rings around the central black spot. Coalition of lesions occurred as the disease developed further and eventually the whole leaf was covered by blackish fungal growth. This also induced defoliation of affected leaves. However, defoliation was also observed when leaves started yellowing, even in the absence of lesions on the leaf surface. Branches and pods were also affected especially in susceptible ones. Figure 4.3.B. shows how resistant and susceptible plants looked like 30 days post inoculation. Mycelial growth along with conidia from the infected leaves was removed with the help of a sterilized scalpel and slides were prepared to observe them under a compound microscope. Conidia were observed primarily, and they were solitary, multicellular, slender, slightly curved, translucent and blackish in colour which confirmed the symptoms within the fenugreek plants was due to *C. traversiana*.

##### **4.3.1.1. Days to first disease appearance:**

Each of the 46 genotypes treated were significantly different ( $p \leq 0.01$ ) in terms of first disease appearance (table 4.1). TPi-3 showed resistant characteristics with a comparatively lower disease score but was one of the two earliest genotypes to show visible symptoms (8.25 days); the other being the susceptible cultivar Tristar. The longest

time for symptom appearance was in PiT-1 (12.5 days). Two other resistant parental genotypes L3717 and PI138687 showed symptoms at 11.75 and 10.75 days respectively.

#### **4.3.1.2. Disease score (AUDPC):**

As the genotypes used for the experiment were  $F_1$ 's generated from the crossing of two resistant and one susceptible parent, our prime objective was to identify and sort-out the susceptible genotypes. Therefore, we proposed a resistant category where genotypes with a mean AUDPC higher than 35 were considered susceptible and genotypes with mean AUDPC equal or lower than 35 were considered resistant and thus retained for further generation analysis. ANOVA (table 4.1) shows that there was a significant difference ( $p \leq 0.01$ ) between the genotypes treated. Tristar with a mean AUDPC (61.00) was the genotype with the highest disease severity. RT-12 showed lowest disease severity with a mean AUDPC value of 20.00 which was even lower than the AUDPC value of its corresponding resistant parent L3717. Five  $F_1$  genotypes RT-2, PiT-2, PiT-3, PiT-4 and PiT-7 showed a susceptible reaction while rest showed a resistant reaction against *Cercospora traversiana*.

#### **4.3.1.3. Lesion diameter and number of lesions:**

A significant difference ( $p \leq 0.01$ ) was observed in the mean AUDPC for number of lesions between the genotypes (table 4.1). The increment in number of lesions was lowest in PiT-6 (28.6698) and highest in genotype PiT-9 (39.0615) which was not significantly different from the susceptible cultivar ‘Tristar’. Results show a similar fashion of increment in number of lesions in all other genotypes. However, the AUDPC of lesion diameter was not significantly different between the genotypes treated.

A separate analysis of data was carried out to determine the change in lesion diameter and number of lesions per leaf over time (at 10, 20 and 30 DAI). Irrespective of the genotypes treated, average size of lesions 10, 20 and 30 days after inoculation was 2.83mm, 3.15mm and 4.35mm respectively showing increment in lesion diameter with time (figure 4.1). The figure also shows diameter increment was more rapid in the period from 20 DAI to 30 DAI than from 10 DAI to 20 DAI. In a similar fashion as lesion diameter, number of lesions increased from 10 DAI (1.15) to 20 DAI (1.41); however, interestingly the number of lesions in 30 DAI (1.32) was lower than that found in 20 DAI. This rapid increase in lesion diameter and decrease in number of lesions from 20 DAI to 30 DAI may be due to coalescing of lesions after 20DAI under favorable conditions.

#### **4.3.1.4. Correlation between components of CLS resistance in whole plant assay:**

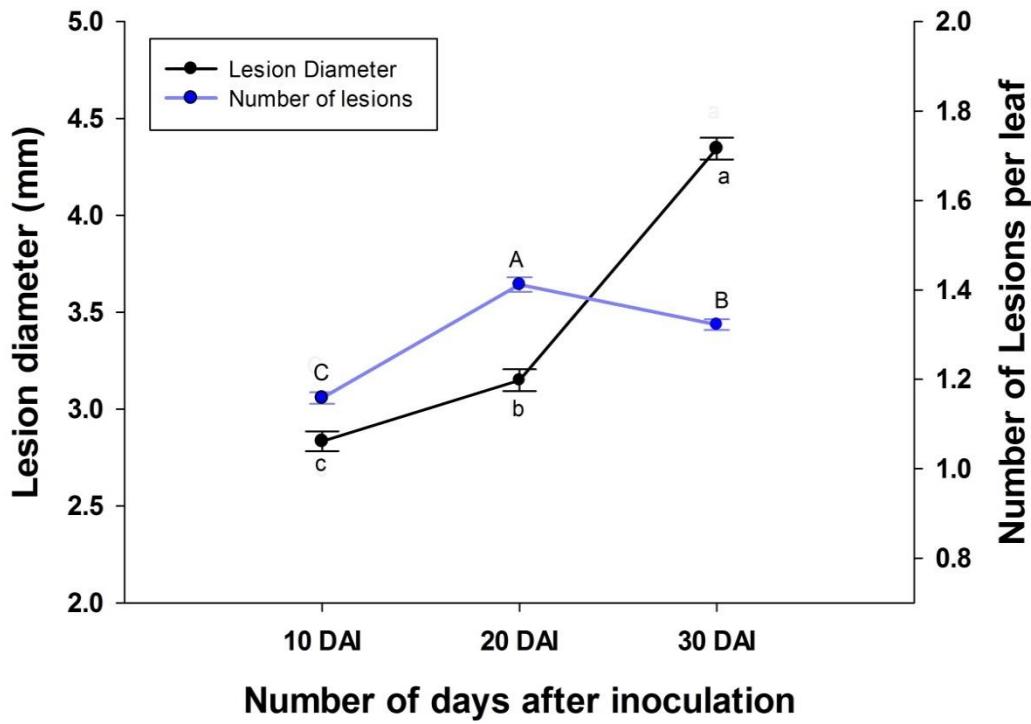
Table 4.2 shows a moderate and inverse correlation ( $p < 0.01$ ) between first disease appearance and AUDPC (disease score), indicating that the lower the incubation period the higher the severity will be. A significant ( $p < 0.01$ ), but low positive correlation was obtained between AUDPC (number of lesions) and AUDPC (disease score). Number of lesions (AUDPC) and first disease appearance time were found to be slightly and negatively correlated ( $p < 0.05$ ). No other significant correlations between the disease components were found.

**Table 4.1:** Mean values for different components of disease resistance after treating individual plants with conidial suspension ( $2 \times 10^5$  conidia/ml) of *Cercospora traversiana*.

Name of Genotypes	AUDPC (Disease score)	AUDPC (Number of Lesions)	AUDPC (Lesion Diameter)	First Disease appearance (days)
<b>TR-1</b>	35.0 <sup>BCDE</sup>	30.5 <sup>DEFGH</sup>	8.54	9.75 <sup>CDEFGH</sup>
<b>TR-2</b>	30.0 <sup>CDEFGH</sup>	30.4 <sup>DEFGH</sup>	8.53	10.25 <sup>BCDEFGH</sup>
<b>TR-3</b>	26.3 <sup>EFGH</sup>	31.1 <sup>DEFGH</sup>	8.12	9.00 <sup>FGH</sup>
<b>TR-4</b>	30.0 <sup>CDEFGH</sup>	30.7 <sup>DEFGH</sup>	8.50	9.00 <sup>FGH</sup>
<b>TPi-1</b>	33.8 <sup>BCDEF</sup>	32.6 <sup>CDEFGH</sup>	8.67	8.50 <sup>GH</sup>
<b>TPi-2</b>	28.8 <sup>DEFGH</sup>	30.4 <sup>DEFGH</sup>	8.68	11.25 <sup>ABCDE</sup>
<b>TPi-3</b>	32.5 <sup>BCDEFG</sup>	29.7 <sup>FGH</sup>	8.74	8.25 <sup>H</sup>
<b>TPi-4</b>	27.5 <sup>DEFGH</sup>	30.4 <sup>DEFGH</sup>	9.09	9.00 <sup>FGH</sup>
<b>PiT-1</b>	33.8 <sup>BCDEF</sup>	30.4 <sup>DEFGH</sup>	8.11	12.50 <sup>A</sup>
<b>PiT-2</b>	37.5 <sup>BCD</sup>	30.7 <sup>DEFGH</sup>	7.78	9.50 <sup>DEFGH</sup>
<b>PiT-3</b>	36.3 <sup>BCDE</sup>	29.2 <sup>GH</sup>	7.87	9.75 <sup>CDEFGH</sup>
<b>PiT-4</b>	36.3 <sup>BCDE</sup>	31.7 <sup>CDEFGH</sup>	8.60	9.50 <sup>DEFGH</sup>
<b>PiT-5</b>	33.8 <sup>BCDEF</sup>	31.9 <sup>CDEFGH</sup>	7.65	9.25 <sup>FGH</sup>
<b>PiT-6</b>	32.5 <sup>BCDEFG</sup>	28.7 <sup>H</sup>	8.31	9.50 <sup>DEFGH</sup>
<b>PiT-7</b>	41.3 <sup>B</sup>	33.9 <sup>BCDEF</sup>	6.87	8.50 <sup>GH</sup>
<b>PiT-8</b>	32.5 <sup>BCDEFG</sup>	33.5 <sup>BCDEFG</sup>	7.47	11.00 <sup>ABCDEF</sup>
<b>PiT-9</b>	33.8 <sup>BCDEF</sup>	39.1 <sup>A</sup>	7.41	9.75 <sup>CDEFGH</sup>
<b>PiT-10</b>	35.0 <sup>BCDE</sup>	35.5 <sup>ABC</sup>	7.75	9.25 <sup>FGH</sup>
<b>PiT-11</b>	22.5 <sup>GH</sup>	33.6 <sup>BCDEF</sup>	7.62	11.25 <sup>ABCDE</sup>
<b>PiT-12</b>	26.3 <sup>EFGH</sup>	32.8 <sup>CDEFGH</sup>	8.05	10.75 <sup>ABCDEF</sup>
<b>RT-1</b>	32.5 <sup>BCDE</sup>	37.7 <sup>AB</sup>	7.07	9.25 <sup>FGH</sup>
<b>RT-2</b>	40.0 <sup>BC</sup>	33.0 <sup>CDEFG</sup>	7.99	9.00 <sup>FGH</sup>
<b>RT-3</b>	30.0 <sup>CDEFGH</sup>	33.6 <sup>CDEFG</sup>	8.22	10.00 <sup>ABCDEF</sup>
<b>RT-4</b>	27.5 <sup>DEFGH</sup>	31.0 <sup>DEFGH</sup>	7.97	11.50 <sup>ABCD</sup>
<b>RT-5</b>	28.8 <sup>DEFGH</sup>	31.7 <sup>CDEFGH</sup>	8.17	10.75 <sup>ABCDEF</sup>
<b>RT-6</b>	32.5 <sup>BCDEFG</sup>	29.9 <sup>EFGH</sup>	6.97	9.25 <sup>FGH</sup>
<b>RT-7</b>	28.8 <sup>DEFGH</sup>	33.4 <sup>BCDEFG</sup>	7.35	9.75 <sup>CDEFGH</sup>
<b>RT-8</b>	20.0 <sup>H</sup>	29.9 <sup>EFGH</sup>	7.45	12.25 <sup>AB</sup>
<b>RT-9</b>	23.8 <sup>FGH</sup>	33.6 <sup>BCDEF</sup>	8.84	10.75 <sup>ABCDEF</sup>
<b>RT-10</b>	30.0 <sup>CDEFGH</sup>	33.8 <sup>BCDEF</sup>	8.05	9.25 <sup>EFGH</sup>
<b>RT-11</b>	28.8 <sup>BCDE</sup>	31.9 <sup>CDEFGH</sup>	7.07	10.75 <sup>ABCDEF</sup>
<b>RT-12</b>	20.0 <sup>H</sup>	33.6 <sup>BCDEF</sup>	7.99	11.25 <sup>ABCDE</sup>
<b>RT-13</b>	31.3 <sup>BCDEFG</sup>	34.1 <sup>BCDE</sup>	8.22	10.00 <sup>CDEFGH</sup>
<b>RT-14</b>	30.0 <sup>CDEFGH</sup>	31.5 <sup>CDEFGH</sup>	7.97	10.50 <sup>ABCDEF</sup>
<b>RT-15</b>	26.3 <sup>EFGH</sup>	32.5 <sup>CDEFGH</sup>	8.17	10.75 <sup>ABCDEF</sup>
<b>RT-16</b>	23.8 <sup>FGH</sup>	30.0 <sup>EFGH</sup>	6.97	11.50 <sup>ABCD</sup>
<b>RT-17</b>	32.5 <sup>BCDEFG</sup>	31.0 <sup>DEFGH</sup>	7.35	10.25 <sup>BCDEFGH</sup>
<b>RT-18</b>	32.5 <sup>BCDEFG</sup>	30.8 <sup>DEFGH</sup>	7.45	9.75 <sup>CDEFGH</sup>
<b>RT-19</b>	27.5 <sup>DEFGH</sup>	33.9 <sup>BCDEF</sup>	8.84	10.75 <sup>ABCDEF</sup>
<b>RT-20</b>	32.5 <sup>BCDEFG</sup>	32.9 <sup>CDEFGH</sup>	9.51	11.00 <sup>ABCDEF</sup>
<b>RT-21</b>	27.5 <sup>DEFGH</sup>	31.3 <sup>CDEFGH</sup>	9.00	9.75 <sup>CDEFGH</sup>
<b>RT-22</b>	32.5 <sup>BCDEFG</sup>	34.5 <sup>BCD</sup>	7.77	9.75 <sup>CDEFGH</sup>
<b>RT-23</b>	33.8 <sup>BCDEF</sup>	32.9 <sup>CDEFGH</sup>	8.57	9.50 <sup>DEFGH</sup>
<b>L3717</b>	26.3 <sup>EFGH</sup>	32.5 <sup>CDEFGH</sup>	8.98	11.75 <sup>ABC</sup>
<b>PI138687</b>	28.8 <sup>DEFGH</sup>	31.2 <sup>CDEFGH</sup>	8.72	10.75 <sup>ABCDEF</sup>
<b>Tristar</b>	61.3 <sup>A</sup>	37.3 <sup>AB</sup>	8.07	8.25 <sup>H</sup>
<b>LSmean</b>	<b>10.63</b>	<b>4.31</b>	<b>1.61</b>	<b>2.16</b>
<b>F-value</b>	<b>2.99**</b>	<b>2.05**</b>	<b>1.05<sup>NS</sup></b>	<b>1.81**</b>
<b>CV</b>	<b>18.54</b>	<b>9.53</b>	<b>14.12</b>	<b>15.32</b>
<b>Standard error</b>	<b>3.800</b>	<b>1.544</b>	<b>0.576</b>	<b>0.772</b>

Note: NS-Non-Significant, \*\*- Significant at 1% level of probability

Means with different superscripts within the same column are significantly different from each other.



**Figure 4.1:** Changes in Cercospora leaf spot lesion diameter and number of lesions over time in fenugreek leaves. Error bars represent standard error of the mean and means with different superscripts are significantly different ( $P < 0.05$ ) from each other; a, b, c are for lesion diameter and A, B, C are for number of lesions.

**Table 4.2:** Correlation of different parameters of resistance in relation to Cercospora leaf spot of fenugreek while inoculating whole plants.

Parameters	(AUDPC) Disease Score	(AUDPC) Lesion Diameter	(AUDPC) Number of lesions	First Disease Appearance
<b>(AUDPC) Disease Score</b>	1			
<b>(AUDPC) Lesion Diameter</b>	0.027 <sup>NS</sup>	1		
<b>(AUDPC) Number of lesions</b>	0.261**	0.0139 <sup>NS</sup>	1	
<b>First Disease Appearance</b>	-0.557**	0.0291 <sup>NS</sup>	-0.196*	1

Note: NS-Non- Significant, \*- Significant at 5% level of probability \*\*- Significant at 1% level of probability

#### **4.3.2. Detached leaf assay:**

Inoculation of detached fenugreek leaves with a spore suspension ( $1 \times 10^7$  conidia/ml) of *Cercospora traversiana* resulted in clearly defined lesions which varied significantly among different genotypes, temperatures applied and state of the leaves when scored based on the percentage of leaf area infected. Symptoms were similar to those observed in whole plant assays. The only visible differences in symptoms among WPA and DLA within a leaf were: a) fully grown lesions looked darker in DLA than WPA which we presume was due to presence of moisture within the leaves all of the time and b) most of the leaves in DLA had single lesions while in the case of WPA, most of the leaves had more than one lesion which coalesced later. Figure 4.3.D. shows how leaves of resistant and susceptible plants reacted to *C. traversiana* in DLA.

##### **4.3.2.1 Effect of temperature on disease severity:**

Less than 5% of the plant samples kept in the greenhouse at temperature regime 20/12°C only showed disease symptoms (figure 4.3.E), therefore, they were not subjected to analysis. Even the leaves from the susceptible cultivar 'Tristar' didn't show any symptoms until 45 days after inoculation. Thus, all samples kept at a 20/12°C temperature regime were sidelined and samples kept at a 25/14°C temperature regime were only taken into consideration for data analysis. A repeat of the experiment was conducted to test the reliability of the results, but this time only three parental genotypes were included. Similar results with no symptoms in leaves kept at 20/12°C were obtained. This shows that environmental temperature of 25/14°C can readily initiate disease symptoms and cause damage to the leaves while 20/12°C may not be sufficient for symptoms to initiate in detached leaves.

#### **4.3.2.2. Effect of wounding on disease severity:**

ANOVA (table 4.3a) conducted for samples kept at a 25/14°C temperature regime indicated that wounding ( $p < 0.0001$ ) significantly affected disease severity and time for the first visible symptoms appearance irrespective of genotype. Disease score (table 4.3b) of the leaves with wounds (2.0) was significantly higher than the disease score of the leaves with no wounds (1.7). Furthermore, it took 19.7 days for symptoms to appear in un-wounded leaves (table 4.3b). This was significantly longer time than it took in wounded leaves (18.8 days) for the disease to develop. However, no interaction between genotypes and state (wounding or un-wounding) of leaves in both disease score and first symptoms appearance time were observed.

#### **4.3.2.3. Difference among genotypes for disease severity:**

Disease score of each of the genotypes treated was significantly different ( $p < 0.0001$ ) with each other showing presence of variability within the genotypes (table 4.3c). The disease score was highest (3.9) in the susceptible cultivar whereas, PiT-11 showed the lowest severity with an average disease score of 0.5. Average disease score of the two resistant parents L3717 and PI138687 was 1.125 and 2.00 respectively. Most of the F<sub>1</sub> genotypes showed a resistant reaction with disease score being closer to resistant parents while few of them showed a susceptible reaction. Still, none of them surpassed the disease score of the susceptible cultivar ‘Tristar’. Number of days it took for disease to appear on detached leaves of 46 different genotypes irrespective of state of leaves (wounding and un-wounding) is presented in appendix-1.

**Table 4.3a:** Analysis of variance for detached leaves of 46 genotypes and their state (wounding and un-wounding) in terms of disease score and first disease appearance.

Sources	DF	DS		FDA (days)	
		F value	P value	F value	P value
<b>Genotype</b>	45	14.81	<.0001	4.65	<.0001
<b>State of Leaves</b>	1	39.45	<.0001	17.37	<.0001
<b>Genotype*State of Leaves</b>	45	1.02	0.4440	0.95	0.5678

DF = Degrees of freedom  
DS = Disease score  
FDA = First disease appearance

**Table 4.3b:** Mean disease score and first visible symptoms appearance (days after inoculation) of wounded and un-wounded detached leaves irrespective of genotypes.

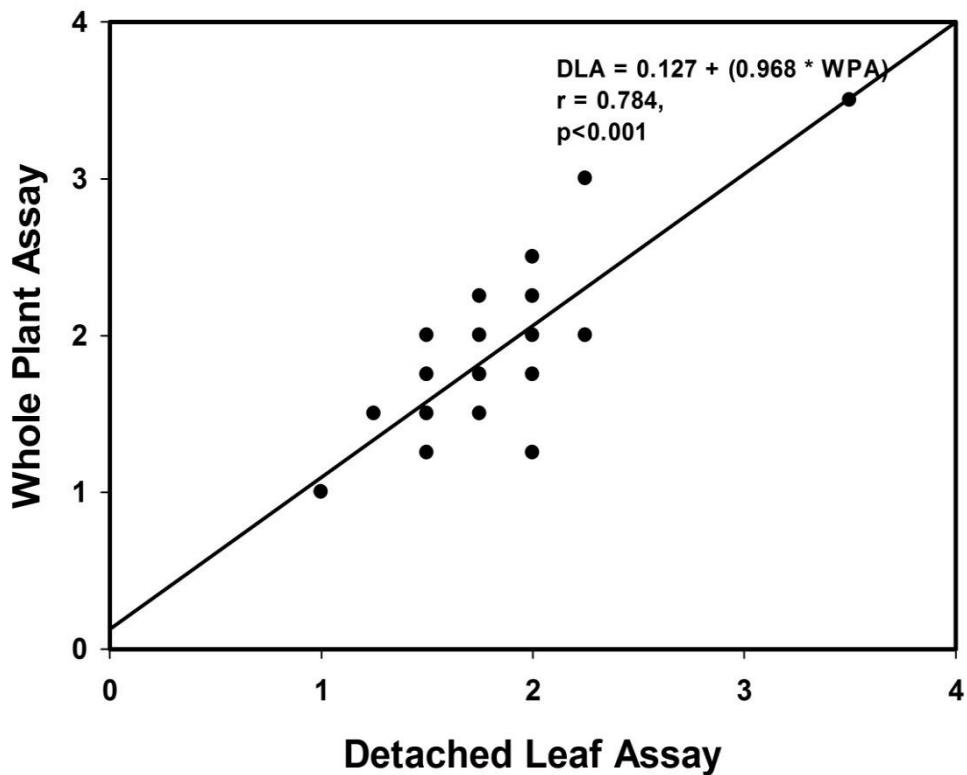
State of leaves	Disease score ± SE	First disease appearance (days) ± SE
<b>Wounded</b>	<b>2.04 ± 0.037<sup>a</sup></b>	<b>18.8 ±0.188<sup>b</sup></b>
<b>un-Wounded</b>	<b>1.71 ± 0.037<sup>b</sup></b>	<b>19.7 ± 0.188<sup>a</sup></b>
Means with different superscripts within the same column are significantly different from each other SE = Standard error		

**Table 4.3c:** Categorisation of 46 different genotypes into resistant and susceptible based on their average disease score obtained after treating detached leaves irrespective of state of leaves (wounding and un-wounding).

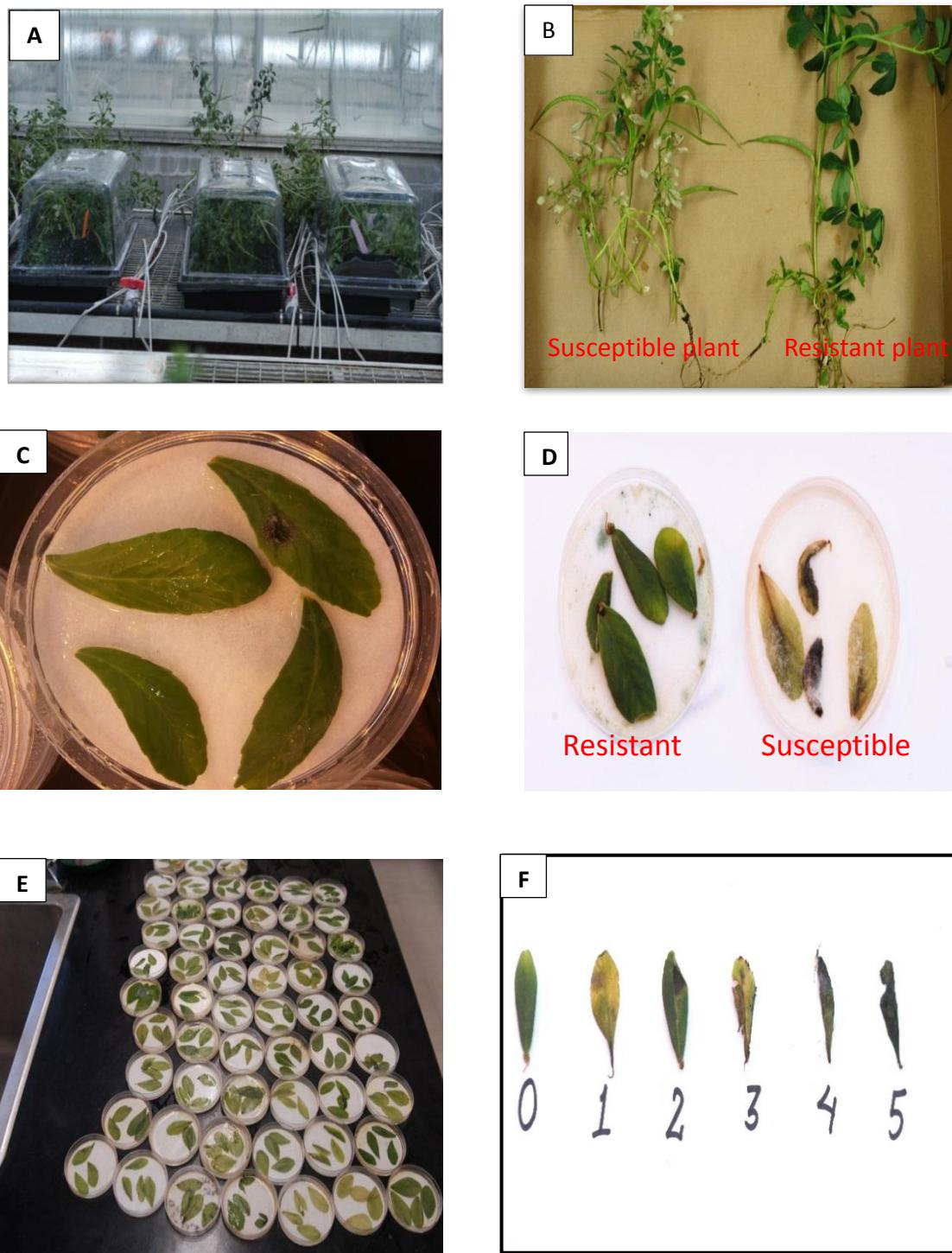
Genotypes	Mean disease score	Resistant Category	Genotypes	Mean disease score	Resistant Category	Genotypes	Mean disease score	Resistant Category
TPi-1	1.50 <sup>IJKL</sup>	R	RT-1	1.25 <sup>KLM</sup>	R	TR-1	2.75 <sup>BC</sup>	S
TPi-2	1.13 <sup>LM</sup>		RT-3	2.00 <sup>EFGH</sup>		RT-2	2.75 <sup>BC</sup>	U
TPi-3	1.86 <sup>FGHI</sup>		RT-5	2.13 <sup>DEFG</sup>		RT-4	2.75 <sup>BC</sup>	S
TPi-4	1.63 <sup>HJK</sup>		RT-6	1.50 <sup>IJKL</sup>		RT-18	2.75 <sup>BC</sup>	C
TR-2	2.38 <sup>CDE</sup>		RT-7	1.13 <sup>LM</sup>		RT-19	2.75 <sup>BC</sup>	E
TR-3	1.38 <sup>JKLM</sup>		RT-8	1.13 <sup>LM</sup>		RT-17	3.00 <sup>B</sup>	P
TR-4	1.38 <sup>JKLM</sup>		RT-9	1.88 <sup>FGHI</sup>		RT-23	3.13 <sup>B</sup>	T
PiT-1	1.75 <sup>GHJ</sup>		RT-10	2.38 <sup>CDE</sup>		Tristar	3.88 <sup>A</sup>	I
PiT-2	2.5 <sup>CD</sup>		RT-11	1.38 <sup>JKLM</sup>				B
PiT-3	2.50 <sup>CD</sup>		RT-12	1.25 <sup>KLM</sup>				L
PiT-4	1.88 <sup>FGHI</sup>		RT-13	0.63 <sup>NO</sup>				E
PiT-5	1.88 <sup>FGHI</sup>	A	RT-14	1.25 <sup>KLM</sup>	A			
PiT-6	1.88 <sup>FGHI</sup>		RT-15	2.13 <sup>DEFG</sup>				
PiT-7	2.13 <sup>DEFG</sup>		RT-16	1.75 <sup>GHJ</sup>				
PiT-8	1.75 <sup>GHJ</sup>		RT-20	1.75 <sup>GHJ</sup>				
PiT-9	1.00 <sup>MN</sup>		RT-21	2.38 <sup>CDE</sup>				
PiT-10	1.38 <sup>JKLM</sup>		RT-22	2.00 <sup>EFGH</sup>				
PiT-11	0.50 <sup>O</sup>		L3717	1.13 <sup>LM</sup>				
PiT-12	2.25 <sup>DEF</sup>		PI138687	2.000 <sup>EFGH</sup>				
Means with different superscripts are significantly different from each other ( p<0.05)								

### **4.3.3. Correlation between WPA and DLA:**

A separate correlation analysis was conducted for the disease scores obtained for all genotypes from whole plant assay and detached leaf assay (unwounded leaves kept at 25/14°C) which is presented in figure 4.2. The figure shows linear relationship between WPA and DLA with equation  $X = 0.127 + 0.968Y$  and correlation coefficient ( $r$ ) = 0.784 ( $p < 0.01$ ).



**Figure 4.2:** Correlation of mean disease score between detached leaf assay (unwounded leaves kept at 25/14°C) and whole plant assay conducted in a controlled environment at Lethbridge Research and Development Center.



**Figure 4.3:** Some glimpse of the experiment: **A**-Maintaining humidity by keeping plants along with pods in a tray containing water and covered by a transparent lid. **B**- Difference between resistant and susceptible plants in WPA 30 days after inoculation. **C**- Detached leaves in a 6cm petri plate **D**-Difference between resistant and susceptible leaves. **E**- Asymptomatic leaf samples after 45 days kept at 20°C. **F**- Disease assessment chart as a percent of leaf area infected.

#### **4.4. Discussion:**

We found that whole plant assay and the detached leaf assay can be used quickly, efficiently and reliably while screening fenugreek for resistance to *C. traversiana*. They can be used to accelerate the fenugreek breeding program without using a large space and workforce (Gurung et al. 2015; Twizeyimana et al. 2007a, b). Furthermore, alteration of environment around the plant as per required, less inoculum requirement, easier disease assessment made these methods superior over the field screening trials (Dong et al. 2017; Foolad et al. 2015). Artificial inoculation results in our experiment indicated that both methods could be used to differentiate the performance of the genotypes. In addition, the correlation coefficient ( $r = 0.784$ ,  $p < 0.01$ ) derived from the mean disease score from each genotype, between WPA and DLA (unwounded leaves kept at 25/14°C) shows that they can be used interchangeably while screening fenugreek for CLS (figure 4.3). Several other studies also have shown a good correlation between DLA and WPA and their usefulness towards differentiating plant genotypes (Hansen et al. 2005; Parke et al. 2002). Congruency of their results with field experiments also has increased their reliability. However, in our case, correlation of WPA and DLA with disease scores of CLS obtained from field trails needs to be determined to see if they mimic actual field situations.

In whole plant assay we observed no such contrasting difference between the genotypes in terms of number of lesions per leaflets and diameter of those lesions. A number of factors may have acted in development of similar sized lesions during this experiment. Firstly, most of the genotypes treated were  $F_1$ 's developed from a cross of resistant and susceptible parents which themselves were not significantly different (table 4.1) in case of AUDPC (lesion diameter). Furthermore, a single isolate of pathogen inoculated using

uniform epidemiological conditions around the plants also may be the reason behind symptom progression in a similar fashion.

Inoculum (conidia suspension) concentrations of  $2 \times 10^5$  and  $1 \times 10^7$  were successfully able to produce disease symptoms in WPA and DLA respectively with the exception of samples of DLA kept at 20/12°C temperature regime. The disease symptoms on fenugreek plants and leaves in this experiment were similar to those described in the previous study undertaken in a controlled environment (Prasad et al. 2014). Disease scores for DLA were slightly higher than WPA when quantified based on the same scoring chart (0-5). The difference presumably results from the higher concentration of conidial suspension used in DLA and favorable environmental conditions (long wetness period and high humidity) available to individual detached leaves compared to plants in WPA. In the case of WPA, a high humidity was maintained only for 7 days as compared to 28 days in DLA. Such an increase in the level of CLS severity with the increase in inoculum concentration and longer humidity period has been noticed in several experiments conducted before (Khan et al. 2009; Wallin and Loonan 1971). Furthermore, Lui et al. (2007) observed a decline in the defensive power of detached leaves as compared to intact ones. Surprisingly, the number of days for first symptoms to appear in fenugreek plants was shorter by around 8 days when whole plants were treated rather than detached leaves. A physiological difference between attached and detached leaves may be the reason behind the disparity in latent period with in the assays. However, further in-depth research is required to confirm these results.

In detached leaf assay, the samples kept under 20/12°C temperature regime were asymptomatic (more than 95%) while almost every leaf sample kept at 25/14°C readily depicted visible symptoms of CLS. The repeated trial with three parental genotypes with

a similar kind of reaction supports the result and implies a large impact of temperature on CLS severity. Several experiments carried out in various plants have identified a temperature around 25°C to be optimum for *Cercospora* species producing maximum symptoms thus yield loss (Alderman and Beute 1987; Cooperman and Jenkins 1986; Paul and Munkvold 2005). Nevertheless, such a drastic change in the appearance of CLS symptoms from 25°C to 20°C while treating whole plants has not been obtained before. Disparity of our results with studies conducted prior can be partially explained by difference in procedures because most of the studies were carried out by treating whole intact plants instead of detached leaf as we used in our study. Reports suggest physiological changes after the detachment of leaves may create differences in disease expression (Felsenstein et al. 1998; Liu et al. 2007). Furthermore, all the studies we mentioned above were conducted by providing constant temperature during day and night which may have led to uniform disease development. However, our experiments although had almost similar day temperature as others, lower temperature (12°C) at night for samples kept in temperature regime 20/12°C could be the reason why detached leaves were symptomless. *C. traversiana* may be sensitive to such temperature fluctuations which may have resulted in a asymptomatic reaction within the plant parts. However, further studies should be carried out in order to confirm if temperature fluctuations can produce discrepancies in disease expression of *C. traversiana*. In contrast to our result, Tooley et al. (2009) obtained disease symptoms at 10°C when detached leaves of *Rhododendron* were treated with *Phytophthora ramorum*. Phytophthora leaf spot being a cool season disease, *P. ramorum* is likely to be adapted in cold temperatures. However, fenugreek being typically a warm season crop, *C. traversiana* is less expected to be adapted to the cold temperatures, thus may have shown such asymptomatic reaction in

our case. A temperature vs pathogen growth study should be conducted to confirm the infectivity conditions of *C. traverisana*. All these results suggest that the same protocol of detached leaf assay may not always be applicable for screening every plant species with different species of pathogen. Setting up small trial experiments with a few samples to identify the most suited condition before starting any DLA could be helpful where knowledge about the host and the pathogen is limited.

One of the pragmatic findings of this experiment is that wounding/physical damage helps increase disease severity. Wounded leaves were affected more with a disease score of 2.04 while un-wounded leaves showed fewer symptoms with a disease score of 1.71. It has been stated that wounds facilitate easier attachment and penetration of fungal mycelia into plants (Adorada et al. 2000; Misaghi 1982). Increase in fungal disease severity due to physical damage (wounding) in different parts a of plant such as stem (Hansen et al. 2005; Sakamoto and Gordon 2006), root (Adorada et al. 2000; Stutz et al. 1985), tubers (Salas et al. 2000), fruits (Oliveira et al. 2014), leaves (De Dobbelaere et al. 2010) have been noticed. A field experiment conducted by Bradley and Ames (2010) found an increased gray leaf spot severity in corns which were physically injured by simulated hail and prevention of yield loss in damaged corn with foliar fungicide was not effective. Our result of higher CLS severity in wounded leaves can be directly related to plants in the field as physical damage to the plant especially leaves can happen through divergent means such as severe weather, insects, machines and animals; this may help farmers to undertake preventive measures against the disease.

Three fenugreek genotypes viz. Tristar (susceptible), L3717 (resistant) and PI138687 (resistant), and 43 different F1s developed by artificial hybridization of those three genotypes were used as plant material for this experiment. Most of the F1 plants showed

a lower susceptibility to CLS disease score similar to the resistant parents. This indicates the dominance of resistance over susceptibility in the case of CLS in fenugreek. However, further generation analysis is necessary to determine the number of genes involved, nature of their inheritance and heritability estimates. Those F<sub>1</sub>'s found resistant to CLS disease by DLA and WPA can be used as germplasm to broaden the genetic base of fenugreek, particularly in western Canada.

## **CHAPTER – 5**

### **EFFECTS OF HOST SUSCEPTIBILITY, PLANT AGE AND INOCULUM CONCENTRATION ON CERCOSPORA LEAF SPOT SEVERITY IN FENUGREEK.**

#### **5.1. Introduction:**

The genus *Cercospora* is a cosmopolitan and destructive group of fungal pathogens recognized to have more than 3,000 species (Pollack, 1987). A wide range of agricultural crops including cereals, legumes, vegetables, forages and tree species such as coffee and mulberry are affected by this pathogen causing significant economic loss (Ghosh et al. 2012; Hasan et al. 2016; Kimber and Paull, 2011; Mani et al. 2017; Mengistu et al. 2012; Silva et al. 2016; Westerveld et al. 2008). A single species (*Cercospora traversiana*) currently is recognized to infect fenugreek all around the world (Acharya et al. 2010; Prasad et al. 2014; Ryley 1989) but no critical studies to examine this disease have been undertaken. A few decades back, the disease was noticed to cause considerable damage to fenugreek fields in the Prairies especially in Manitoba, Canada (Zimmer 1984). No further reports of significant loss were published on this disease after that in Canada although its identification and assessment of loss were carried out in different continents (Bobev et al. 1999; Malhotra 2010; Ryley 1989; Voros and Nagy 1972). Currently, there are reports of this disease showing up again in western Canada (unpublished data). Therefore, developing suitable preventive measures such as identification of host resistance, judicial fungicide treatments or cultural manipulations to cope with the disease is expected to help avert a CLS epidemic in this region.

*Cercospora traversiana* usually infects green parts of the fenugreek plant such as leaves, green shoots, and pods (Prasad et al. 2014; Ryley 1989; Zimmer 1984) and severe

infection may produce dark and diseased seeds as well. The characteristic symptom of CLS is initiation of circular sunken lesions on the leaf surface which progressively turn to black necrotic spots surrounded by a yellowish halo (Prasad et al. 2014). If favorable conditions persist, lesions expand rapidly to coalesce with adjacent lesions resulting in severe blight followed by defoliation and disease progress to stem, pods and seeds. The fungus conidiophores protrude from the plant surface in clusters through the stomata and produce multicellular conidia which are long, slender, colorless to dark, straight to slightly curved (Acharya et al. 2010; Prasad et al. 2014). Transmission of conidia occurs from blowing wind and splashing rain. Infected seeds and plant debris are also reported in relaying the disease over the seasons (Acharya et al. 2010). Some weed species such as *Chenopodium* and *Heliotropium* can also act as a disease reservoir thus facilitating easier spread of the disease (Hasan et al. 1995; Vaghefi et al. 2017). Upon availability of a water film or high humidity around the living plant parts, conidia produce germ tubes and enter the plants through minute fissures such as stomata or wounds (Hasan et al. 1995; Weiland and Koch 2004; Bradley and Ames 2010).

Interaction between three factors primarily determines a plant disease epidemic; host characteristics, nature of the pathogen and environmental conditions available in the area (Keane and Kerr 1997). There are several reports of *Cercospora* spp causing devastation in divergent crops which are primarily attributed to lack of host resistance and presence of an extremely favorable environment (Jacobsen et. al., 2001; Kimber and Paull 2011; Mani et al. 2016; Monfort et al. 2004; Wolf and Verreet 2002, 2003). Along with the level of resistance, several other host factors such as growth stage (Lazarovits et al. 1980; Salzman et al. 1998), growth habit (Freitas 2002; Mesterházy 1995), leaf structure and

orientation (Freeman and Beattie 2008; Russell 1975) affect the disease incidence. *Cercospora spp.* caused maximum damage when temperatures ranged from 25-30°C (Alderman and Beute 1987; Carisse et al. 1993; Cooperman and Jenkins 1986; Paul and Munkvold 2005), relative humidity was > 80% (Ghosh et al. 2012; Khan et al. 2009; Windels et al. 1998), and leaf wetness duration was longer than 36 hours (Wallin and Loonan 1971; Wu et al. 1999). However, for maximum disease severity, it may necessitate that two or more environmental factors are at optimum at the same time even if a susceptible host and adequate inoculum must also be present. For the pathogen, virulence and aggressiveness, ability to adapt to a changing environment and host, mode of spread and survival efficiency are factors determining the level of disease severity (Keane and Kerr 1997).

Although several studies conducted in diverse plants have identified younger plants as being more susceptible than mature ones (Lazarovits et al. 1980; Salzman et al. 1998), the age at which fenugreek plants suffer a maximum CLS infection has not been determined. Research results for *Cercospora spp.* also show that CLS severity can easily surpass the economic threshold level under high temperature and relative humidity even in lower inoculum concentrations when a field is dominated by susceptible plants (Harveson 2013; Jacobsen et al. 2004b; Paul and Munkvold 2005). Deployment of efficient management practices is hindered due to lack of concrete information regarding host and environmental factors influencing disease severity. Furthermore, no recommendations related to resistant cultivars and fungicidal treatments for western Canadian conditions have been reported. Information regarding several factors influencing disease severity not only helps disease control through best management

practices to avoid disease progress but also helps to standardise fungicide application and to implement suitable breeding strategies (Uysal and Kurt 2017).

This study was undertaken with an objective of identifying the effects of host susceptibility, plant age and inoculum concentration in different components of CLS resistance in fenugreek. In the meantime we were able to determine a suitable inoculum concentration to be applied for differentiating resistant and susceptible genotypes through whole plant assay of fenugreek conducted in controlled environment conditions.

## **5.2. Material and Methods:**

### **5.2.1. Host plants:**

Two fenugreek genotypes L3717 (resistant) and L3721 (highly susceptible) were used for all experiments (Prasad et al. 2014). Plants were raised in plastic pots 10 cm × 10 cm × 12 cm filled with soil mixture containing fine-sand, soil and Cornell mix (Basu et al. 2008) in equal proportions. Three seeds per pot were sown which were later thinned to two healthy plants per pot two weeks after emergence. Temperature in the greenhouse was maintained at 22°C during the day and 14°C at night. Photo period was adjusted to 16 hours. Pots were watered regularly as per requirement.

### **5.2.2. Experimental design:**

A 2×3×4 factorial RCBD with 6 replications was considered for the experiment where treatment was comprised of two fenugreek lines (L3717 and L3717), three growth stages (20 DAS, 40 DAS and 60 DAS) and four inoculum concentrations ( $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$  conidia/ml). Each pot containing two plants was considered the experimental unit, while the two plants within each pot were considered to be subsamples. Data obtained from those two plants were averaged for several parameters measured which represented a single experimental unit. Seeds were sown at 20 days interval to obtain plants at three growth stages during the time of inoculation. At the time of inoculation, 20-day-old plants were at the vegetative growth stage, 40-day-old plants were at flowering, and 60-day-old plants were at the pod filling stage. Six extra pots containing two plants for each plant age were kept as control.

### **5.2.3. Inoculum preparation and inoculation:**

A frozen vial of live pure culture of *Cercospora traversiana* (isolate - 1M1 318080) was used for sub culturing which was initially obtained from CAB International (CABI), United Kingdom. Initially, for multiplication of inoculum, the frozen inoculum was streaked into a PDA plate and incubated in darkness at room temperature. After 21 days, sub culturing from same media plate was done into several PDA plates and incubated at room temperature maintaining darkness for 28 days. A spore suspension was prepared in distilled deionized (dd) water with a few drops of tween 20 (non-ionic surfactant) added into it. Conidial suspensions of four different concentrations ( $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  conidia/ml) were prepared in four different beakers after counting conidia in the suspension with the help of haemocytometer. The suspensions were then sprayed in mist form with the help of hand-held sprayers until runoff from each leaf in the plant. Inoculated plants were covered with transparent polyethylene bags immediately after inoculation to allow infection by maintaining humidity around the plants. Control plants were sprayed with distilled water and covered with polyethylene bags as other treatments. After 3 days, polythene bags were removed and the plants were kept in the greenhouse set as before. Temperature and lighting were constant as described in section 5.2.1. Plants were sprayed twice a day (morning and evening) with tap water.

### **5.2.4. Disease assessment:**

Inoculated plants were observed daily after five days for symptoms expression. Disease scoring was done based on the scale of 0-9 where; 0 = no infection; 1 = 1 to 5% leaves infected; 2 = 6 to 10% leaves infected, 3 = 11 to 20% leaves infected; 4 = 21 to 30% leaves infected; 5 = 31 to 40% leaves infected; 6 = 41 to 50% leaves infected; 7 = 51 to

60% leaves infected; 8 = 61 to 80% leaves infected and 9 = 81 to 100% leaves infected.

First disease scoring was done after seven days of inoculation and thereafter three more scorings were done in 7 days intervals. Areas under disease progress curve (AUDPC) values were calculated using following formula;

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left\{ \frac{Y_i + Y_{i+1}}{2} \right\} \times (T_{i+1} - T_i)$$

Where,

$Y_i$  = disease severity on the  $i^{\text{th}}$  date,

$T_i$  = time on which  $Y_i$  was recorded and

$n$  = number of times observations were taken.

Defoliation was calculated by expressing the number of completely dead and fallen leaves as a percentage of the number of leaves present initially (one day before inoculation) at pre-marked section which was measured on the stem between 10 to 20 cm above the soil surface.

Pod infection was calculated by expressing the number of pods showing disease symptoms as a percentage of total number of pods formed till maturity.

Mature plants were cut 2 cm above the soil surface and air dried at 60°C for 2 days. Seed yield was obtained by separating and weighting the dry seeds. Seed yield loss was calculated by deducting seed yield of the disease treated plants from the average seed yield of control plants and was expressed as a percentage of average seed yield of the control plants. Forage yield was the remaining biomass weight after the removal of seeds. Percentage of forage yield loss was calculated in a similar manner as seed yield loss.

### **5.2.5. Data analysis:**

Data for different parameters were analyzed using PROC MIXED (SAS Institute, Inc., 2010) with host susceptibility, plant age and inoculum concentration as fixed factors and replication as a random factor. Treatment mean comparisons were made using Tukey's Honestly Significant Difference test and the probability level was set at 5% level. Linear and non-linear models were tested on AUDPC- inoculum concentration relationships for two genotypes in three different plant ages. Based on the significance of the models ( $p < 0.05$ ) and coefficient of determination ( $r^2$ ) values, non-linear (cubic) equations ( $y = ax^3 + bx^2 + cx + d$ ) were chosen to best fit the relationship. Relationships between different parameters were modeled using regression analysis.

### **5.3. Results:**

Visible symptoms of CLS in fenugreek initiated after about 9 days with the appearance of sunken spots on the lower leaves which progressed towards upper leaves gradually. Sunken spots developed into blackish lesions 1-3mm diameter in size which later coalesced later and invaded whole leaves mostly in susceptible plants. Defoliation of leaves and infection of pods and stems were evident with advancement of the disease. Symptoms were almost similar as described by Prasad et al. (2014). Confirmation of the disease was performed by observing conidia obtained from infected leaves under a compound microscope. Conidia were solitarily placed, multicellular, long and slender, slightly curved, translucent and darkish. Each of the parameters responded differently according to the level of resistance present in plants, their age and inoculum concentration sprayed over them.

Plants kept as control exhibited very low disease incidence (<5%) with no defoliation and pod infections thus were not considered for data analysis. Effects of host susceptibility, plant age and inoculum concentration and their interactions on various CLS components were closely observed by analysing them separately.

**Note:** Significant means  $p < 0.05$  and non-significant means  $p > 0.05$ .

**Table 5.1:** Analysis of variance (ANOVA) conducted for various components of CLS resistance.

Sources	P-value					
	AUDPC	FDA	% Defoliation	% Pod infection	% Seed yield loss	% Forage yield loss
<b>Genotype <sup>a</sup></b>	<.0001	<.0001	<.0001	<.0001	<.0001	0.0096
<b>Stage <sup>b</sup></b>	<.0001	NS	NS	<.0001	<.0001	NS
<b>Genotype×Stage</b>	<.0001	NS	<.0001	<.0001	<.0001	NS
<b>Concentration<sup>c</sup></b>	<.0001	<.0001	<.0001	<.0001	<.0001	0.0263
<b>Genotype×Concn</b>	<.0001	0.0309	<.0001	0.0035	<.0001	NS
<b>Stage×Conc</b>	<.0001	NS	NS	<.0001	<.0001	NS
<b>Genotype×Stage×Concn</b>	0.0030	NS	NS	<.0001	<.0001	NS

FDA- Days for first disease appearance

AUDPC- Area Under Disease Progress Curve

NS- Non Significant ( $P > 0.05$ )

<sup>a</sup> Two genotypes: L3717 and L3721 were used.

<sup>b</sup> Three fenugreek growth stages: 20DAS, 40DAS and 60 DAS were used.

<sup>c</sup> Four conidia concentrations:  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  were used.

### **5.3.1. Area under disease progress curve (AUDPC):**

Results of the analysis of variance (ANOVA) for AUDPC after treating fenugreek plants with different levels of *C. traversiana* conidial concentration in various aged plants showed a significant difference among host genotypes, plant age, inoculum concentration, genotype × plant age, genotype × inoculum concentration, plant age × inoculum concentration and genotype × plant age × inoculum concentration interactions (table 5.1). Comparison of means between 24 different treatments for disease severity (AUDPC) in fenugreek plants is presented in table 5.2. AUDPC for susceptible plants (52.5) was significantly higher than the resistant plants (31.8) regardless of the plant age and inoculum concentration. In both resistant and susceptible genotypes, disease progress was significantly slower at  $10^3$  conidia/ml as compared to three higher concentrations, although a statistical difference was obtained for each concentration level. This suggests that an inoculum concentration of  $10^3$  conidia/ml may not be sufficient to generate full symptoms within the plants and there was no significant difference between genotypes at this concentration. AUDPC was highest in 60-day-old plants and lowest in 20-day-old plants in both genotypes for all conidial concentrations applied (table 5.2). This depicted increasing trend of disease severity with increase in plant age. Maximum AUDPC value was obtained for 60-day-old susceptible plants treated with  $10^6$  conidia/ml (85.75) while, minimum AUDPC value was obtained for 20-day-old resistant plants treated with  $10^3$  conidial/ml suspension (16.33).

**Table 5.2:** AUDPC values of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

Area Under Disease Progress Curve (AUDPC) values.								
Concentration (Conidia/ml)	L3717 (Resistant)				L3721(Susceptible)			
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean
<b>10<sup>3</sup></b>	16.33 b, y	18.08 d, y	21.00 c, x	<b>18.47 C</b>	25.08 c, y	25.38 c, y	30.63 d, x	<b>27.03 C</b>
<b>10<sup>4</sup></b>	27.13 a, z	36.17 b, y	43.46 b, x	<b>35.58 B</b>	42.86 b, z	56.86 b, y	68.54 c, x	<b>56.10 B</b>
<b>10<sup>5</sup></b>	27.13 a, z	41.70 a, y	47.54 a, x	<b>38.79 A</b>	41.42 b, z	67.38 a, y	77.00 b, x	<b>61.93 A</b>
<b>10<sup>6</sup></b>	28.29 a, y	30.92 c, y	43.75 b, x	<b>34.32 B</b>	48.13 a, z	60.96 b, y	85.75 a, x	<b>64.94 A</b>
<b>Stage Mean</b>	<b>24.72 Z</b>	<b>31.72 Y</b>	<b>38.94 X</b>	<b>31.79 (II)</b>	<b>39.38 Z</b>	<b>52.65 Y</b>	<b>65.48 X</b>	<b>52.5 (I)</b>

DAS= Days after sowing

Numbers in columns (AUDPC) followed by different letters (a, b, c and d) for each concentration differed significantly at  $p < 0.05$  within a plant age.

Means (AUDPC) in columns followed by different letters (A, B and C) for each concentration differed significantly at  $p < 0.05$ .

Numbers in rows (AUDPC) followed by different letters (x, y and z) for each plant age differed significantly at  $p < 0.05$  within an inoculum concentration.

Means (AUDPC) in rows followed by different letters (X, Y and Z) for each plant age differed significantly at  $p < 0.05$ .

Means (AUDPC) for two different genotypes differed significantly at  $p < 0.05$  and are separated by letters I and II.

### **5.3.2. First disease appearance (Incubation Period):**

Only genotype, inoculum concentration, and interaction between them had a significant effect on incubation period (table 5.1). Disease appeared earlier in susceptible genotypes (9.6 days) as compared to resistant genotypes (11.2 days) irrespective of inoculum concentration and plant age (table 5.3). Incubation period didn't differ significantly with plant age while conidial concentration had a marginal but significant effect. Latest disease appeared in the resistant genotype (12.4 days) when inoculated with a suspension of  $10^3$  conidia/ml and it was a significantly longer period than the other three higher conidial concentrations with no significant differences among themselves for incubation period. In contrast, earliest disease appeared in the susceptible genotype (8.8 days) when treated with a suspension of  $10^6$  conidia/ml which was significantly different from the other three lower conidial concentrations which were all similar to each other (table 5.3).

**Table 5.3:** Incubation period (number of days) of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

First Disease appearance/Incubation period (number of days)								
Concentration (Conidia/ml)	L3717 (Resistant)				L3721(Susceptible)			
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean
<b>10<sup>3</sup></b>	12.83	12.50	12.00	<b>12.44 A</b>	10.17	10.50	10.00	<b>10.22 A</b>
<b>10<sup>4</sup></b>	10.83	10.67	11.00	<b>10.83 B</b>	9.50	9.83	9.67	<b>9.67 A</b>
<b>10<sup>5</sup></b>	11.17	11.00	10.67	<b>10.94 B</b>	9.67	9.83	9.33	<b>9.61 A</b>
<b>10<sup>6</sup></b>	10.33	11.50	10.17	<b>10.67 B</b>	8.67	8.67	9.00	<b>8.78 B</b>
<b>Stage Mean</b>	<b>11.29</b>	<b>11.42</b>	<b>10.96</b>	<b>11.22 (II)</b>	<b>9.50</b>	<b>9.70</b>	<b>9.50</b>	<b>9.57 (I)</b>

DAS= Days after sowing

Means (number of days for first disease appearance) in columns followed by letters A and B for each concentration differed significantly at p < 0.05.

Mean (number of days for first disease appearance) for two different genotypes differed significantly at p < 0.05 and are separated by letters I and II.

### **5.3.3. Defoliation:**

Resistant (37.3%) and susceptible (60.8%) genotypes differed significantly for defoliation percentage while plant age didn't show any effect on it (table 5.4). However, genotypes and plant age showed a significant interaction effect. In the resistant genotype, highest defoliation (41.37%) was obtained when plants were treated 60 DAS which differed significantly with plants treated while they were in earlier stages (20 and 40 DAS). In contrast, defoliation within the susceptible genotype was lowest (56.71%) at 60 DAS as compared to 20 and 40 days old plants with 62.86 % defoliation combined. There was a significant difference in defoliation among the plants treated with different inoculum concentrations regardless of genotype and plant age. In resistant genotype, conidial concentration  $\geq 10^4$  produced a similar effect for defoliation and they were significantly higher than defoliation caused by conidial concentration of  $10^3$  conidia/ml. However, in the susceptible genotype, highest defoliation was recorded when plants were treated with a concentration of  $10^6$  conidia/ml which differed significantly from  $10^5$  and  $10^4$  conidia/ml. Lowest defoliation was obtained when plants were treated with a concentration of  $10^3$  conidia/ml.

**Table 5.4:** Defoliation (%) for CLS affected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

Concentration (Conidia/ml)	Defoliation percentage							
	L3717 (Resistant)				L3721(Susceptible)			
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean
10 <sup>3</sup>	27.42	30.17	27.30	<b>28.30 B</b>	45.73	39.74	37.69	<b>41.05 C</b>
10 <sup>4</sup>	35.26	37.43	45.67	<b>39.45 A</b>	60.95	68.24	64.40	<b>64.53 B</b>
10 <sup>5</sup>	36.63	38.91	47.99	<b>41.18 A</b>	66.18	73.37	58.43	<b>65.96 B</b>
10 <sup>6</sup>	36.74	39.87	44.50	<b>40.37 A</b>	71.16	77.50	66.33	<b>71.66 A</b>
<b>Stage Mean</b>	<b>34.01 Y</b>	<b>36.56 Y</b>	<b>41.37 X</b>	<b>37.32 (II)</b>	<b>61.00 X</b>	<b>64.71 X</b>	<b>56.71 Y</b>	<b>60.80 (I)</b>

DAS= Days after sowing

Means (% Defoliation) in columns followed by different letters (A, B and C) for each concentration differed significantly at p < 0.05.

Means (% Defoliation) in rows followed by letters X and Y for plant age differed significantly at p < 0.05.

Mean (% Defoliation) for two different genotypes differed significantly at p < 0.05 and are separated by letters I and II.

### **5.3.4. Pod Infection:**

Analysis of variance (ANOVA) revealed that the two genotypes, three different plant ages and four conidial concentrations differed significantly for pod infection within their respective treatments regardless of the other two treatments. Furthermore, significant differences were observed among all possible interactions viz: genotype  $\times$  plant age, genotype  $\times$  inoculum concentration, plant age  $\times$  inoculum concentration and genotype  $\times$  plant age  $\times$  inoculum concentration (table 5.1). Pod infection was significantly higher in susceptible genotypes (48.55%) compared to resistant genotypes (33.19%). 60-day old plants in both genotypes for all inoculum concentrations showed a significantly higher pod infection than 40-day old plants. Lowest pod infection was recorded when plants were 20 days old. This shows that application of inoculum in the reproductive stage of fenugreek leads to the highest pod infection. The highest pod infection difference between resistant and susceptible genotypes was apparent when the plants were 60 days old (~ 24%). Inoculum concentrations  $\geq 10^4$  conidia/ml produced similar results for pod infection at all three plant ages for both resistant and susceptible genotypes which produced a significantly higher infection as compared to  $10^3$  conidia/ml sprayed into the plants. Maximum pod infection was obtained for 60-day-old susceptible plants treated with  $\geq 10^4$  conidia/ml while minimum pod infection was obtained for 20 days old resistant plants treated with  $10^3$  conidia/ml.

**Table 5.5:** Pod infection (%) of CLS infected fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

Pod infection percentage									
Concentration (Conidia/ml)	L3717 (Resistant)				L3721(Susceptible)				Concentration Mean
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean	
<b>10<sup>3</sup></b>	7.12 b, z	17.03 b, y	43.21 b, x	<b>22.45 B</b>	10.79 c, z	41.85 b, y	55.19 b, x	<b>35.94 C</b>	
<b>10<sup>4</sup></b>	17.59 a, z	41.78 a, y	52.12 a, x	<b>37.16 A</b>	20.66 b, z	52.12 a, y	80.05 a, x	<b>50.94 B</b>	
<b>10<sup>5</sup></b>	12.38 a, z	43.91 a, y	52.59 a, x	<b>36.30 A</b>	33.57 a, z	53.97 a, y	80.05 a, x	<b>55.86 A</b>	
<b>10<sup>6</sup></b>	14.78 a, z	43.62 a, y	52.12 a, x	<b>36.84 A</b>	19.33 b, z	55.39 a, y	79.63 a, x	<b>51.45 B</b>	
<b>Stage mean</b>	<b>12.97 Z</b>	<b>36.59 Y</b>	<b>50.01 X</b>	<b>33.19 (II)</b>	<b>21.09 Z</b>	<b>50.83 Y</b>	<b>73.73 X</b>	<b>48.55 (I)</b>	

DAS= Days after sowing.

Numbers in columns (% Pod Infection) followed by different letters (a, b, c and d) for each concentration differed significantly at  $p < 0.05$  within a plant age.

Means (% Pod Infection) in columns followed by different letters (A, B and C) for each concentration differed significantly at  $p < 0.05$ .

Numbers in rows (% Pod Infection) followed by different letters (x, y and z) for each plant age differed significantly at  $p < 0.05$  within an inoculum concentration.

Means (% Pod Infection) in rows followed by different letters (X, Y and Z) for each plant age differed significantly at  $p < 0.05$ . Means (% Pod Infection) for two different genotypes differed significantly at  $p < 0.05$  and are separated by letters I and II.

### **5.3.5. Seed yield loss:**

Genotype, plant age, inoculum concentration and all possible interactions from them had a significant effect on seed yield loss incurred after disease treatment (table 5.1). As anticipated, susceptible plants suffered more seed yield loss (36.95%) than resistant ones (23.09%) regardless of plant age and inoculum concentration. Seed yield loss was significantly higher when the inoculum was applied 60 DAS followed by 40 DAS. Least seed yield loss was obtained when plants were 20 days old. In resistant genotype, 20 and 40-day old plants were no different in terms of seed yield loss; however, significant differences were observed at all three plant ages for seed yield loss in susceptible genotype. In both genotypes, for all three plant ages, no significant difference in seed yield loss was observed when inoculated with  $10^3$  conidia/ml while conidial concentrations  $\geq 10^4$  produced a significant difference. Irrespective of genotype and plant age, conidial concentrations  $\geq 10^4$  produced a similar result for seed yield loss which was significantly higher than  $10^3$  conidia/ml. All three different aged plants of susceptible genotype and 60-day-old plants from resistant genotype followed the same trend, but all four concentrations produced no difference when applied to 20 and 40-day-old resistant plants. This collectively shows that with increasing inoculum densities and plant age, the seed yield in susceptible genotype seems to be more strongly affected than in the resistant genotype. There was also strong relationship between pod infection and seed yield loss ( $r^2 = 0.78$ ,  $p < 0.0001$ ) regardless of plant age and inoculum applied in the susceptible genotype. Resistant genotype however showed a lower correlation ( $r^2 = 0.15$ ,  $p < 0.0009$ ) between pod infection and seed yield loss (figure 5.1.).

**Table 5.6:** Seed yield loss (%) for fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

Seed yield loss percentage								
Concentration (Conidia/ml)	L3717 (Resistant)			L3721(Susceptible)				
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean
<b>10<sup>3</sup></b>	20.18 a, x	19.94 a, x	13.38 b, x	<b>17.83 B</b>	14.51 a, x	19.25 b, x	19.70 b, x	<b>16.58 B</b>
<b>10<sup>4</sup></b>	20.10 a, y	20.31 a, y	30.53 a, x	<b>23.95 A</b>	15.59 a, z	39.65 a, y	75.03 a, x	<b>43.42 A</b>
<b>10<sup>5</sup></b>	21.98 a, y	22.83 a, xy	29.69 a, x	<b>24.83 A</b>	14.94 a, z	40.64 a, y	74.64 a, x	<b>43.41 A</b>
<b>10<sup>6</sup></b>	21.55 a, y	23.65 a, y	32.02 a, x	<b>25.74 A</b>	15.52 a, z	40.33 a, y	77.34 a, x	<b>44.40 A</b>
<b>Stage Mean</b>	<b>21.18 Y</b>	<b>21.68 Y</b>	<b>26.40 X</b>	<b>23.09 (II)</b>	<b>15.14 Z</b>	<b>34.97 Y</b>	<b>61.68 X</b>	<b>36.95 (I)</b>

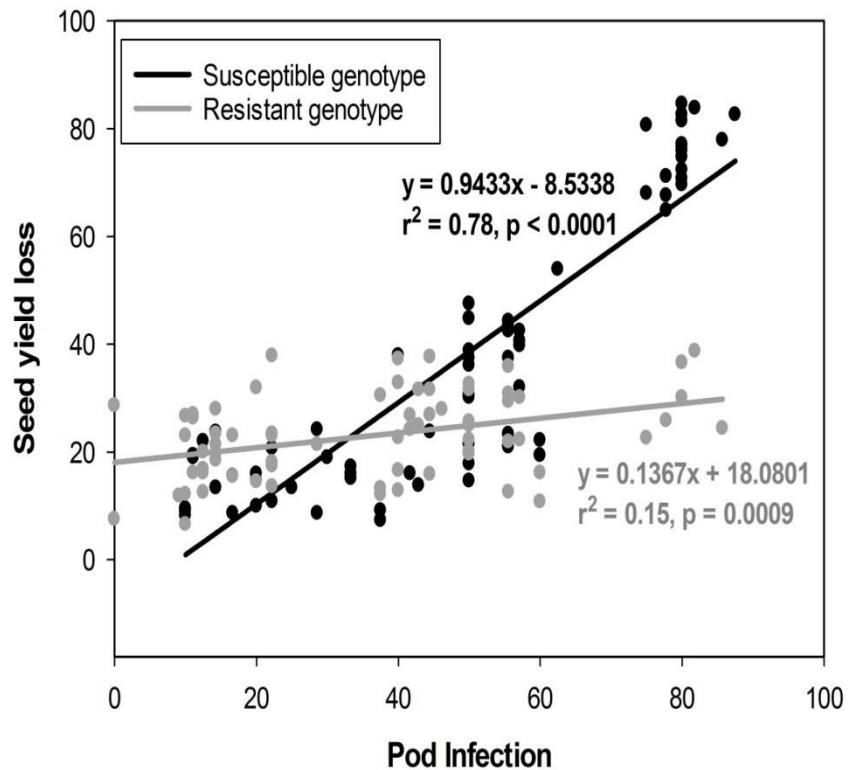
DAS= Days after sowing

Numbers in columns (seed yield loss %) followed by letters a and b for each concentration differed significantly at  $p < 0.05$  within a plant age.

Means (seed yield loss %) in columns followed by letters A and B for each concentration differed significantly at  $p < 0.05$ .

Numbers in rows (seed yield loss %) followed by different letters (x, y and z) for each plant age differed significantly at  $p < 0.05$  within an inoculum concentration.

Means (seed yield loss %) in rows followed by different letters (X, Y and Z) for each plant age differed significantly at  $p < 0.05$ . Means (seed yield loss %) for two different genotypes differed significantly at  $p < 0.05$  and are separated by letters I and II.



**Figure 5.1:** Relationship of percent seed yield loss of fenugreek to percent pod infection due to Cercospora leaf spot for resistant and susceptible genotypes.

### **5.3.6. Forage Yield loss:**

Only the genotypes and inoculum concentration showed an effect on forage yield loss while plant age and all possible combinations had no significant effect. Susceptible plants (25.08%) suffered a higher forage yield loss compared to resistant plants (20.75%). When data were divided into resistant and susceptible genotypes, all four conidial concentrations applied to susceptible genotypes led to a similar forage yield loss, while in resistant genotypes inoculum concentrations of  $10^3$  and  $10^6$  conidia/ml only differed from each other. All three plant ages for both genotypes did not differ for forage yield loss.

**Table 5.7:** Forage yield loss (%) for fenugreek plants as affected by genotypes, plant ages and inoculum concentrations.

Forage yield loss in percentage								
Concentration (Conidia/ml)	L3717 (Resistant)				L3721(Susceptible)			
	20 DAS	40DAS	60DAS	Concentration Mean	20DAS	40DAS	60DAS	Concentration Mean
<b>10<sup>3</sup></b>	14.07	14.87	20.09	<b>16.34 B</b>	22.06	19.81	21.23	<b>21.03</b>
<b>10<sup>4</sup></b>	19.18	21.26	24.79	<b>21.74 AB</b>	26.39	28.93	25.51	<b>26.94</b>
<b>10<sup>5</sup></b>	20.68	21.16	22.05	<b>21.30 AB</b>	25.57	28.33	21.88	<b>25.26</b>
<b>10<sup>6</sup></b>	21.31	19.76	29.74	<b>23.60 A</b>	28.27	25.34	27.65	<b>27.09</b>
<b>Stage Mean</b>	<b>18.81</b>	<b>19.26</b>	<b>24.17</b>	<b>20.75 (II)</b>	<b>25.57</b>	<b>25.60</b>	<b>24.07</b>	<b>25.08 (I)</b>

DAS= Days after sowing.

Means (forage yield loss %) in columns followed by different letters (A and B) for each concentration differed significantly at p < 0.05.

Mean (forage yield loss %) for two different genotypes differed significantly at p < 0.05 and are separated by letters I and II.

#### **5.4. Discussion:**

In this study, effect of host resistance, plant growth stage, and concentration of inoculum was investigated on whole fenugreek plants under greenhouse conditions. Each of those factors acted independently or in combination to cause variations in different components of disease severity such as incubation period, AUDPC, defoliation, pod infection, seed yield loss and pod yield loss. Visible symptoms of CLS in our experiment appeared between 9 and 13 days depending on the host characteristics and inoculum concentration. This incubation period is in congruency with earlier growth chamber experiment carried out by Prasad et al. 2014 where first symptoms of *C. traversiana* infection appeared around 10 days post inoculation when fenugreek plants were treated with a conidial suspension of  $10^4$  conidia/ml. Symptoms depicted by *C. traversiana* infected fenugreek plants in this study were also similar to those described by previous researchers. (Acharya et al. 2010; Prasad et al. 2014; Ryley 1989; Zimmer 1984).

This study showed that plant genotypes with a high level of resistance can incur less disease than susceptible ones, even in the presence of a favorable infection environment and high disease pressure. In the resistant genotype L3717, the incubation period was longer than for the susceptible genotype L3721 at every plant stage treated with four different inoculum concentrations (table 5.3). In contrast, disease score (AUDPC), pod infection, defoliation, seed yield loss and forage yield loss were notably higher in susceptible genotypes compared to resistant genotypes. This shows that the presence of host resistance can suppress CLS development thereafter reducing the proportion of affected plant parts. Apparent infection rate (disease progress) of CLS caused by *C. beticola* was lower by almost 50% in resistant sugar beet cultivars compared to susceptible ones (Rossi 1995). Delayed CLS onset and slower disease progress was

evident in resistant cultivars of carrot, celery, peanut, sugarbeet and strawberry (Berger 1976; Gaurilcikiene et al. 2006; Kaiser et al. 2010; Nathalie et al. 1995; Rossi 1995; Waliyar et al. 1993; Lacy et al. 1996; Westerveld et al. 2008; Wolf and Verreet 2005). Several resistance components such as longer incubation period, lower frequencies of infection, smaller lesion diameter, and reduced density of lesions could be the reasons behind resistant cultivars being less affected by CLS than susceptible ones (Rossi et al. 1999). In our case, only the incubation period for *C. traversiana* was assessed which followed a similar trend to that of other *Cersospora* spp.

In general, earlier growth stages of the plant suffer more from diseases than the later stages (Lazarovits et al. 1980; Salzman et al. 1998). However, in our experiment, increase in disease severity (AUDPC value) with the plant age was observed for both genotypes and all inoculum concentrations applied (table 5.2). This result contradicts earlier studies that show more disease tolerance of mature plants compared to young plants and other studies suggesting susceptibility increases with plant age (Canova 1959; Rossi et al. 1990; Schneider et al. 1976; Vloutoglou and Kalogerakis 2000; Weiland and Koch 2004). No exact cause for susceptibility of mature plant/leaves towards the fungal pathogen has been identified so far; however; sometimes passive mechanisms such as reduction in thickness of the epicuticular wax layer in mature leaves (Tewari and Skoropad 1976) or increase in stomatal density in mature leaves (Gwinn et al. 1987; Paul and Munkvold 2005) can facilitate easy entry of pathogens into plants and leave plants at vulnerable state. Large differences between the AUDPC value in matured and younger plants in this study were also caused by less availability of a secondary inoculum that can be produced and transmitted from the fenugreek plant. Most of the leaves infected

through primary inoculum (directly sprayed suspension inoculum) defoliated at all growth stages; however, arrival of fresh leaves in 20-day-old plants was more rapid than in the mature plants and those leaves were without spots or had fewer spots. This led to lowering the disease score as time passed and thereafter the AUDPC value in 20-day-old plants. This may be because of insufficient moisture on the leaf surface or relative humidity in the environment after the removal of the plastic cover used and transfer of plants to normal greenhouse conditions. Reduced secondary cycle of *Cercospora* sps within the plants under unfavorable condition is not unexpected (Hasan et al. 1995; Khan et al. 2009; Paul and Munkvold 2005). Nevertheless, the circumstances for plants grown in field conditions may be different because of continuous availability of primary inoculum throughout the growing period and abundance of secondary inoculum under favorable environmental conditions (Paul and Munkvold 2005). This may produce slightly different results than we observed in our greenhouse experiment. Furthermore, resumption of disease progress was evident after the availability of a favorable environment for various *Cercospora* sps (Paul and Munkvold 2005; Rathaiah 1977), but confirmation of such behavior in *C. traversiana* needs further experimentation.

Lower pod infection in 20-day-old plants also supports the reduced availability of secondary inoculum in our experiment. Higher pod infection led to a higher seed yield loss in 40 and 60-day-old plants which can be related to the inoculum directly sprayed on to the pods which caused more lesions to appear. In congruency with our results, Schuh (1992) in a greenhouse experiment observed pods infected by *Cercospora kikuchii* in soybean when plants were sprayed with a suspension inoculum in the small and large-pod stages. However, he found no pod infection when plants were in the flowering stage.

Pod infection in this study was the most important component of disease severity because of its strong relationship ( $r^2 = 0.78$ ,  $p < 0.0001$ ) with seed yield loss regardless of plant age and inoculum applied in the susceptible genotype. Resistant genotype however showed a lower correlation ( $r^2 = 0.15$ ,  $p < 0.0009$ ) between pod infection and seed yield loss. This is because of less seed infected in the resistant genotype although they depicted some symptoms in the pods. No such significant correlation was observed between other parameters. It was anticipated that high defoliation would cause high forage yield loss, but we observed no significant difference in forage yield loss for three different plant ages although a difference in defoliation was evident. This may be because of the small size and light weight of fenugreek leaves which did not have a large effect on forage yield loss.

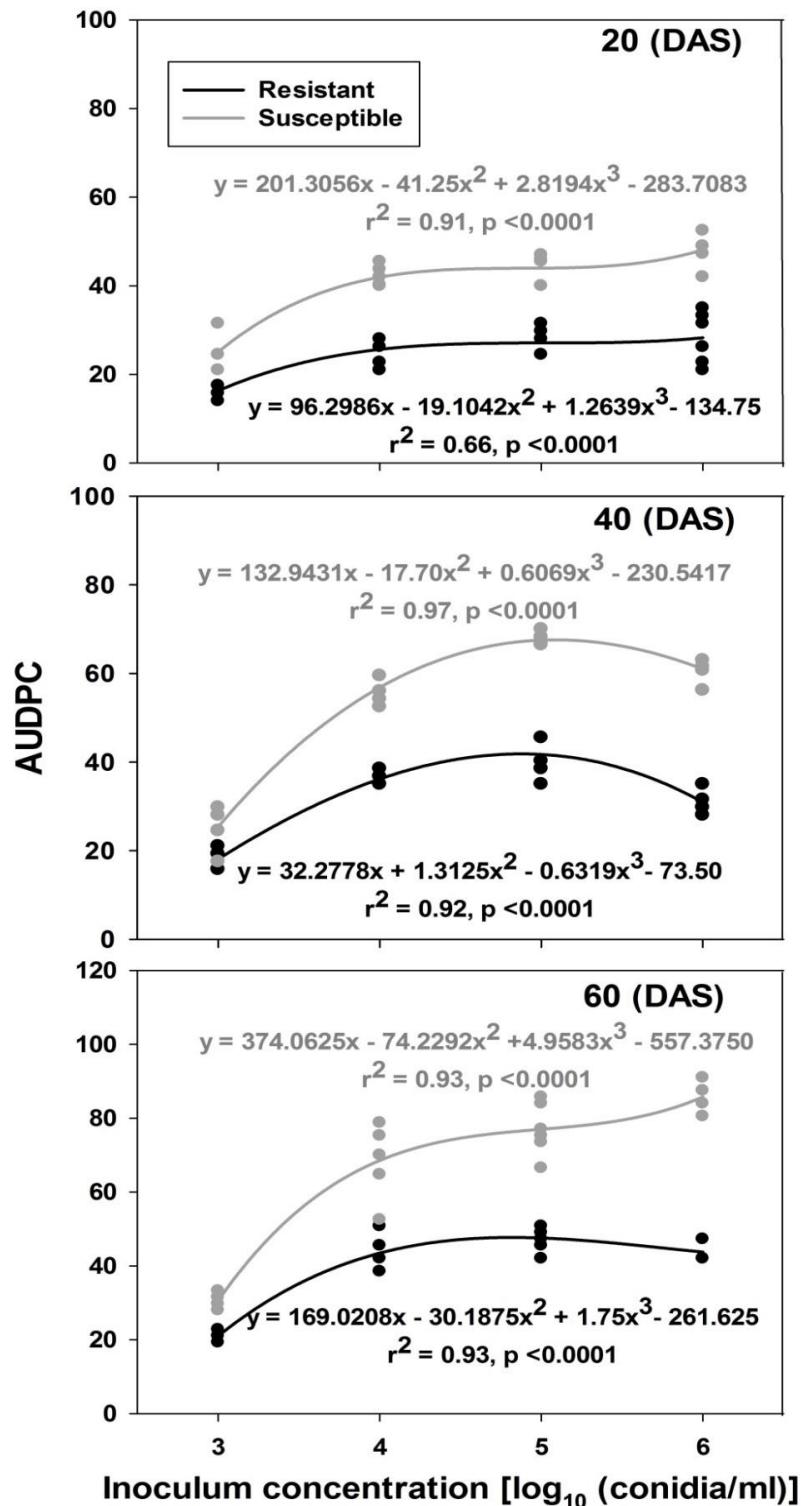
As has been reported previously (Hasan et al. 1995; Khan et al. 2009; Pundhir and Mukhopadhyay 1987; Tedford et al. 2018), inoculum concentration in this study also affected disease severity along with other parameters that were accessed. Regression model for the effect of inoculum concentration on disease severity (AUDPC) of *C. traverisana* tends to follow a nonlinear (cubic) trend for every plant age in both genotypes (Figure 5.2). Conidial concentration of  $10^3$  conidia/ml produced some symptoms, but it was discovered as not sufficient to develop symptoms to a full extent even with a favorable environment around the fenugreek plants. This is supported by AUDPC values of  $10^3$  conidia/ml which didn't allow sufficient distinction between resistant and susceptible genotypes (table 5.2). However, with the increase in level of inoculum concentration ( $\geq 10^4$  conidia/ml), disease severity for both resistant and susceptible plants was almost concurrent with what was previously described by Prasad

et al. (2014). A rapid rise in disease severity was observed when inoculum concentration increased from  $10^3$  to  $10^4$  conidia/ml while disease severity stayed almost constant or slightly increased depending upon the genotype and plant age when the inoculum increased from  $10^4$  to  $10^6$  conidia/ml. This suggests that a conidial concentration of at least  $10^4$  conidia/ml is required for *C. traversiana* to cause sufficient infection in fenugreek to produce symptoms in order to differentiate between resistant and susceptible genotypes against *C. traversiana* in greenhouse conditions. Prasad et al. (2014) also used  $10^4$  conidial/ml while screening 53 fenugreek accessions against *C. traversiana* in a growth chamber with similar temperature conditions and were successfully able to categorize those accessions based on their degree of resistance. Hasan et al. (1995), who tested *Heliotropium* (a weed species) against *C. heliotropii-bocconii*, also observed an inoculum concentration of  $10^4$  was able to kill the plants within one month and drastically reduced plant survival. An inoculum concentration  $\geq 10^4$  conidia/ml also showed an increasing trend with a marginal difference for defoliation, seed yield loss, and forage yield loss, while there were inconsistent results for incubation period and pod infection which may have arisen due to interaction effects and variability in microenvironment among the replicates. Results from this study only indicate optimum inoculum concentration under controlled environmental conditions in a greenhouse and may or may not be applicable for field conditions when artificial inoculation is done. Development of optimum inoculum under field condition will require a separate set of field experiments.

Interactions of factors for several parameters were evident in our experiment. AUDPC, pod infection and seed yield loss were higher in 60-day-old plants compared to other

younger plants even when inoculum concentrations were low ( $\sim 10^4$  conidia/ml) in both genotypes excluding the concentration of  $10^3$  conidia/ml. Similarly, 60-day-old resistant plants had more pod infection (50.01%) than 20-day-old susceptible plants (21.09%) and almost similar pod infection as compared to 40-day-old susceptible plants (50.83%). This practically implies that CLS infestation in the pod filling stage of fenugreek can be detrimental even if the field is dominated by resistant plants which may demand few fungicide applications before severe damage is incurred.

This study was undertaken in the greenhouse to identify the most vulnerable growth stage of fenugreek to *C. traversiana* infection and quantify the fungus optimal inoculum concentration required to differentiate fenugreek plants based on the degree of CLS resistance they possess. Several components of CLS resistance such as AUDPC, incubation period, defoliation, pod infection, seed and forage yield loss were assessed. This study identified for the first time that CLS severity in fenugreek is strongly dependent on the degree of resistance present in a host, their age and inoculum concentration. However, disease epidemics also depend on several other factors such as temperature, relative humidity, leaf wetness period that were kept constant in our experiment. We do not yet have sufficient understanding of how variation in these factors affects CLS severity. Therefore, other greenhouse and field experiments are deemed necessary in order to broaden the knowledge base on *C. traversiana* and thus can help in predicting disease epidemiology in fenugreek fields.



**Figure 5.2:** Non-linear (cubic) relationship between AUDPC values with inoculum concentration [ $\log_{10}$  (conidia/ml)] for resistant and susceptible genotypes 20, 40 and 60 days after sowing (DAS).

## **CHAPTER-6**

### **SUMMARY AND FUTURE DIRECTIONS**

#### **6.1. Summary:**

Fenugreek is a new crop to western Canada whose integration into the cropping system can provide new agronomic as well as economic opportunities to the farmers. Tristar, one of the five cultivars of fenugreek developed in Canada, is gaining popularity for its high yielding nature and dryland adaptations. However, Tristar and other cultivars were categorised as susceptible to Cercospora leaf spot (CLS), a disease which causes severe yield loss through foliar damage, defoliation, and pod and seed infections. Furthermore, limited knowledge about the epidemiology of CLS has also hindered deployment of efficient management practices. Therefore, this research project was employed for developing new germplasms of fenugreek through artificial hybridization of Tristar with resistant plant introductions (L3717 and PI138687), and evaluation of their resistance to Cercospora leaf-spot and their adaptation to western Canada growing conditions. In addition to that, identifying various epidemiological factors affecting CLS severity was our second objective. The mechanism of CLS resistance was determined for the first time in fenugreek. Results from our research show that CLS is governed by a single dominant gene suggesting easier gene transfer from parents to progenies. Likewise, narrow sense heritability was moderate indicating early generation selection to be fruitful while undertaking CLS resistant breeding projects. This information will be helpful for future researchers to develop an efficient breeding strategy.

Epidemiological studies conducted in the greenhouse revealed that various factors such as temperature, physical damage, host susceptibility, plant age and inoculum

concentration are important in determining the proportion of disease within a plant. A longer incubation period, lower disease severity and reduced seed and forage yield loss was evident in resistant plants as compared to susceptible ones even when the environment was favorable for disease proliferation showing importance of the resistant cultivars especially when leaf spot disease epidemic is forecasted. Physical damage (wounding) was also found to enhance CLS. Increasing concentrations of *Cercospora traversiana* inoculum corresponded with increasing severity of CLS in fenugreek. Nevertheless, an inoculum concentration of  $10^4$  conidia/ml was efficient for differentiating fenugreek genotypes in greenhouse conditions. Mature plants in our experiment depicted more symptoms than younger ones and they incurred more pod infection and seed yield loss. All this information can be utilised while developing suitable CLS control methods. To sum up, this study has provided a valuable information for future researchers as well as fenugreek producers in understanding the genetics behind CLS resistance and the factors that may be exploited to reduce CLS occurrence in fenugreek.

## **6.2. Future directions:**

Although, great progress is being made in understanding the suitable agronomical practices for fenugreek all over the world, there are still many unknowns regarding pathological problems of fenugreek especially Cercospora leaf spot which is of concern as this disease has already proven to be a serious threat to fenugreek producers. Prasad et al. (2014) identified two potential genotypes; L3717 and PI138687, as a source of CLS resistance genes in fenugreek. We determined that CLS resistance in those genotypes is governed by a single dominant gene which is moderately heritable. This result can be

confirmed by modern molecular techniques. Furthermore, identification of phenotypic markers as well as molecular markers which are closely related to CLS resistance can aid in the selection process. The qualitative nature of resistance observed in our study implies incorporation of CLS resistance in adopted cultivars can be carried out with relatively easier pathways, but there always remains a risk that new virulent races of pathogens may arise which may again render the genotype susceptible to disease. Alternatively, for long-term solution, studies can be undertaken to identify several new sources of genes conferring CLS resistance in fenugreek and along with the two potential sources that we identified, they can be pyramided within a single cultivar. The heritability estimates in our study were calculated through greenhouse experiments with uniform environmental condition. Confirmation of our estimates can be done through more detailed observations after subjecting plants to CLS inoculum in field situations. Moreover, further generation analysis other than F<sub>2</sub> generation can be done to obtain more accurate heritability estimates.

We have identified the suitability of WPA and DLA while screening fenugreek genotypes for *C. traversiana* under greenhouse conditions. It is, however important to investigate if the results of both methods corroborate with the results obtained from field screenings. Congruency of their results will increase their reliability of screening fenugreek genotypes, thereby save huge space, time, money and workforce required as compared to field screenings.

Through greenhouse experiments, we have reported physical damage, plant age, host susceptibility and inoculum concentration to affect CLS severity in fenugreek. These results can be further validated through field experiments which can mimic actual

situations that farmers face in the field. In addition to that, there are several other factors such as temperature, relative humidity, leaf wetness period that affect CLS severity in fenugreek. Greenhouse house, as well as field experiments, can be set up to standardize such factors thus provide some valuable information for CLS forecasting. In a situation of limited knowledge about CLS and its causing agent *C. traversiana*, there are divergent avenues that need to be explored which can help characterize the disease and suggest some promising solutions.

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## APPENDICES

**Appendix-1:** First disease appearance in detached leaves of 46 different genotypes irrespective of state of leaves (wounding and un-wounding).

Genotype	FDA <sup>a</sup> (days)	Letter Group <sup>b</sup>	Genotype	FDA (days)	Letter Group
TPi-1	18.625	H I J K L M N	R T - 4	19.125	E F G H I J K L M
TPi-2	20.625	B C D E F G H	R T - 5	18.875	F G H I J K L M N
TPi-3	20.750	B C D E F G	R T - 6	20.750	B C D E F G
TPi-4	19.250	E F G H I J K L M	R T - 7	19.000	E F G H I J K L M N
TR-1	18.000	J K L M N O	R T - 8	17.250	M N O
TR-2	19.000	E F G H I J K L M N	R T - 9	19.000	E F G H I J K L M N
TR-3	17.250	M N O	R T - 10	19.875	C D E F G H I J
TR-4	17.000	N O	R T - 11	19.250	E F G H I J K L M
PiT-1	17.500	L M N O	R T - 12	18.625	H I J K L M N
PiT-2	21.000	B C D E	R T - 13	21.375	B C D
PiT-3	20.750	B C D E F G	R T - 14	20.125	C D E F G H I
PiT-4	16.125	O	R T - 15	18.625	H I J K L M N
PiT-5	19.125	E F G H I J K L M	R T - 16	19.500	D E F G H I J K L
PiT-6	21.875	B C	R T - 17	17.000	N O
PiT-7	17.375	M N O	R T - 18	17.625	K L M N O
PiT-8	20.125	C D E F G H I	R T - 19	18.500	I J K L M N
PiT-9	24.000	A	R T - 20	19.000	E F G H I J K L M N
PiT-10	16.000	O	R T - 21	19.625	D E F G H I J K
PiT-11	19.250	E F G H I J K L M	R T - 22	20.875	B C D E F
PiT-12	18.750	G H I J K L M N	R T - 23	18.375	I J K L M N
R T - 1	22.625	A B	L 3717	20.375	C D E F G H I
R T - 2	18.500	I J K L M N	P I 138687	20.625	B C D E F G H
R T - 3	18.625	H I J K L M N	Tristar	19.000	E F G H I J K L M N
Standard error = 0.75903 LSMean = 2.09308 a = First disease appearance b = FDA values with different letter groups are significantly different from each other (p<0.05)					

**Appendix-2:** Mean and range for different morphological traits of 43 F<sub>2</sub>-families along with three parents (Tristar, L3717 and PI138687) when grown in LRDC field.

F <sub>2</sub> -Family	Flowering time		Maturity time		Plant Height		No. of Branches		No. of pods		Seed yield		Forage yield	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
<b>TPi-1</b>	42-47	44.2	117-122	118.6	45.5-67.4	53.12	6-8	7	43-65	52.8	17.3-35.2	35.2	29.8-42.4	35.5
<b>TPi-2</b>	42-47	44	117-123	120	39.3-68.3	52.78	5-8	7	27-67	49	25.3-55.1	55.1	35.4-84.3	60.5
<b>TPi-3</b>	44-47	45.4	112-122	117.8	41.6-65.8	52.3	4-8	6	23-65	44.6	27.4-54.4	51.4	36.9-50.3	43.6
<b>TPi-4</b>	42-45	43.6	116-122	118.8	46.3-63.5	51.4	4-9	6.2	32-76	49	18.3-53.4	53.4	35.6-56.8	46.0
<b>TR-1</b>	35-42	39	96-109	101.9	37.6-48.2	44.08	4-9	5.5	34-54	42.2	26.9-41.3	41.3	35.4-69.3	51.0
<b>TR-2</b>	36-43	40.2	95-108	99.1	37.4-56.4	45.42	4-8	5.6	33-86	52.6	17.4-45.6	45.6	33.5-63.5	47.7
<b>TR-3</b>	37-42	39.4	99-116	104.2	35.7-55.8	42.39	5-7	6	35-67	51.4	15.3-65.3	65.3	36.5-71.5	56.0
<b>TR-4</b>	37-40	38.6	96-108	101.6	35.8-47.3	40.98	4-11	6.4	23-54	39.8	32.5-61.3	61.3	30.1-52.3	41.5
<b>PiT-1</b>	42-44	42.4	111-123	115.3	34.1-59.4	45.22	5-11	8.6	35-35	58.6	32.5-66.3	66.3	65.395.3	79.9
<b>PiT-2</b>	43-46	44.2	107-119	112.6	34.1-65.3	48	5-9	7.2	3-76	48.6	14.4-55.4	55.4	49.4-76.1	67.5
<b>PiT-3</b>	40-46	43	113-121	116.2	39.1-53.1	44.62	5-10	7.8	34-87	66	25.4-54.2	54.2	48.4-99.1	79.4
<b>PiT-4</b>	43-47	44.3	106-117	110.7	41.2-56.4	50.88	5-11	7.8	32-98	57	19.4-63.4	63.4	45.4-90	66.1
<b>PiT-5</b>	41-41	44	108-117	112	40.1-58	50.38	5-9	7.2	45-86	64	25.4-52.2	52.2	39.0-75.6	52.9
<b>PiT-6</b>	40-45	42.8	103-116	110	39.5-53.9	45.66	6-9	7.4	59-94	72.8	24.4-52.4	52.4	36.9-94.1	63.4
<b>PiT-7</b>	41-47	43.1	109-117	113	37.6-51	43.96	5-9	7.3	48-87	68.2	14.5-54.3	54.3	25.4-63.7	46.9
<b>PiT-8</b>	40-45	41.8	111-117	112.8	38.5-52.1	44.96	6-11	8	45-69	60	27.4-45.6	45.6	56.6-94.3	75.6
<b>PiT-9</b>	41-45	42.8	113-121	117	48.3-62.4	53.08	7-9	8	56-96	71.2	32.2-56.1	56.1	36.8-94.3	63.7
<b>PiT-10</b>	41-46	43.1	111-121	118.2	41.2-59	50.48	7-13	10.4	56-107	85.8	31.2-44.3	44.3	43.7-98.3	71.0

<b>PiT-11</b>	41-47	43.8	107-116	111.4	42.5-56.3	48.8	6-11	9	45-78	66.4	13.3-55.4	55.4	36.8-86.0	67.5
<b>PiT-12</b>	40-45	42	110-119	113.4	38-56.6	44.36	7-9	8	56-96	71.2	22.5-44.7	44.7	45.7-99.4	75.3
<b>RT-1</b>	35-42	37.4	97-114	104.7	34.8-56.8	43.9	4-8	6.4	45-88	62.8	19.4-55.6	55.6	36.5-75.9	51.9
<b>RT-2</b>	39-44	40.6	98-106	102.6	26.9-42.6	35.68	6-8	6.8	45-45	60	12.4-45.0	45.0	42.6-55.6	48.0
<b>RT-3</b>	34-39	35.2	100-114	105.6	34.7-49.7	40.86	4-8	6.2	32-76	57	28.4-65.8	65.8	36.5-85.7	52.9
<b>RT-4</b>	34-40	35.8	94-105	100	36.5-51.9	43.96	5-7	5.8	24-54	43.2	24.9-63.1	63.1	29.9-62.6	42.0
<b>RT-5</b>	33-41	36	96-106	102.8	34.9-43.7	38	3-7	4.8	31-46	39.2	16.3-66.5	66.5	28.4-94.3	56.4
<b>RT-6</b>	35-44	38.8	99-105	102.2	31.2-55.2	39.64	2-7	4.4	23-44	37	36.2-88.4	88.4	36.2-81.3	58.9
<b>RT-7</b>	34-42	36.7	96-107	101.4	25.9-51.2	36.86	4-5	4.4	32-54	41.2	34.7-49.7	49.7	35.6-65.8	47.1
<b>RT-8</b>	35-42	37.4	100-114	105.8	34.5-38.4	36.46	4-8	5.6	23-54	39.4	34.6-61.4	61.4	44.5-97.7	71.0
<b>RT-9</b>	36-44	40.6	98-111	104.8	35.1-44.9	39.52	4-7	5.2	34-62	47.8	41.3-55.3	55.3	49.4-82.0	66.3
<b>RT-10</b>	34-43	38.4	97-110	102.4	39.7-49.4	45.16	3-7	4.8	31-46	39.2	17.4-46.3	46.3	35.4-76.6	54.1
<b>RT-11</b>	36-41	38.2	98-106	102	36.8-56.4	43.78	4-9	6.4	34-56	44.8	28.6-52.4	52.4	49.6-85.6	65.4
<b>RT-12</b>	35-44	39.6	98-104	101.8	38.8-51.9	44.42	5-8	6	22-56	40	25.3-46.6	49.6	44.7-74.3	61.9
<b>RT-13</b>	36-44	40.6	97-106	100.2	37.5-55.1	46.36	4-8	6.2	43-79	59.6	36.5-62.9	62.9	39.3-75.6	56.1
<b>RT-14</b>	36-41	38.2	98-104	99.9	35.1-55.9	42.98	4-9	7	43-88	62.4	24.4-52.4	52.4	36.3-95.6	62.2
<b>RT-15</b>	35-43	39.4	95-104	98.2	38.8-57.7	48.78	5-8	6.2	35-64	51	14.4-48.7	48.7	36.9-98.6	62.6
<b>RT-16</b>	37-43	39	99-106	102.4	38.4-49.5	43.84	4-9	6.6	43-78	53.6	23.7-65.5	65.5	42.4-98.7	63.7
<b>RT-17</b>	34-43	38	98-107	103	37.4-55.1	43.64	4-7	5.6	44-71	54.4	19.7-47.3	47.3	46.9-91.3	64.5
<b>RT-18</b>	36-43	38.6	100-117	110	25.946.1	38.38	3-9	5.4	43-67	54.4	31.5-39.5	39.5	44.7-75.6	57.0

<b>RT-19</b>	34-41	37.2	97-108	103.4	36.4-56.4	43.2	4-6	5	34-75	49.8	18.6-42.4	42.4	49.5-74.6	61.9
<b>RT-20</b>	33-41	36.6	100-108	104.2	29.4-52.8	39.14	4-8	6.4	42-85	54.2	22.5-65.3	65.3	46.9-74.4	61.2
<b>RT-21</b>	34-41	37.4	98-109	103	36-56.4	44.28	6-8	7.2	23-54	40.6	35.2-51.3	51.3	47.6-65.5	54.4
<b>RT-22</b>	35-42	38.8	99-106	103	31.7-55.4	41.94	3-8	5.6	21-50	38.6	14.0-44	44.0	32.0-45.4	36.9
<b>RT-23</b>	36-44	39.8	98-102	99.6	38.4-56.4	44.2	5-8	6.4	41-81	61.4	15.4-42.4	42.4	25.4-53.0	42.1
<b>Tristar</b>	42-44	43	119-124	122.2	45.5-68.2	54.34	7-9	8	64-88	76.2	36.9-55.6	55.6	49.4-97.4	79.9
<b>PI138687</b>	45-47	46.6	114-121	118	42.5-57.6	50.196	5-8	6.2	54-68	61.6	34.8-46.3	46.3	61.9-86.2	75.2
<b>L3717</b>	35-36	35.8	97-100	98.4	34.1-44.2	39.26	3-6	4.4	44-64	54.2	37.5-43.2	43.2	34.5-55.4	44.6