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Identification of high seed yielding and stable fenugreek mutants

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IDENTIFICATION OF HIGH SEED YIELDING AND STABLE FENUGREEK MUTANTS

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Abstract

Fenugreek (*Trigonella foenum-graecum* L.) was recently introduced to western Canada as a forage crop. To reach its full potential, high yielding, early maturing fenugreek cultivars that produce good seed yield and quality within 100 frost free days need to be developed. In this study, mutation breeding approach was used on five locally adapted fenugreek genotypes to generate variants showing improved seed yield and yield attributing traits that can be used for cultivar development. Mutant generations of these plants were evaluated in multi-location, multi-year trials, and individual plants were selected for high seed and biomass yield. Seeds from a tetraploid fenugreek line and its diploid parent Tristar were grown under multiple environments to understand effect of environment on seed oil content. In addition, mold resistant fenugreek genotypes were identified by screening a collection of fenugreek accessions against a destructive fungal pathogen *Cercospora traversiana*.

Dedication

To my Parents.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
AB	Alberta
ADF	Acid detergent fibre
ANOVA	Analysis of Variance
Ba	Barium
BC	British Columbia
Br	Bromine
Ca	Calcium
CDCS	Crop Diversification Centre South
cm	centimeter
Co	Cobalt
CP	Crude Protein
Cu	Copper
CV	Coefficient of Variation
DF	Degrees of Freedom
DNA	Deoxyribonucleic Acid
EMS	Ethyl Methane Sulphonate
Fe	Iron
g	gram
GPS	Global Positioning System
ha	hectare
HSD	Honestly Significant Difference

IR	Irrigation/Irrigated
kg	kilogram
Leth	Lethbridge
LRC	Lethbridge Research Centre
LR-NMR	Low Resolution Nuclear Magnetic Resonance
m	meter
M	Molar
m^3	cubic meter
MCPB	Methyl Phenoxy Butanoic Acid
Mg	Magnesium
Mn	Manganese
Na	Sodium
NDF	Neutral Detergent Fibre
^oC	Degree Celsius
p	Probability
PC 1	First Principal Component
PC 2	Second Principal Component
PDA	Potato-Dextrose Agar
PGRC	Plant Genetic Resources of Canada
r	Correlation coefficient
RF	Rain-fed
SE	Standard Error
SEM	Scanning Electron Microscopy

USA	United States of America
Zn	Zinc

Chapter One: Introduction

1.1 Introduction

The annual crop fenugreek (*Trigonella foenum-graecum* L.) is a member of the family *Fabaceae* (formerly known as *Papilionaceae*) within the order *Leguminosae*. It is a self-pollinating dicotyledonous plant with branched stems, trifoliate leaves, which bears white flowers and produces golden yellow seeds (Acharya et al. 2010a; Petropoulos 2002). Although fenugreek cultivation is mostly concentrated in Asia and the Mediterranean region, it is now widely cultivated in northern Africa, central Europe, North America and Australia (Fotopoulos 2002).

Fenugreek is primarily used as a spice in countries where it is grown (Acharya et al. 2006). Especially in India and countries in the Mediterranean regions both seed and leaves of fenugreek are widely used as a culinary spice to enhance the taste of many meat, poultry and vegetable dishes (Mary 2009). Seeds and leaves of fenugreek are well characterized with a distinctive, pungent scent that has made it highly desirable in culinary applications (Max 1992). The seed is frequently used in Indian sub-continental cuisine as an ingredient of various curry powders, and in the preparation of pickles, and pastes (Srinivasan 2006). Fenugreek seed is the main condiment in Yemen and the Arabian Gulf (Weiss 2002). In south Asia and Egypt young leaves and sprouts of fenugreek are eaten as green vegetables while dried leaves are used to flavor soups and curries (Duke 1981). Adding fenugreek leaves in dishes is a tradition of Iranian cooking. In Ethiopian and Eritrean cuisine the plant is used as a supplement in wheat and maize flour for bread making while in India fenugreek seed powder is one of the ingredients in

making a specialty type of bread (Leela and Shafeekh 2008; Al-Habori and Raman 2002). Fenugreek plant products are used more for coloring food than flavoring in China. Leafy stems of fenugreek are ground to produce an organic powdered food color product to color steamed pastries (Hu 2005).

Fenugreek is also grown for forage. It is regarded as traditional forage in Egypt, India, Turkey and the Mediterranean region (McCormick et al. 2009). Many researchers have suggested that it has been used extensively in the past as hay, green fodder and silage, and as a supplement with other animal feed (Smith 1982; Hardman 1969; Rouk and Mangesha 1963). In addition fenugreek, mixed with cottonseed is fed to weaning cows to increase flow of milk (Hidvegi et al. 1984). It is also used to mix with inferior hay and sour hay (mildewed hay) to increase palatability (Petropoulos 2002). It is recommended as alternative leguminous forage in alfalfa based cattle farms since it can prevent bloating in cattle which is a disadvantage associated with use of alfalfa fodder (Acharya et al. 2007). Fenugreek is reported to provide similar rumen conditions, digestibility and weight gain in cattle in comparison to alfalfa (Mir et al. 1998). Acharya et al. (2008) stated that fenugreek forage yield is identical to two cuts of alfalfa. As well, it is capable of retaining its quality profile throughout the season.

Fenugreek has a long history of use as a medicinal herb. It is extensively used in both Indian Ayurvedic medicines and traditional Chinese medicines (Tiran 2003). In herbal medicine it is used in the treatment of diabetes (Leela and Shafeekh 2008). The crop species has long been used as a galactogogue to promote lactation in weaning mothers and to promote weight-gain in women (Rgubi and Belahsen 2006; Tiran 2003). In early times, it has been used to get diverse medicinal benefits that include wound-

healing, aid in digestion, treatment of sinus and lung congestion, inflammation and infection mitigation, hair treatment, bust enhancement and aphrodisiac effects (Leela and Shafeekh 2008; Tiran 2003). Advances in nutraceuticals and demand for phytonutraceuticals and functional foods have renewed interest in fenugreek as a functional food. These have led to identification of specific health benefits of this novel crop through extensive research and clinical trials (Acharya and Thomas 2007). Health benefits that can be obtained using fenugreek comprise anti-inflammatory (Langmeade et al. 2002), anti-carcinogenic (Raju and Bird 2006; Amin et al. 2005), anti-nociceptive (Hibasami et al. 2003; Sur et al. 2001); antioxidant (McCue and Shetty 2003; Langmeade et al. 2002), anti-microbial (Thomas et al. 2006), anti-ulcer (Pandian et al. 2002), anti-obesity (Handa et al. 2005), anti-hyperglycemic (Ruby et al. 2005; Basch et al. 2003), anti-diabetic (Saxsena and Vikram 2004) and hypocholesterolemic (Basch et al. 2003) effects. Most of the laboratory studies and clinical trials have focused on three bioactive compounds found in fenugreek namely galactomannan, diosgenin and 4-hydroxyisoleucine. The results of these studies indicate that most health benefits of fenugreek can be attributed to these three key biochemical components.

Fenugreek as a crop provides other environmental, economic and social benefits (Acharya et al. 2011; Moyer et al. 2003). It is a dry-land adaptive crop which can reduce water requirement during cultivation. It can passively prevent contamination of ground water and soil run-off by irrigation water. As a legume it binds nitrogen in root nodules. Incorporation of the plant into the soil after harvest can serve as a nitrogen source for subsequent crops; thus fenugreek also can lower the need for application of nitrogenous

fertilizers in the field; it can successfully be used in short term rotations to help maintain soil productivity (Acharya et al. 2008; 2006; Moyer et al. 2003).

The fixed oil in fenugreek seed has a tenacious aroma. Fenugreek oil is used in flavoring for many canned food and syrups. Artificial maple syrup is flavored with fenugreek seed extract as it imitates a maple syrup smell (Acharya et al. 2008). It also has attracted the interest of the perfume trade. It is considered as a secret ingredient in some French perfumes (Petropoulos 2002).

Since 1992, fenugreek has been grown commercially in western Canada. Initially it was introduced to Canada for its use as a spice and "AC Amber" was the first Canadian fenugreek cultivar developed by Agriculture and Agri-Food Canada, Manitoba. Currently five fenugreek cultivars are being grown commercially in Canada. "Tristar", developed by Agriculture and Agri-Food Canada, Lethbridge, is the most recent cultivar developed among the five and, was the first fenugreek cultivar developed for forage use (Basu et al. 2009). It is well adapted to semi-arid prairie regions of western Canada and can produce a high biomass yield. However, the released cultivars and adapted fenugreek germ plasm are unable to support consistent high quality seed production in temperate climates. As a crop originating from tropical regions, fenugreek is reported to mature in 130-140 days under tropical climate conditions. Although "Tristar" is a good cultivar for use as forage, it does not consistently produce a high quality and quantity of seeds when grown in western Canada where, on average, there are only 100 frost free days available for cultivation of the plant; *i.e.*, "Tristar" requires about 120 days to produce mature seed in the temperate climate found in western Canada.

Most fenugreek have an indeterminate growth habit where the plants grow continuously at the shoot apex and keep producing new shoots, flowers and seed pods that mature slowly (Basu *et al.* 2009). However, some fenugreek plants can exhibit a determinate growth habit where the shoot apices fail to branch and redirect plant resources into flowering and seed development resulting in early plant maturation. In this study, mutation breeding was used to generate mutants with increased population variability that can be used to select for early maturity and a determinate growth habit. Five germplasms adapted for growth in western Canada, namely AC Amber, F70, F80, F86 and Tristar were treated with EMS to produce mutant plants that can be used for cultivar improvement. It was hypothesized that production of a diverse group of mutant plants with a range of genetic backgrounds will better facilitate development of new fenugreek cultivars, than previous mutant breeding studies that employed only a single cultivar of the plant.

Increase in ploidy levels of somatic chromosomes often results in an increase in plant vigour, size of the plant and seeds due to larger cell size (Fehr 1987). Basu (2006) treated “Tristar” fenugreek with colchicine. He reported that the tetraploid plants produced had larger leaves, a longer pod length, vigorous growth and larger seeds in comparison of untreated “Tristar” plants. In my study it is hypothesized that as the seeds of the tetraploid plants are larger in size than the diploid plants, the seeds may contain more oil than the seeds of diploid plants. If these tetraploid seeds are found to contain more oil, the tetraploid plants may be used to develop a cultivar suitable for commercial use for its oil.

Fenugreek crop is affected by many pathogenic fungi. Among the fungal diseases of fenugreek, Cercospora leaf spot disease caused by *Cercospora traversiana* is considered one of the most serious, destructive and widespread disease. Although this disease is not a yield limiting factor in Canada, the disease has been reported in Canada in a previous study (Zimmer 1984). The author also reported that the pathogen inflicted yield up to 80% in that year. As fenugreek is gaining more recognition in Canada and other countries where it is introduced recently, the acreage of fenugreek will eventually increase in these countries. With the increases of acreage the pathogen may prevail in these areas. So, identification of resistant genotypes may be very useful to develop tolerant cultivars in future.

Objectives of this research study project were:

i. To produce more variability in the populations through mutation breeding using five adapted germplasm and characterization of the mutant in their early generations.

ii. To advance the mutant generations and their evaluation in multi-location trials.

iii. To select plants on the basis of their phenotype from different mutant generations to generate new potential lines.

iv. To assess the oil content in seeds of tetraploid plants.

v. To screen for resistant/tolerant genotypes against *Cercospora traversiana* among the fenugreek world accessions, and to re-characterize the disease.

1.2 Background

1.2.1 Taxonomy of Fenugreek

Fenugreek (*Trigonella foenum-graecum* L.) is a diploid, annual plant that is strongly scented. Vasil'chenko (1953), placed the genus "*Trigonella"* within the family *Leguminosae* (currently known as *Fabaceae*). Heywood (1967), Hutchinson (1964) and Sinskaya (1961) also described fenugreek as part of this family. Hutchinson (1964) described the genus "*Trigonella"* as one of the six genera of the subfamily *Trifoliae* of the family *Fabaceae* within the order *Leguminales.* Tutin and Heywood (1964) have investigated the genus *Trigonella* and divided it into three subgenera, namely *Trigonella, Trifoliastrum* and *Foenum-graecum* and, placed fenugreek under the subgenus *Foenum-graecum*. Though the scientific name *Trigonella foenum-graecum* (L.) is well established now for fenugreek, in much of the published literature it was addressed with different synonyms for its scientific name; e.g., Mathé (1975) identified nine synonyms for *Trigonella foenum-graecum* (L.); *i.e., Buceras foenum-graecum* (L.), *Foenum-graecum sativum* Medik, *Foenum-graecum officinale* Moench, *Foenum-graecum officinale* spp. *cultum* Alef, *Folliculigera graveolens* Pasq, *Medicago foenu-graeca* Ehz Krause, *Telis foenum-graecum* (L.) O.ktze, *Trigonella graeca* St. Lag. non Boiss and *Trigonella ensifera* Trautv (Petropoulos 2002).

The taxonomic rank of fenugreek is given below:

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

1.2.2 Origin and centre of diversity of fenugreek

Fenugreek is a diverse species. Authors have widely debated the probable ancestry of *Trigonella foenum-graecum* (L.), although the divergent schools of opinion identify three probable centers of origin for the plant (Acharya et al. 2008). Vavilov (1951, 1926) suggested that fenugreek originated from the Mediterranean region. However, according to Fazli and Hardman (1968), and De Candolle (1964) fenugreek has an Asian/Indian center of origin. Dangi et al. (2004) also proposed that the species originated in Turkey. Collections of fenugreek from different countries have been made for the purposes of taxonomic investigation and characterization. Results of these studies have revealed other probable centers of diversity for fenugreek; e.g., Serpukhova (1934)

proposed that Yemen and Abyssinia are centers of diversity for fenugreek, while Moschini (1958) suggested that Sicily, Tuscany and Morocco are centers of diversity for fenugreek. In another study, Yemen, the Transcaucasia region of Eurasia, Africa, Afghanistan, the China-Iran region, and India also have been proposed as diversity centers for fenugreek (Furry 1950).

The exact number of species of the genus *Trigonella* is also unsettled. Petropoulos (2002) indicated that Linneaus suggested that about 260 species of fenugreek exist, while 97 species and 128 species have been described by Fazli (1967) and Vasil'chenko (1953) respectively. Hutchinson (1964), and Rouk and Mangesha (1963) placed about 70 species under the *Trigonella* genus. This decline in specie's number between the 18th and 20th centuries suggests that many species of *Trigonella* may have been lost due to lack of conservation and species domestication. Recently, Acharya et al. (2008) suggested that a total of 18 different currently recognized species of fenugreek may still exist.

1.2.3 Regions of cultivation

Fenugreek is now cultivated in all habitable continents of the world. Some of these continents have a long history of use, while other continents only started cultivating the crop during the past 2-3 decades. Asia is positioned in 1st place among continents in terms of fenugreek production and acreage with India leading in fenugreek seed production, producing about 90% of the world fenugreek grown (Acharya et al. 2008, 2007). Among other Asian countries; Iran, Israel, China and Pakistan also have high levels of production. Asia is followed by the continent of Africa in terms of fenugreek production and acreage as well as richness in genetically distinct fenugreek germplasm

(Petropoulos 2002). In Africa, fenugreek production is mostly concentrated in Egypt, Ethiopia, Kenya and Morocco. Spain, Turkey, Greece and Germany are notable for cultivation of fenugreek in Europe, whereas Argentina is important for production in South America. Within the literature Italy has a history of cultivation of fenugreek for use as fodder, although a limited amount of fenugreek cultivation currently is practiced in this country (Fazli and Hardman 1968; Moschini1958). In Canada fenugreek has been cultivated commercially for the past two decades and, most of its cultivation is concentrated in prairie regions of western Canada (Acharya et al. 2010b, 2008, 2007). It is also a new crop to the USA and different accessions have been evaluated for cultivation in the mid-western United States (Berti et al. 1993). McCormick et al. (2009, 2006) have reported cultivation of fenugreek as a minor crop in south-eastern regions of Australia since the mid 1980s.

Fenugreek is known by its English name in countries where fenugreek is introduced as a new crop in recent years. But interestingly it has retained its local name(s) in countries where it has been cultivated for many years. Therefore, presence of a local name for fenugreek may be an indication of a long history of use for the plant in a respective country (Petropoulos 2002). Names of countries that cultivate fenugreek as a crop along with its local names are presented in Table 1.1.

Table 1.1. Countries involved in cultivation of fenugreek and the local names used to identify it.

Name of continent	Name of country of cultivation	Local name(s) of fenugreek
Africa	Egypt	Hulba, Hulabah, Hhelbah, Hhelbeh
	Ethiopia	Abish
	Kenya	Hulba, Hulabah, Hhelbah, Hhelbeh
	Morocco	Hulba, Hulabah, Hhelbah, Hhelbeh
	Sudan	Hulba, Hulabah, Hhelbah, Hhelbeh
	Tanzania	Hulba, Hulabah, Hhelbah, Hhelbeh
	Tunisia	Hulba, Hulabah, Hhelbah, Hhelbeh
Asia	Bangladesh	Methi
	China	K'u-Tou, Hu Lu Ba
	India	Methi
	Iran	Schemlit
	Israel	
	Japan	Koroba, Koroha
	Lebanon	Hulba, Hulabah, Hhelbah, Hhelbeh
	Pakistan	Methi
	Russia	Pazhitnik, Pazsitnyik, Grezsezki szeno
	Turkey	Çemen
Ocenia	Australia	
Europe	Austria	
	England	
	France	Fenugrec, Senegre
	Germany	Griechisch Heu, Griechisches Heu, Bockshornklee, Kuhhornklee, Bisamklee
	Greece	Trigoniskos, Tsimeni, Tintelis, Moschositaro, Tili, Tipilina
	Portugal	Alforva
	Russia	Pazhitnik, Pazsitnyik, Grezsezki szeno
	Spain	Alholya
	Switzerland	Bockhornsklover
	Ukraine	
North America	Canada	
	United States of America	
South America	Argentina	

Source: Acharya et al. 2008; Berti et al. 1993; McCormick et al. 2006; Petropoulos 2002

1.2.4 Biological and morphological features of fenugreek

The genus *Trigonella* is made up of many species, many of which are diploid. Darlington and Wylie (1945) reported that the haploid chromosome number (*n*) of most species of the genus *Trigonella* is 8, 9, 11 or 14. However, *T. hamosa* from Egypt was found to have 16 and 44 chromosomes; *T. geminiflora* from Iran and Asia Minor, and *T. grandiflora* from Turkey also have 44 chromosomes, while *T. polycerata* which was collected in areas around the Mediterranean and in Asia was reported to have 28, 30 and 32 chromosomes, and *T. ornithopodioides* was reported to possess 18 chromosomes in its genome (Petropoulos 2002). According to Darlington and Wylie (1945) fenugreek has a diploid chromosome number of 2*n* = 16 chromosomes. Other *Trigonella* species also show some deviation from the normal chromosome number. Singh and Singh (1976) isolated primary trisomics along with five double trisomics from the progeny of autotetraploid fenugreek which had 18 (2*n*+1+1) chromosomes. Joshi and Raghuvanshi (1968) looked for the presence of ß-chromosomes in fenugreek and demonstrated that chromosome number in the plant can increase due to their presence. Roy and Singh (1968) also produced tetraploid fenugreek by treating shoot apices with colchicine, while Basu (2006) also reported that he had produced tetraploid fenugreek (2*n* + 2*n* = 32) by treating seeds with colchicine.

Fenugreek seeds require 3-10 days for germination and, follow an epigeal process where in the presence of sufficient moisture, oxygen and heat, the emergent cotyledon is bent over as it is pulled from under the surface of the soil during germination; i.e, imbibitions of water into the seed results in swelling of the seed endosperm. As the cells absorb water they elongate, extending the radicle to form a

primary root in the soil which eventually will develop secondary roots. According to Petropoulos (2002) protrusion of the radicle by more than 5 mm is considered a sign of fenugreek seed germination. As the hypocotyl elongates, the cotyledons are pulled above the soil to form a seedling; an epicotyl is characteristically absent. Release of the cotyledons leads to growth of the first simple leaf, followed by emergence of the first trifoliate leaf. Development of stems, flowers, pods and seeds occurs after seed germination and growth of the seedling (Petropoulos 2002).

Fenugreek stems are erect, hollow, and circular to slightly quadrangular. The color of the stems is dark green or dark bluish-green due to anthocyanin accumulation. Two types of stems are found in fenugreek; a monostalk without secondary shoots, and a multistalk where many shoots extend from the basal and higher nodes. In some cases where secondary shoots extend from the basal nodes, the main shoot does not differ markedly from the secondary shoots.

In general, two types of flowering shoots are observed in fenugreek. The most common type shows an indeterminate growth habit and bears axillary flowers; occasionally “blind shoots” form which produce both axillary and terminal flowers which develop seeds at the tip (Petropoulos 2002).

Leaves from fenugreek are simple, distinctly petiolate and consist of three leaflets. The leaflets of fenugreek are slightly dented over the edge, oval to orbicular in shape and green in color. The leaves are arranged in an alternate array throughout the plant (Slinkard et al. 2006). The root system of the fenugreek plant consists of a tap root and branched side roots arising from the main root. Like other leguminous plants

fenugreek is also capable of forming root nodules containing nodule-forming bacteria (Acharya et al. 2006).

The flowers of fenugreek are complete, papilionaceous and triangular in shape. The genus name '*Trigonella*' comes from Latin, meaning 'little triangle' in reference to the triangular shape of the flowers (Rosengarten 1969). The calyx consists of five sepals fused together to form a calyx tube with five teeth about as long as the tube. The corolla consists of a large standard or banner, two lateral wing petals, and two fused petals that form the keel. Each flower contains ten stamens and one pistil. The stamens are arranged on a stamen tube where nine stamen anthers are grouped and one stamen anther is free. Flower setting in fenugreek leaf axils is generally paired but, more rarely solitary and usually starts 35 – 40 days from seed sowing. Two types of flowers are observed in fenugreek; (i) cleistogamous flowers and (ii) aneictogamous flowers. A majority of fenugreek flowers are cleistogamous in which the keel of the flower remains closed in order to favor self-pollination. Aneictogamous flowers represent less than 1% of the total number of flowers found on a plant, and offer opportunities for cross-pollination since parts of the corolla remain open (Petropoulos 2002).

The fruit structure in fenugreek is the pod. Fenugreek pods are long, erect, pointed, sickle-shaped and slender. They have a sharp beak, 2-3 mm in length at the end (Duke 1981). The pods are green in color during growth of the plant but, turn yellow to yellowish brown at maturity. Fenugreek plants can be divided into two classes on the basis of number of pods per node. When there is only one pod per node the plants are considered to posses a "single" or "solitary pod", whereas if there are two pods per node a plant is referred to as having a "double" or "twin pod". In "double podded" plants the

two pods project in opposite directions from the same node of the stem. In ancient Greece, fenugreek was called “ox horn” or “goat horn”, probably because of the two seed pods projecting in opposite directions which resembles an ox or goat horn (Rosengarten 1969). Each pod carries approximately 10-20 seeds and, each pod-bearing branch can produce about 2-8 pods (Petropoulos 2002).

Fenugreek seeds are yellow to golden-yellow in color when mature, although some varieties can produce mature seeds which are green or yellow-green in color (McCormick et al. 2009). The seeds have a rectangular, square or irregular rhomboidal shape with grooves situated between the radicle and the cotyledons (Slinkard et al. 2006). Fenugreek seeds are surrounded by a seed coat which is separated from the embryo by a dark translucent endosperm (Fazli and Hardman 1968). A single layer of living tissue known as the aleurone layer lies between the seed coat and the endosperm. In mature seeds, a majority of the cytoplasm found within cells of the endosperm is made up by storage reserves consisting of ‘galactomannan’ (Petropoulos 2002). Botanical and morphological characteristics of fenugreek are summarized in Table 1.2.

1.2.5 Agronomic aspects of fenugreek

Agronomic practices are very important in growing a crop successfully. The agronomic requirements of a crop species often are unique and can differ from one crop species to another. As a well known cultivated crop, fenugreek has specific agronomic requirements for its successful production.

Table 1.2. Botanical and morphological aspects of fenugreek.

Feature	Parts of the organ	Short description	Dimension
Ploidy level		Mostly diploid ($2n = 16$)	
Germination		Epigeal. Hypocotyl pulls cotyledons above the soil	
Plant habit		Erect, straight or profusely branched	20-130 cm in length
Root	Main root and side roots	Tap root system	15-30 cm in length
Stem	Primary shoot, primary shoot and secondary shoots	Branched, circular to quadrangular, green or bluish green	0.5-1.0 cm in diameter
Leaf	Leaf lamina	Simple, trifoliate, toothed on tip	1.5-4.5 cm × 0.8-1.5 cm
	Petiole	Pale green, often anthocyanin tinged, very small	0.5-1.1 mm
Flower	Calyx	Consist of five fused green sepals, green	6-8 mm × 13-19 mm
	Corolla	Papilionaceous, white, papery	1.5-1.9 cm
	Stigma	Pale green, pale bluish green, ten stamens (9+1)	1.5-2.1 mm
	Style	Pale green, pale bluish green	0.2-0.5 mm
Pod		Long, slender, erect, beaked at the end	0.2-0.6 cm × 10-19 cm
Seed		Rectangular to oval, pale brown to golden yellow in color	3-5 mm × 2-3mm

1.2.5.1 Environment and soil

Fenugreek is cultivated on every continent. The wide distribution of its cultivation reflects to its adaptation to a wide range of growing environments and climatic conditions. Fenugreek likes a temperate and cool growing season, but does not like extreme temperatures. Moderate to low rainfall is favorable for its cultivation. Fenugreek is fairly drought resistant.(Del' Gaudio 1952) and will do poorly during a wet summer (Petropoulos 2002). In India fenugreek is normally grown as a winter annual crop (Petropoulos 2002). In European countries, it is grown both as winter crop in areas with a mild winter and as a spring crop in areas where the soil remains moist in summer (Petropoulos 2002). Seeding of fenugreek in late-April to mid-May has been successful in western Canada (Acharya et al. 2008).

Fenugreek grows best on well-drained loamy and/or sandy soils. Heavy clay and wet soils limit the growth of fenugreek (Petropoulos 2002). Slightly alkaline soils are ideal for successful fenugreek cultivation. A pH of 8-8.5 is favored by the crop. Fenugreek is fairly salt tolerant and, can be grown in low to moderate saline soils (Abdelmoumen and Idrissi 2009).

1.2.5.2 Seeding and seed rate

After seeding, germination of fenugreek may be affected if the seed is sown too deep, if there is inadequate moisture, very hard soil or freezing conditions (Petropoulos 2002). For seeding, the soil should have a fine granular consistency, not powdery. Fenugreek seed should be at least 95 per cent pure, have an 80 % germination rate (Heeger 1989) and, is best planted in rows to maximize yield (Petropoulos 2002). Most

favorable productivity has been obtained when fenugreek seeds are planted using a row spacing of 15 – 30 cm (Baswana and Pandit 1989; Gill et al. 2001; Korla and Saini 2003; Slinkard et al. 2006).

Various seeding rates have been suggested for fenugreek ranging from 15 to 25 kg/ha (Petropoulos 2002; Duke 1981). In western Canada a seeding rate of 27 – 33 kg/ha has been suggested for satisfactory yield results (Slinkard et al. 2006).

Legumes are well known for their ability to fix atmospheric nitrogen using nitrogen-fixing bacteria. As a legume fenugreek seeds must be inoculated with an appropriate nodule forming bacteria to maximize growth potential. Inoculation of fenugreek seeds with a suitable strain of *Rhizobium,* an aerobic, non-sporulating Gram negative nodule-forming bacterium, can improve quality and quantity of seed produced by the plant (Abdelgani et al. 1999).

1.2.5.3 Cultural practices

Soil nutrient management is an important practice to achieve high crop yields from fenugreek. Poor supply of plant nutrients often results in insufficient plant growth, slow growth, poor grain quality and low biomass yields. These constraints can be removed by adequate application of a fertilizer and/or manure to the soil. Fenugreek is sensitive to mineral deficiencies mainly of nitrogen (N) and phosphorous (P) (Yadav and Kumawat 2003; Petropoulos 1973). Deficiencies in boron (B), magnesium (Mg), and manganese (Mn) can also result in a poor or reduced seed and biomass yield (Petropoulos 2002). Fenugreek yield can be increased by applying manure and other organic and inorganic sources of nitrogen and phosphorous to the soil (Khiriya and Singh 2003;

Detoroja et al. 1995). When fenugreek crops are properly inoculated with *Rhizobium meliloti*, a small amount of nitrogen can be applied as 'infantile nitrogen" at the same time as the seed is sown. This infantile nitrogen dose helps promote rapid growth of fenugreek seedlings until the root nodules can form and the *Rhizobium* bacteria in the nodules are able to fix atmospheric nitrogen in large quantities (Petropoulos 2002). Deora et al. (2009) also reported that application of 20 kg/ha nitrogen in areas of Rajasthan, India significantly increased yield and nitrogen uptake by the crop, while Singh et al. (2008) suggested application of 40 kg/ha nitrogen significantly improved growth, seed yield and nitrogen uptake. Slinkard et al. (2006) reported that nitrogen requirement for fenugreek is similar to that of lentil in Canada.

Phosphorous can play an important role in crop management of fenugreek as it accelerates maturity of the crop and hence lessens risk from frost damage in the fall in cold areas. It also promotes transfer of substances from the stalks, leaves and other growing parts of the plant to the seed increasing their size at maturity (Petropoulos 2002). The rate of application of phosphorous fertilizer depends mainly on the amount of available phosphorous in the soil (Petropoulos 2002). High yields of fenugreek have been obtained by application of 40 to 50 kg inorganic P_2O_5 /ha to the soil (Deora et al. 2009; Tiwari et al. 2006). Sulphur (S) levels of 50 kg/ha also can significantly increase plant biomass and seed yield.

Although fenugreek is fairly drought resistant, higher yields are obtained (Lee 2009) when irrigation is used to supplement dry land farming and to avoid plant stress promoting smooth and continuous growth during the period of active growth of the crop. Irrigation is significant for cultivating fenugreek in arid or semi-arid areas, especially in

situations when rainfall plus the residual water does not cover the water requirement of this crop (Petropoulos 2002). Baricevic and Zupancic (2002) reported lower diosgenin yield when fenugreek was cultivated under drought stressed condition. However, when fenugreek was grown under optimal irrigation regime, diosgenin yield increased in comparison to normal irrigated plants. These results suggest that the plant species does well under minimal irrigation. Irrigation requirements for fenugreek production are dependent on some variables that include soil texture and depth, degree of evaporation, temperature and cropping practices. According to Del' Gaudio (1952) if the rainfall from September to April is less than 400 mm, irrigation of fenugreek is necessary. Heavy watering is not needed for fenugreek as the crop species possesses a shallow root system. Irrigation with application of 200 m^3/ha water every time for sandy soils, and 250 m^3/ha for heavier soils replicated every two weeks have been stated sufficient for a successful fenugreek crop (Petropoulos 2002). The number of irrigation varies depending on the region where the crop is being cultivated. Petropoulos (2002) reported that application of irrigation for five times for the whole growing period of fenugreek under Indian conditions is sufficient for a successful crop.

Studies on weed competition in genus *Trigonella* have been mostly concentrated to fenugreek. Fenugreek is vulnerable to weed intrusion particularly during the seed germination and seedling establishment phases, as fenugreek grows slowly after emergence (McCormick et al. 2006; Moyer et al. 2003; Petropoulos 2002). Tripathi and Govindra (1993) investigated the critical crop-weed competition period in fenugreek. From their field trials in India they concluded that the crop-weed competition period extends over the first 30 days after sowing of fenugreek. Their experiment also showed

that if the emerged weeds were removed soon in this period caused only 14.2% yield reduction, while if the weeds were left for the entire cropping season reduced yield to 69%. Weeds compete with fenugreek seedlings for available nutrients, available space and moisture that results in yield losses of fenugreek (Petropoulos 2002). Studies also showed that weed interference also causes changes in quality of the plant. It was found that ensiled fenugreek forage with low weed content had a lower neutral NDF and ADF, and CP content in cometparison to samples with higher degree of weed infestation (Moyer et al. 2003).

A range of weed species may be found in fenugreek depending on the geographical region, soil type and agro-ecologic environment. These weed species are divided into two groups; (i) winter species and (ii) spring species. Among winter species *Sinapsis* spp. (e.g., wild mustard), *Melilotus* spp., *Trifolium* spp. are found critical in fenugreek. *Chenopodium* spp., *Poa annua*, *Echinochloa crus-galli*, and *Setaria* spp. can be serious for spring-sown fenugreek (Petropoulos 2002). Certain weed species such as *Imperata cylindrica* L. and *Argemone mexicana* L. have the potential to reduce fenugreek germination and growth through allelopathy (Inderjit-Dakshini 1991; Leela 1981). Weed competition in fenugreek can be very strong if there is a heavy infestation by highly competitive and fast-growing perennial weeds. Perennial species like *Convolvulus arvensis*, *Cyperusrotundus*, and *Cyndon dactylon* can create a very strong competition with fenugreek (Petropoulos 2002). In Indian conditions, parasitic flowering plant *Orobanche indica* Ham. was found to parasitize fenugreek roots (Bhargva et al. 1976). In Southern Albarta region of Canada, fenugreek has been reported to be heavily infested by weed species such as *Setaria viridis* L., *Avena fatua* L., and *Amaranthus retroflexus* L.

(Moyer et al. 2003). Contamination of fenugreek fields with alfalfa, flax, canaryseed and wheat can be problematic as this can cause difficulties in separating the seeds after harvest (Slinkard et al. 2006).

Prevention of weed competition during the first 30 days should be the primary objective on any weed control program in fenugreek. Maliwal and Gupta (1989) concluded, on the basis of field trials in India, that hand weeding on the 20th and 40th days after sowing were sufficient for a maximal seed yield. Similarly, hand weeding at 15th and 30th days after sowing resulted in the highest seed yield (Mandam and Maiti 1994). Richarrdson (1979) identified suitable pre-emergence and post-emergence herbicides for fenugreek. Post-emergence herbicides such as bentazon, MCPB, diclofop-methyl and alloxydim-Na were found well tolerated by fenugreek, while chlorthal-dimethyl, propyzamide, butam, propachlor, trifluralin, tri-allate and chlorpropham were found suitable among pre-emergence herbicides. Mandam and Maiti (1994) found application fluchloralin at 3 kg/ha followed by hand weeding to be the best treatment. Field trials conducted in Sardarkrushinagar, India revealed that pre-emergence application of pendimethalin at a rate of 0.75 kg/ha followed by hand weeding at 20th and 40th days after sowing was statistically at same level with weed free checks (Mehta et al. 2010). In another study, Tiwari et al. (2006) found that application of pendimethalin at a rate of 1 kg/ha followed by hand weeding at the 25th day of sowing reduced the weed population successfully. In Canada, currently there is no registered herbicide for use on fenugreek (Slinkard et al. 2006). Moyer et al. (2003) conducted field trials in Alberta, Canada and found that treatment with imazamox/imazethapyr or mixes of imazamox/imazethapyr with ethalfluralin, annual weeds were restricted to 5% of dry matter production without

fenugreek injury or yield loss. The same investigators also revealed that weeds can represent 37% to 86% of dry matter if no herbicidal control of weeds is done in fenugreek.

1.2.6 Diseases and pests of fenugreek

A number of investigations have reported the appearance of pest attacks and diseases in fenugreek crops that can affect both yield and quality of the plant adversely. Fenugreek production is affected by both biotic and abiotic agents.

Abiotic diseases or disorders are mainly physiological condition related and are often caused by a deficiency in nutrients, extremes in temperature, moisture, soil acidity or alkalinity, an excess of certain micronutrients within the soil and toxic impurities in the atmosphere (Acharya et al. 2008; Petropoulos 2002). Sinskaya (1961) reported yellowing of some fenugreek plants under field conditions due to mineral deficiencies. In western Canada, exposure of fenugreek crops to very dry and hot conditions has resulted in stunted growth and yellowing, with occasional loss of leaves from the plant (Acharya et al. 2010a).

Diseases caused by living or biotic agents (pathogens) are often infectious (Acharya et al. 2010a). The most important diseases of fenugreek are caused by plant pathogenic fungi. Bacterial diseases are next in degree of importance, followed by viral diseases (Jongebloed 2004; Petropoulos 2002; Weiss 2002; Fogg et al. 2000; Prakash and Sharma 2000; AAFRD 1998). The two most common and serious fungal diseases infecting fenugreek are Cercospora leaf spot and powdery mildew (Acharya et al. 2010a; AAFRD 1998). Powdery mildew on fenugreek, caused by *Erysiphe polygoni*, can

seriously reduce crop yield (Jongebloed 2004; Prakash and Sharma 2000) and has the potential to affect biomass and seed yield in crops grown under moist agroclimatic conditions in North America. Cercospora leaf spot caused by *Cercospora traversiana* is becoming a major fenugreek disease concern (Acharya et al. 2010a; Weiss 2002). Other well-known fungal diseases observed to be associated with fenugreek are wilt, downy mildew, spring black stem and leaf spot, collar rot, rust, leaf spot and pod spot diseases (Bretag and Cunnington 2005; Jongebloed 2004; Lakra 2003, 2002; Petropoulos 2002).

McCormick and Hollaway (1999) and Fogg et al. (2000) found that infection of fenugreek with *Pseudomonas syringae* resulted in bacterial blight. It has also been verified that the bacterium *Xanthomonas alfalfa* can infect fenugreek (Petropoulos, 2002). Bean yellow mosaic virus, alfalfa mosaic virus, cowpea mosaic virus, soybean mosaic virus, pea mosaic virus, potato virus A and Y, and clover vein mosaic virus are all common viral infections of fenugreek (Petropoulos 2002; Bhasker and Summanwar 1982). Yellow mosaic potyvirus (Singh 1969) and pea streak carla virus (Hagedorn and Walker 1949) have also been reported on fenugreek.

Although fenugreek appears very resistant to attacks by insect pests, numerous insects are reported to attack fenugreek. In Australia, insects such as thrips, podborers and *Heliothis* spp. can cause serious damage to forage yield in fenugreek (Lucy 2004). *Aphis craccivora* and *Myzodes persicae* have caused damage to fenugreek crops from west Asia to India, while various Thysanoptera (thrips), including *Scirtothrips dorsalis*, have been found on almost all fenugreek crops grown from the Mediterranean to India (Petropoulos 2002; Weiss 2002). There have also been reports of *Tetranychus cucurbitae* attacks on fenugreek in India (Weiss 2002). *Pachymerus pallidus*, a seed beetle, is a

major pest of fenugreek in the Sudan (Weiss 2002). A number of polyphagous caterpillars including *Diacrisia oblique*, *D. orichalcea* and *Prodenia litura*, and especially *Maruca testulalis* have been reported to affect fenugreek in India (Weiss 2002). In southern Alberta, Canada, a low level of insect pests such as Lygus bugs and, to a lesser extent, alfalfa plant bugs and aphids, western flower thrips (especially severe under greenhouse conditions), alfalfa looper, alfalfa weevil and *Sitona* spp. were found attracted to standing fenugreek crops under field conditions (Acharya et al. 2010a). Various nematodes can damage fenugreek roots (Jongebloed 2004; Weiss 2002). The soilborne nematode *Meloidogyne incognita* has been shown to cause root rot and the death of immature fenugreek plants in Australia (Jongebloed 2004).

1.2.7 Uses

1.2.7.1 Ancient use of fenugreek

Historical uses of fenugreek have been reported by many authors. In the tomb of the Egyptian Pharaoh, Tuthankhamun (1333 BC to 1324 Bc), seeds of fenugreek were found. The Egyptians also used the leaves of fenugreek as one of the components of holy smoke in fumigation and embalming rites (Fazli and Hardman, 1968). Yoshikawa et al. (1997) mentioned that fenugreek was used as an aid to induce labor during childbirth and delivery in ancient Rome.

1.2.7.2 Fenugreek as a spice

Fenugreek has long been used as a spice in South Asia, the Mediterranean, and in Africa. Both seed and leaves of fenugreek are widely used as a culinary spice to enhance the taste of many meat, poultry and vegetable dishes (Acharya et al. 2010a b). Fenugreek

seed is often used in the Indian subcontinent cuisine, and in the preparation of pickles, curry powders, and pastes. The fresh or dried leaves are used to flavor dishes in many parts of India. In Egypt and Asia, sprouts of fenugreek and the young leaves are eaten as green vegetables. Fenugreek seed is a natural source of galactomannan gum. This property of fenugreek seed has provided the food industry with an opportunity to use seed extracts as thickening agent in foods or as food emulsifier (Slinkard et al. 2006). In India and China, the seeds are also a source of a food coloring dye and industrial mucilage. In Chinese cuisine, fenugreek plant products are more used for coloring than flavoring. In China, there is an organic green powdered food color product commercialized with the name of 'fenugreek herb' prepared from grounded leafy stems of fenugreek to give a green color to steamed pastry (Acharya et al. 2010ab; Hu 2005). Iran has a particularly rich tradition cooking with fenugreek leaves. In Africa, fenugreek seeds are mixed with bread to prepare a traditional food. Moreover, boiled seeds are taken with honey as a snack in many African countries. In Yemen and Arabian Gulf, fenugreek seed is considered as the main condiment (Weiss 2002). Fenugreek seeds are an important constituent of many unique and traditional spice mixes. Fenugreek seed constitutes an essential part of Bengali five spice mixture- panch foron; Georgian spice mixture- khmeli suneli; Ethiopian spice mixture- berbere; Bulgarian spice mixture- sharena sol (Acharya et al. 2010ab).

1.2.7.3 Fenugreek uses for remedy and medicinal purposes

Fenugreek has been documented as a medicinal plant in ancient herbal publications, religious scriptures, travel records, and anecdotes. The medicinal properties of fenugreek were recorded by the Egyptians and Hippocrates (Lust 1974). Its use as a

traditional medicine is also referred in Indian Ayurvedic, Greek, Chinese and Arabian medicines (Evidente et al. 2007; Sur et al. 2001). A wide range of medicinal properties has been attributed to fenugreek such as wound-healing, bust enhancement, enhanced lactation in weaning mothers, as an aphrodisiac, anti-diabetic, anti-hyperthyroidism, anticancer, gastro-protective, antioxidant, antipyretic, antimicrobial, anthelmintic, antisterility, antiallergy, antiinflammatory effects (Acharya et al. 2008; Krishnaswamy 2008). Historical accounts indicate that fenugreek leaves and seeds were used for many medicinal purposes, such as treating mouth ulcers and chapped lips, cure of baldness, in alleviation of abdominal and abscesses pain, in alleviation of cardiovascular and hepatic disorders, treating arthritis, dropsy, heart disease, spleen and liver enlargement, kidney ailments among several others, in the subcontinent of India, Greece, Arab and China (Acharya et al. 2010a; Tiran 2003; Weiss 2002).

Fenugreek (mostly seed) is a valuable source of many bio-chemical components that are attributed to various biological and pharmacological actions. Fenugreek seeds are also source of many minerals and vitamins. The seeds are found rich in Mg, Ca, Fe, Zn, Mn, Co, Ba, Cu and Br (Duke 1981; Picci 1959). Fenugreek is considered one of the few natural sources of steroidal sapogenins (Skaltsa 2002; Petropoulos 2002). Fenugreek is rich in flavonoids (Skaltsa 2002). Trigonelline, a methylbetaine derivative of nicotinic acid is one of the major alkaloids found in fenugreek seeds (Skaltsa 2002). The principal free amino acid of fenugreek is 4-hydroxy-isoleucine. It represents up to 80% free amino acid in fenugreek dry seeds, but it is absent from seed reserve proteins (Skaltsa 2002; Sauvaire et al. 1996).

In general, fenugreek contains three important phytochemicals with rich medicinal properties: 1) galactomannans, 2) sapogenins; and 3) 4-hydroxy isoleucine. Due to the presence of these chemicals fenugreek is currently rated higher among most commonly competing and well recognized nutraceuticals or health food products (Srichamroen et al. 2009; Acharya et al. 2008). Fenugreek galactomannan emerged to be beneficial in controlling type 2 diabetes in animals (Vats et al. 2003, 2002; Puri et al. 2002) and in humans (Puri et al. 2002; Raghuram et al. 1994). Bordia et al. (1997) showed that taking 2.5 g of fenugreek per day for three months can significantly reduce human cardiac risks. McAnuff et al. (2002) reported that steroidal sapogenins are extremely efficient in controlling hypocholesterolaemia. Sapogenins are also appear to selectively inhibit the growth of tumor cells and cancer prevention (Liagre et al. 2004; Raju et al. 2004). The amino acid isoleucine, a precursor of 4-hydroxyisoleucine has been reported for regulating insulin secretion, controlling blood sugar, and obesity prevention (Handa et al. 2005; Broca et al. 1999; Bordia et al. 1997).

1.2.7.4 Fenugreek as animal food

Although fenugreek is mostly known as a spice crop, the species name *foenum-graecum* refers to “Greek hay” supporting its use as a forage crop in early years (Acharya et al. 2008). It is used as green fodder and hay for cattle in India and Turkey (Petropoulos 2002). In Japan, it is used as silage. Petropoulos (2002) reviewed fenugreek as an alternative to alfalfa or forage peas. Fenugreek seeds are also used as feed for lactating cattle as it increases the flow of milk (Duke 1981; Hidvegi et al. 1984). Its ability to provide high quality forage at all stages of growth has made fenugreek a desirable forage (Acharya et al. 2008). It does not show a marked decline in quality even after

reproductive growth has been initiated. Mir et al. (1998) have shown that fenugreek forage has comparable nutritive value to early-bloom alfalfa and can successfully be used in beef industries. Acharya et al. (2008) reviewed that this legume is highly palatable and does not cause bloating in beef cattle. Mir et al. (1998) suggested fenugreek as a close substitute for alfalfa forage as they observed similar rumen conditions, digestibility, and weight gain in cattle. Goel et al (2007) have shown increased efficiency of fermentation in the rumen resulting in lower methane production. This observation suggests that fenugreek diet for cattle may be environmentally beneficial by reducing methane production by cattle. Montgomery et al. (2008) have shown its uses in the dairy industries. Alemu and Doepel (2011) studied the effect of fenugreek haylage to dairy cattle and observed similar digestibility although intake of fenugreek was slightly lower. Petropoulos (2002) mentioned that the ancient Greek and Romans used fenugreek hay as horse feed.

1.2.7.5 Agricultural and others uses

As a legume, fenugreek has the ability to fix atmospheric nitrogen in the soil by harboring nitrogen-binding bacteria in its roots. The crop requires a minimal amount of nitrogen fertilizer for its growth, and reduces the need for nitrogen fertilizers for subsequent crops (Acharya et al. 2010b). Fenugreek is considered a dryland crop thus water requirement of this crop is low. Use of fenugreek in arid and semi-arid environments, and in regions with limited water supply can reduce the cost of irrigation, reduce the potential for eutrophication of surface water and limit contamination of groundwater sources (Acharya et al. 2008; Basu 2006). Fenugreek is a good choice to

incorporate in short-time crop rotation schemes, especially with non-leguminous crops (McCormick et al. 2006).

Fenugreek possesses insecticidal, nematicidal, molluscicidal and antimicrobial properties (Acharya et al. 2008; Zia et al. 2001). Fenugreek itself is an insect tolerant crop; moreover, it provides effective control against stored grain insect pests. Fenugreek seed and leaf extracts were reported to have widespread antimicrobial activity against both gram positive and gram negative bacteria (Bhatti et al. 1996).

The aroma and flavor of fenugreek are attributed to volatile constituents it comprises. Fenugreek seed contains 0.02–0.05% volatile compounds (Petropoulos 2002). The major components in this group are heptanoic acid, n-hexanol, dihydroactiniolide, dihydrobenzofuran, tetradecane, a-muurolene, b-elemene and pentadecane (Leela and Shafeekh 2008). Fenugreek seeds hold 7.5% lipids on a dry matter basis. The total lipids consisted of 84.1% neutral lipids that mostly consisted of triacylglycerols, 10.5% phospolipids, and 5.4% glycolipids (Skaltsa 2002). The seeds contain about 7% fixed oil consisting mainly of linoleic, oleic and linolenic acids (Leela and Shafeekh 2008). Extractable oil from fenugreek seed carries a particular smell. Being strongly scented, the oil is used as an insect repellent for wooden furniture and cloths (Duke 1981). Fazli and Hardman (1968) referred to the use of fenugreek oil in perfumes. Petropoulos (2002) mentioned the presence of fenugreek oil as a secret ingredient in a very famous perfume of France. In Europe and North America, fenugreek is familiar as a component in artificial flavoring such as maple and butterscotch, while this aroma of fenugreek seed is mostly attributed to fenugreek oil (Slinkard et al. 2006).

1.2.8 Improvement of fenugreek

The improvement of a crop that is grown under a wide range of soil and climatic conditions is a dynamic challenge due to its wide diversity. Fenugreek is one of these crops. Several important considerations, such as the genetic variation, reproductive behavior, environmental adaptability, mode of inheritance of desirable characters, and economical importance, determine the objectives and methods chosen for the genetic improvement of a crop. Fenugreek is normally diploid in nature and that is an advantage for genetic development of this plant species, as diploid genetics has been evaluated extensively. Plant breeding has provided a large number of varieties of fenugreek. The need for fenugreek varieties with higher productivity, increased vigor, elevated amount of essential biochemical products mainly with higher diosgenin content, has driven more breeding efforts in this crop (Petropoulos 2002). Fenugreek is a highly self-pollinated plant, though cross-pollination is reported to occur in a very little extent. Most of the breeding endeavor for the genetic improvement of fenugreek has mainly concentrated on three approaches namely selection, hybridization and mutation used separately or in combination (Petropoulos 2002, 1973; Green et al. 1981).

1.2.8.1 Selection

Selection is a basic process in plant breeding, most suitable for improvement of diploid plants like fenugreek (Busbice et al. 1975; Marques de Almeida 1940). Selection approach consists of choosing the outstanding types and discarding those that are undesirable because of certain characteristics. Unless the qualities of the superior types of plants can be readily detected improvement by selection method is not possible. For

different inherited traits of fenugreek, suitable morphological and physiological characters as an index of selection can provide a reliable basis for genetic improvement through selection. Petropoulos (2002) has reported that in fenugreek presence of twin pods is an indication of a high diosgenin content in the seeds. This readily detected phenotypic selection is useful to improve fenugreek cultivar with higher diosgenin content. Knowledge of dominant and recessively inherited traits is considered very important as it would have a direct impact on progeny behavior of selected plants. Ahmed et al. (1989) produced a variety with higher diosgenin content by passively selecting for plants bearing 'twin pods'. Two procedures are commonly used for the process of selection to develop improved varieties of fenugreek: the individual (also called pedigree and pure line selection) or simple plant selection and the mass selection.

Selection method is very important for producing varieties in an area where the plant is recently introduced. The first North American forage fenugreek cultivar "Tristar" has been developed by selecting suitable genotype among fenugreek germplasm, and subsequent adapting the selected genotype in North American conditions (Acharya et al. 2008a). A group of investigators have evaluated fenugreek landraces for the assessment of drought tolerance in Iran. Suitable genotype(s) may produce good drought tolerant fenugreek cultivar in Iran by selection and adaptation in areas of fenugreek cultivation in Iran (Sadeghzadeh-Ahari 2010; 2009). In Australia where fenugreek is a minor crop, McCormick et al. (2009) have evaluated a germplasm collection of 205 fenugreek accessions for a range of phenotypic traits including seed yield. Selection approach has produced many fenugreek cultivars for various desired traits (as reviewed in Petropoulos 2002). The cultivar RH 2701 and RH 2698 with higher diosgenin content, RH 3128 with

higher seed yield, the cultivar RH 2699 with higher percent protein, the cultivar RH 2701 for higher amount of fixed oil were created by continuous selection process among the mother cultivars (Petropoulos 2002, 1973).

1.2.8.2 Hybridization

Hybridization process is very important in cultivar development. Hybridization is used to incorporate desired traits into a population, and to create populations with genetic variability. Fehr (1993) suggested that hybridization is more successful when it is done between parents with different genetic backgrounds. Hybridization involves crossing two or more varieties of genetically different individuals. Common methods of hybridization can involve a 2-parent cross, a 3-parent cross, a 4- parent cross, a back cross, a complex cross, a convergent cross and/or a polycross (Fehr 1993).

Hybridization offers high probability for increasing variability for further selection and the greatest possibilities for improvement of fenugreek (Petropoulos 2002). Fenugreek is a highly self-pollinated in nature, and because of its flower structure (cleistogamous) hybridization is tedious, laborious in this plant species. In fenugreek, crosses are normally made by hand. Before pollination the flowers are emasculated to avoid the risk of self-pollination, and this step is considered an important step for hybridization in fenugreek. Emasculation and manual pollination has been used effectively for crossing different lines of fenugreek (Petropoulos 2002). The fenugreek flower should be emasculated at the end of the first floral developmental stage when the stigma of the pistil is beginning to be receptive while the anthers of the stamens are closed and lower than the stigma. Immediate after manual pollination, a bag should be

placed over the fenugreek flowers to avoid any chances of unrestricted out-crossing (Cornish et al. 1983).

To find individual plants that possess unique and desirable combinations of characters, at times hundreds of crosses need to be made in order to generate a successful hybrid. Many fenugreek varieties have been produced by hybridization (Petropoulos 2002; Saleh 1996; Edison 1995). For instance, the high seed yielding genotypes RH 3109/32, RH 3110/37, RH 3105/15 and 3111/8, and the genotypes RH 3109/42 and RH 3110/66 with high diosgenin content were produced by crossing Fluorescent and Kenyan cultivars (Petropoulos 1973). However, this method has a major disadvantage in that undesirable character combinations often are created; these can be difficult to select out and can take many generations of selection or back crossing to eliminate.

1.2.8.3 Mutation breeding

Mutation breeding has been used in many crop species to generate new genetic variability. Mutation breeding can be used when there is little variation in an existing gene pool for a certain trait (Fehr 1993). Four types of mutations can occur and those are 1) genome mutation; 2) structural mutations; 3) gene mutations; and 4) extranuclear mutations. Among the above mentioned mutation types, gene mutations are most desired in plant breeding (Yadav et al. 2007). Gene mutations cause an alteration of gene expression by substituting one nucleotide base for another or by adding or deleting a nucleotide to a gene. Potential lines that are generated from these mutations are used to generate mutant cultivars which can be inbred in order to stabilize a new trait, and then used in hybridization programs to introduce the trait to other plants. Mutations can either

occur spontaneously or may be induced artificially and, are a valuable tool for crop improvement. Mutation breeding has been successfully utilized to bring desirable genetic changes in cultivars of legume crops (Toker et al. 2007; Sigurbjornsson 1983; Sigurbjornsson and Micke 1974); e.g., Manha et al. (1994) used mutation breeding to increase the diosgenin content in *T. corniculata* (a close relative of fenugreek).

Various mutagenic chemicals or irradiation processes can be used to introduce mutations into plants. Ionizing irradiation that includes electromagnetic radiation and particulate radiation (for example, x-rays, gamma rays, alpha particle and beta particle), is used to artificially increase the rate of spontaneous mutations. The chemical mutagens belong to different groups such as base analogs, acridine dyes, nitrous acid, hydroxylamine and alkylating agent. Mutation breeding is more adaptable for inducing recessive genes than dominant genes (Toker et al. 2007). Micke and Donini (1993) suggested that as mutations are mostly recessive, they cannot be selected for until the second generation, whereas dominant mutations occur at low frequencies and can be selected for in the first generation. Although mutations are beneficial for producing variability in populations, the treatments themselves can be detrimental and can cause a reduction in germination, growth rate, plant vigor, and pollen and ovule fertility in a plant. Singh (2005) stated that mutations can be recurrent and that the same gene(s) of a plant species may be induced to mutate again and again. Moreover, mutations generally have pleiotropic effects due to closely linked gene(s).

Mutation breeding has been utilized to improve fenugreek genotypes for various traits. Both spontaneous and induced mutations have been exploited to generate suitable cultivars of fenugreek. A number of fenugreek mutants from spontaneous mutations have

been isolated and are in use all over the world (Laxmi and Datta, 1987; Laxmi et al., 1980; Singh and Singh, 1974; Petropoulos, 1973). The spontaneous mutant RH 3129, characterized with high proportion of twin pods and with high diosgenin content, were isolated from the Moroccan cultivar. The cultivar RH 3112 with higher diosgenin content, RH 3112 with higher seed yield, the cultivar RH 3112 and RH 3118 with higher percent protein, cultivars with higher amount of fixed oil and cultivars with early maturity trait were created by induced mutation process (Petropoulos 2002, 1973; Laxmi et al. 1980). Basu (2006) has generated mutant populations by treating seeds from the North American cultivar “Tristar” with EMS. These populations were reported to exhibit variability in height, seed yield, seed number per pod, biomass yield, total number of pods and number of twin pods.

Chapter Two: Mutation Breeding

2.1 Introduction

Fenugreek is gaining more and more attention from an economical, agronomical and environmental view points in countries where the crop was absent in their cropping systems and in countries where the crop has been recently introduced. As a crop species, fenugreek is fairly new to Canada. Since 1992, fenugreek has been grown commercially in western Canada. The fenugreek genotypes that are adapted to the climatic conditions of western Canada are few. Cultivars that are currently available in this country are the result of selection among the world accessions that were introduced to this region. Further genetic improvement in this species through breeding is very much limited since the adapted genetic pool is very narrow.

Mutation breeding can be used to generate new genetic variability when there is little variation in an existing gene pool for a certain trait (Fehr 1993). Mutation breeding can give rise to many different alleles with different degree of trait modification in crop species (Chopra 2005). Mutations are theoretically all changes which occur in DNA sequence and result in changes in the genetic code. Gene mutation is mostly desired in plant breeding as this adds to variability from which selection can be made (Yadav et al. 2007). Mutation breeding is found to be well suited for genetic improvement of grain legumes that is also suggested by their evolutionary selection history (Adekola and Oluleye 2007). Significant progress has been reported on the improvement of nutritional quality of some legumes by inducing mutation (IAEA 1977, 1991). Yadav et al. (2007) reported improvement of nutritional properties in *Lens culinaris* (L.) through induced

mutation. In *Vigna unguiculata* (L.), improved lines with high amount of protein content and dry matter content, and reduced amount of moisture content were obtained using induced mutation (Adekola and Oluleye 2007). Mutation breeding has been utilized to improve fenugreek genotypes for various traits. Both spontaneous and induced mutations have been exploited to generate suitable cultivars of fenugreek (Laxmi and Datta 1987; Petropoulos 1973). The spontaneous mutant RH 3129, which has been characterized as having a high proportion of twin pods with a high diosgenin content, was initially isolated from a Moroccan cultivar. Cultivars with a high diosgenin content, higher seed yield, a high percent protein, a high amount of fixed oil, and cultivars with an early maturity trait have been created by introduction of mutations to fenugreek (Petropoulos 2002, 1973; Laxmi et al. 1980).

Although both physical and chemical mutagens are used in plant breeding for inducing variability, many researchers reported a number of chemical mutagens to be more effective and efficient than physical mutagens to produce variability (Begum and Dasgupta 2010; Basu et al. 2008; Ganapathay et al. 2008). EMS, a chemical mutagen, has been shown more effective than radiation in inducing polygenic variability in studies with wheat (Gaul and Aastveit 1966), *Arabidopsis thaliana* (Brock 1971) and cowpea (Girija and Dhanavel 2009). Several investigators investigated the effectiveness of chemical mutagens and physical mutagens (radiations) to induce mutation in legume species lentil (*Lens culinaris* L.) (Solanki 2005; Sarker and Sharma 1989). Their studies revealed that chemical mutagens were more efficient than physical mutagens for inducing mutations in lentil. Moreover, in their studies it was also found that among the chemical mutagens, morphological mutation frequency was obtained higher with EMS. As an alkylating

mutagen, EMS alkylates phosphate groups, purine and pyrimidine bases in addition to allelic mutations, small deletions and other chromosomal rearrangements. Henikoff and Comai (2003) stated that in most cases EMS alkylates guanine bases and leads to mispairing or mismatch pairing (particularly pairing of G bases with T in place of C) within the genome. So, it is possible that point mutation in a gene or genes induced by EMS has the potential to activate morphometric and reproductive changes in the fenugreek base populations. Basu (2006) investigated the effectiveness of different level of doses of EMS on Tristar fenugreek. This author found that EMS was very effective to induce variability in the fenugreek base population, and the mutants generated with 300 µM EMS generated the best possible combination of characters among the mutant plants. Yadav et al. (2007) suggested that more than one genotype should be used in a mutation breeding program since response to mutagens is different from a variety to another variety. The same authors also stated that the genotype selected for mutagenesis should in particular be one of the best adapted genotypes. This is supported by the study of Begum and Dasgupta (2010) where significant varietal effect was found on yield and yield attributed characters in mutant generation of *Sesamum indicum* (L.).

To produce variability in the available gene pool in this study, mutation breeding using EMS was applied to some adapted fenugreek genotypes. As mutation breeding produces variability in a base population, five adapted genotypes, namely Amber, F70, F80, F86 and Tristar were used as base populations to produce more variability for regional cultivar improvement. It is hypothesized that as mutant plants conserve a major portion of the base population DNA, use of a wider variety of mutant plants derived from more than one adapted genotype as a base population could provide more variability for

development of new cultivars with desired seed yield, biomass yield, plant height, seed number per pod, total number of pods and number of twin pods.

The objective of this mutation study was:

> To produce variability in adapted genetic material to help improvement in this crop through breeding for early maturity and high seed yield.

2.2 Materials and Methods

2.2.1 Seed material

The five fenugreek (*Trigonella foenum-graecum* L.) genotypes used in this study were Amber, Tristar, F70, F80 and F86. The cultivar Amber was developed by selection at Agriculture and Agri-Food Canada, Morden, Manitoba, Canada. The cultivar Tristar was the first Canadian forage-type fenugreek cultivar, and was developed by selection at Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada. The genotypes F70, F80 and F86 were obtained from the United States Department of Agriculture/ Agriculture Research Station, Pullman, Washington, and further selected for their adaptability and seed yield traits at the Crop Diversification Centre South (CDCS), Brooks, Alberta, Canada. According to Lee (2009), the genotype Amber appeared to be the best performing genotype for diosgenin productivity, while Tristar was found to be the best genotype for production of a high 4-hydroxyisoleucine content, and the genotype F86 produced relatively high seed yield and galactomannan content among ten genotypes evaluated in a multi-location trial conducted in Canada. In another multi-location study conducted by Basu (2006) within the Canadian prairie region, Tristar and F70 were recognized as the best genotypes for forage production among the genotypes tested.

2.2.2 Treatment with mutagen

Fenugreek seeds of each genotype were presoaked in distilled water for 4 h (for effective imbibition) and then treated with EMS (Sigma-Aldrich) for 12 hours at a concentration of 300 µM made up in distilled water. For each genotype 100 seeds (M_0) were treated every time. Each treatment was done three times. In Basu (2006), the

treatment duration and concentration was found to be the best combination for producing mutants with desirable characters in fenugreek.

2.2.3 Growing M_1 plants

Treated seeds from each treatment were planted individually in the greenhouse in 6 inch plastic pots containing a non-sterile, soil-free mix (LRC Soiless mix/Cornell mix) and were designated as M_1 plants. Separate control blocks comprised of 50 plants of untreated Amber, F70, F80, F86, and Tristar fenugreek were planted for comparison of survival rates to their treated counterpart in the greenhouse. The soil free mix was composed of a 3.8 cubic foot bale of sphagnum peat moss, an 18.6 kg bag of medium horticultural grade vermiculite, 1000 g of calcium carbonate flour, 1500 g of 18-6-12 Osmocote (Southern Agricultural Insecticides, Inc.), 1200 g 0-21-0 fertilizer, 20 g of "Fritted" trace elements, 15 g of 13.2 % (W/W) chelated iron, 7 g of 14 % (W/W) chelated zinc and 30 L of washed mortar sand (The Scotts Co.). After maturation, seeds were collected from the plants that survived from mutant treatment. Seeds from each base population were bulked and kept separate from others.

2.2.4 Growing M_2 plants

The seeds harvested from M_1 plants grown in the greenhouse were planted in the field to produce M_2 generation. The M_1 seeds originating from each base population were seeded in separate rows. The seeds were sown in 2 meter long rows at a rate of 100 seeds/row. Two rows of untreated Tristar seeds were planted at the two edges as controls. At maturation, seeds were harvested from M_2 plants. Mutant seeds obtained from each genotype as base population were bulked and kept separate. During the growing period,

the field was visited frequently and examined for the presence of any weeds. If any weeds were found they were pulled out by hand. All surviving plants (Table 2.2) in the M_2 generation were examined individually to gather data on plant height, pod number per plant, double pod number per plant, seed weight per plant and biomass weight per plant. Plant height was measured from the ground to the top most point of the plant. Plants were cut at about 2.54 cm above ground, and then kept for drying. Individual plants was weighed for dry biomass after drying. Seeds were extracted manually from each plant and then weighed.

2.2.5 Growing M_3 plants

From each treatment group (groups based on each genotype used as base population for inducing mutation) of M_2 seeds, 64 seeds were planted individually in the greenhouse in 6 inch plastic pots containing a non-sterile, soil-free mix (LRC Soiless mix/Cornell mix) and were designated as M_3 plants. Separate control blocks composed of 50 plants of untreated Tristar fenugreek were planted for comparison of survival rates in the greenhouse. After maturation M_3 seeds were collected from the surviving plants. Seed from each genotype (the base population) was bulked and kept separate from others. Mutant seeds obtained from each base population genotype (designated as a group) were bulked and kept separate. All surviving plants (Table 2.2) in the M_3 generation were measured individually to gather data on plant height, pod number per plant, double pod number per plant, seed weight per plant and biomass weight per plant.

2.2.6 Statistical analysis

The mutation breeding study was not amenable to statistical manipulation as the objective was to produce variability in mutant population. However, means and standard errors were calculated using Microsoft Excel 2007 (Microsoft Corporation). GGE Biplot Softwear (Version 6.7) was used to identify optimum trait values and environments among the mutant generations (M_2 and M_3), and among mutant categories (Amber, Tristar, F70, F80 and F86) according to the base population used.

2.3 Results

The EMS treated plants (M_1) based on their treatment groups (groups assigned based on the genotypes used as base populations for EMS treatments) exhibited a distinctive pattern of survival rates compared to their respective control groups (Table 2.1). The rate of survival of M_1 plants among the different EMS treated groups varied from 1.33% to 8.00%, which demonstrated a very low level of survival compared to the control groups. The survival rate among the control (untreated) groups varied from 40.00% for F70 to 86% Amber (Table 2.1). Among the mutant groups, the group F86 showed the best survival rate (8.00%) followed by the group Amber (5.33%) and F80 (5.33%) in M_1 generation. For the Tristar and F70 groups, the rates of M_1 plant survival were very poor; i.e., 2.33% and 1.33%, respectively. The survival rate of F70 genotype (40.00%) was also the lowest among the control plants. The survival rate for the Amber control group (86.00%) was the best among the control plants followed by F86 (72.00%), Tristar (62.00%) and F80 (60.00%) (Table 2.1).

The M_2 generation was grown in the field to expose the plants to natural selection. No other selection pressure was put in the M_2 generation. Selection pressure in early generations increases chances of eliminating potential plants with suitable characteristics in successive generations (Fehr 1993). This early mutant generation (M_2) was exposed to an outside environment so that mutant plants that could not deal with a typical crop-environment of western Canada could be eliminated automatically by nature. Thus the number of detrimental mutants that were not suitable for growth in a temperate climate was reduced significantly in the M_3 generation by natural selection in M_2 generation. The

Table 2.1. Survival of M_1 (EMS treated) and untreated plants for each base population .

Genotype used as base populations	Number of M_1 plants survived	Percentage of M_1 plants survived	Number of control plants survived	Percentage of control plants survived
Amber	16	5.33	43	86
F70	4	1.33	20	40
F80	16	5.33	30	60
F86	24	8	36	72
Tristar	7	2.33	31	62

M_3 generation was grown under controlled environment (greenhouse) to make sure that segregating mutants for different plant characters that might be useful in successive generations were not lost.

The survival rates of M_2 plant groups differed from the survival rates of M_3 plant groups. The mutant groups showed an increased level of survival with an increase in the number of the generations. Each mutant group performed differently in every generation in term of survivability. The survival rate of the M_2 plants among the different treatment groups varied from 9.2% to 28.9%, whereas the survival rate among the M_3 plant groups varied between 34.4% to 50% (Table 2.2). The mutant group for Amber showed the best survival rate (28.9%) followed by the group F70 (17.7%) and F80 (14.8%) among M_2 groups. The lowest survival rate was observed for the F86 group (9.2%) treatment group. The survival rate of the F70 group (50%) was highest among M_3, whereas the mutant group F80 was the poorest (34.4%) in survivability. The plant groups Amber and Tristar also showed good survivability in the M_3 generation; i.e., 43.8% and 42.2%, respectively (Table 2.2.). An interesting observation was that the mutant groups F86 and F70 had the highest and the lowest survival rates in M_1 generation, but in the M_3 generation the F70 group had the highest survival rate while F86 showed the poorest survival rate (Table 2.1, 2.2). A number of mutants showing dwarfness, albinism, virina xanthescens and other abnormal phenotypes were observed across all three generations ($M_1 - M_3$). However, many of these plants either died before maturity or did not produce any seed.

Table 2.2. Survival of M_2 plants in the field and M_3 plants in greenhouse conditions for each group.

Genotype used as base populations	Number of seeds planted to grow M_2 plants	Number of M_2 plants survived	Percentage of M_2 plants survived	Percentage of control (Tristar) survived used in M_2	Number of seeds planted to grow M_3 plants	Number of M_3 plants survived	Percentage of M_3 plants survived	Percentage of control (Tristar) survived used in M_3
Amber	246	71	28.86		64	28	43.75	
F70	136	24	17.65		64	32	50	
F80	418	62	14.83	72	64	22	34.38	76
F86	541	50	9.24		64	24	37.5	
Tristar	150	21	14		64	27	42.19	

The M_2 and M_3 plants also showed a wide range of variability in different morphometric parameters; *i.e.*, in height, number of pods, number of double pods, seed weight and biomass weight (Table 2.3, 2.4 and Figure 2.1).

In the M_2 generation the highest mean plant height was obtained in the Tristar group (41.6±2.0 cm) followed by F86 group (41.6±1.3 cm), while the lowest mean plant height was observed in F80 group (36.8±1.2 cm) (Table 2.3). In the M_3 generation, the highest mean plant height was 50.5±1.5 cm and the lowest mean plant height was 33.5±1.8 cm for the groups F70 and Amber, respectively (Table 2.3). For the M_2 generation, the tallest plant (66.8 cm) was found in the F86 treatment group, while the Amber and Tristar groups produced the shortest plants (18.3 cm) (Table 2.4). For the M_3 generation, the tallest plant (73.4 cm) was found in the Tristar group, and the shortest plant (15.2 cm) was found in the F80 group (Table 2.4). The overall mean plant height in the M_3 generation for the groups except for the Amber and the Tristar groups increased in comparison to that of the M_2 generation (Table 2.3). The mean height for the Amber treatment group in the M_3 generation decreased profoundly from what was seen in the M_2 generation, while the mean height for Tristar remained almost the same in both generations.

The mutant groups (treatment groups for F70, F80, F86, Amber and Tristar) relative to the mutant generations (M_2 and M_3) were analyzed using GGE biplot methodology (Yan and Hunt 2003) for selected agronomic traits. The "which wins where or which is best for what" view of the biplot identified the winning mutant groups for the selected agronomic traits over the mutant generations. The biplot is constructed by plotting principle component scores for both genotype group (entries) and generation

Table 2.3. Different morphometric parameters (mean ± SE) of field grown M_2 plants and greenhouse grown M_3 plants.

Genotype used as base populations	M_2 generation						M_3 generation					
	Plant height (cm)	Number of pods per plant	Number of double pods per plant	Percent plants producing double pods	Seed weight per plant (g)	Biomass weight per plant (g)	Plant height (cm)	Number of pods per plant	Number of double pods per plant	Percent plants producing double pods	Seed weight per plant (g)	Biomass weight per plant (g)
Amber	38.25 ± 0.42	10.70 ± 0.93	0.76 ± 0.19	25.35	0.90 ± 0.08	10.78 ± 0.45	33.50 ± 0.82	8.71 ± 1.20	3.36 ± 0.83	53.57	1.18 ± 0.18	5.28 ± 0.44
F70	40.18 ± 0.81	18.25 ± 2.02	0.97 ± 0.49	16.66	1.96 ± 0.31	10.30 ± 0.68	50.57 ± 0.58	24.34 ± 1.47	11.06 ± 1.04	96.88	2.73 ± 0.19	12.97 ± 0.50
F80	36.70 ± 0.47	10.31 ± 1.37	0.61 ± 0.18	19.35	0.54 ± 0.10	9.72 ± 0.42	37.51 ± 1.01	10.86 ± 1.70	3.18 ± 0.93	45.45	1.46 ± 0.23	6.79 ± 0.71
F86	41.57 ± 0.52	9.02 ± 1.14	0.56 ± 0.21	16.00	0.64 ± 0.11	8.49 ± 0.45	48.13 ± 0.53	17.88 ± 1.31	8.75 ± 1.13	87.50	1.9 ± 0.18	10.22 ± 0.66
Tristar	41.63 ± 0.86	13.24 ± 1.83	0.38 ± 0.22	14.29	1.11 ± 0.21	12.85 ± 0.81	41.24 ± 0.96	9.59 ± 0.85	1.11 ± 0.37	29.63	1.2 ± 0.11	6.37 ± 0.61

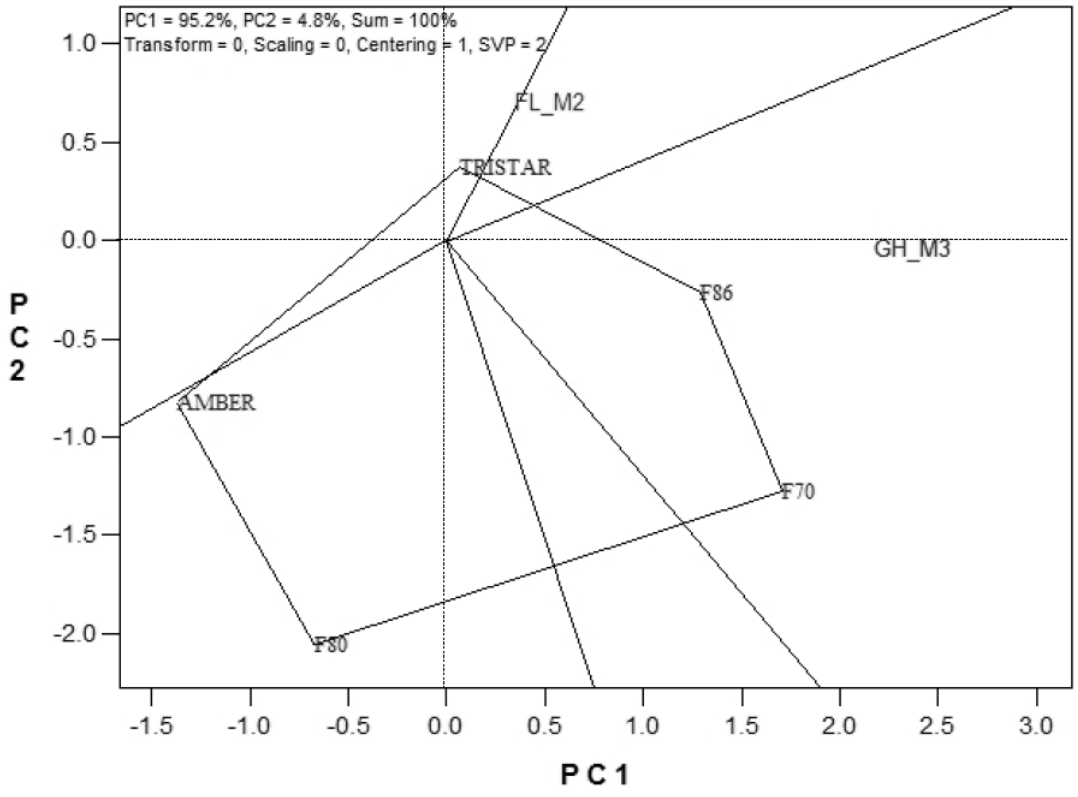

Figure 2.1. The "which wins where" view of GGE biplot for assessment of variation in plant height of the mutant groups over two mutant generations (FL_M2 = field grown M2¬ generation, and GH_M3 = greenhouse grown M3 generation). Variation in plant height was assessed using a Principle Component Analysis of mutated plants from five different genotypes (Tristar, Amber, F80, F70, and F86) over two generations (FL_M2 and GH_M3). Highly positive PC 1 scores indicate superior performance, whereas PC 2 scores close to 0.0 indicate trait stability.

(testers) simultaneously to create a two-dimensional diagram, using the PC1 scores as the abscissa (horizontal axis), and the PC2 scores as the ordinate (vertical axis). 'Entries' with a high PC1 score (positive) indicate superior performance while those that have a PC2 score close to zero indicate stability (Yan and Hunt 2003). The polygon-view or the "what-wins-where" view of the GGE biplot presents a polygon drawn by connecting all the genotypes located farthest from the biplot origin. Genotypes positioned at the vertices of the polygon are referred to as vertex genotypes having longest vectors in their respective directions. The vertex genotypes are the most responsive in their respective directions, while those contained in the polygon are less responsive.

The GGE biplot analysis for plant height relative to mutant groups and mutant generations identified Tristar, Amber, F80, F70 and F86 as the most responsive groups, given their positions at the vertices of the polygon (Figure 2.1). The mutant group F70 was the superior genotype for plant height, given its high PC 1 scores, followed by the mutant group F86. The mutant group F86 was the most stable in terms of plant height given its low PC 2 (close to zero) scores. The mutant groups F70 and F86 also performed well in the greenhouse grown M_3 generation (revealed by their close proximity to GH_M_3 in the biplot), while Tristar and Amber groups performed well in field grown M_2 generation. The given scores for PC 1 and PC 2 showed that the M_3 generation was superior and more stable than the M_2 generation.

The mean pod number per plant and mean double pod number per plant were calculated for each group in the M_2 and M_3 generations. In the M_2 generation, the highest mean pod number per plant was obtained for the F70 treatment group (18.3±2.02), while the lowest mean pod number per plant was observed in F86 group

(9.0±1.14) (Table 2.3). In the M_3 generation, the highest mean pod number per plant was 24.3±1.47 and the lowest mean pod number per plant was 8.7±1.2 for the groups F70 and Amber, respectively.

The F70 group mutant plants had higher number of pods compared to other mutant groups in the M_2 generation (Fig. 2.2). For the M_2 generation, the plant producing maximum number of pods (55) was found in the F80 group, while the plant producing the maximum number of pods (43) was found in F70 group for the M_3 generation (Table 2.4). The overall mean pod number per plant in the M_3 generation for all groups except for the Amber and Tristar treatments groups increased in comparison to that of the M_2 generation (Table 2.3). The GGE biplot analysis for pod number/plant for the mutant treatment groups over mutant generations in a "which wins where" view (Figure 2.3) showed that pod number/plant was responsive in all mutant groups, given their positions at the vertices of the polygon. However, the location of the F70 group at the farthest right of the polygon (high PC 1 score) indicates superior performance for this trait, while the location of the Amber group at the farthest left of the polygon (low PC 1 score) indicates poor performance. The mutant groups Amber and F70 were found more stable groups (low PC 2 scores) for this trait. The mutant group F70 performed well in the greenhouse grown M_3 generation, as well as in the field grown M_2 generation (revealed by its close proximity to GH_M3 and FL_M2 in biplot). The given scores for PC 1 and PC 2 showed that the M_3 generation was superior and more stable than the M_2 generation for this trait.

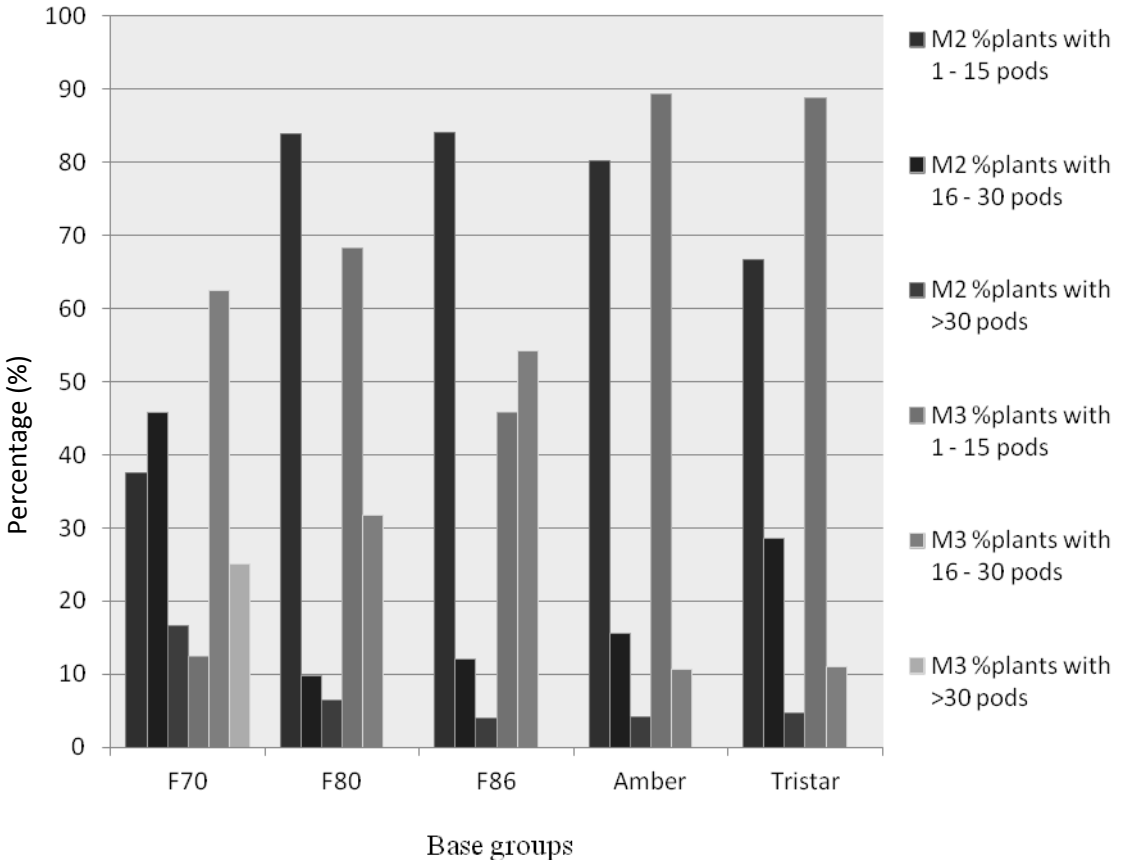

Figure 2.2. Percentage of plant producing different number of pods for M2 and M3 mutant generations for each base population.

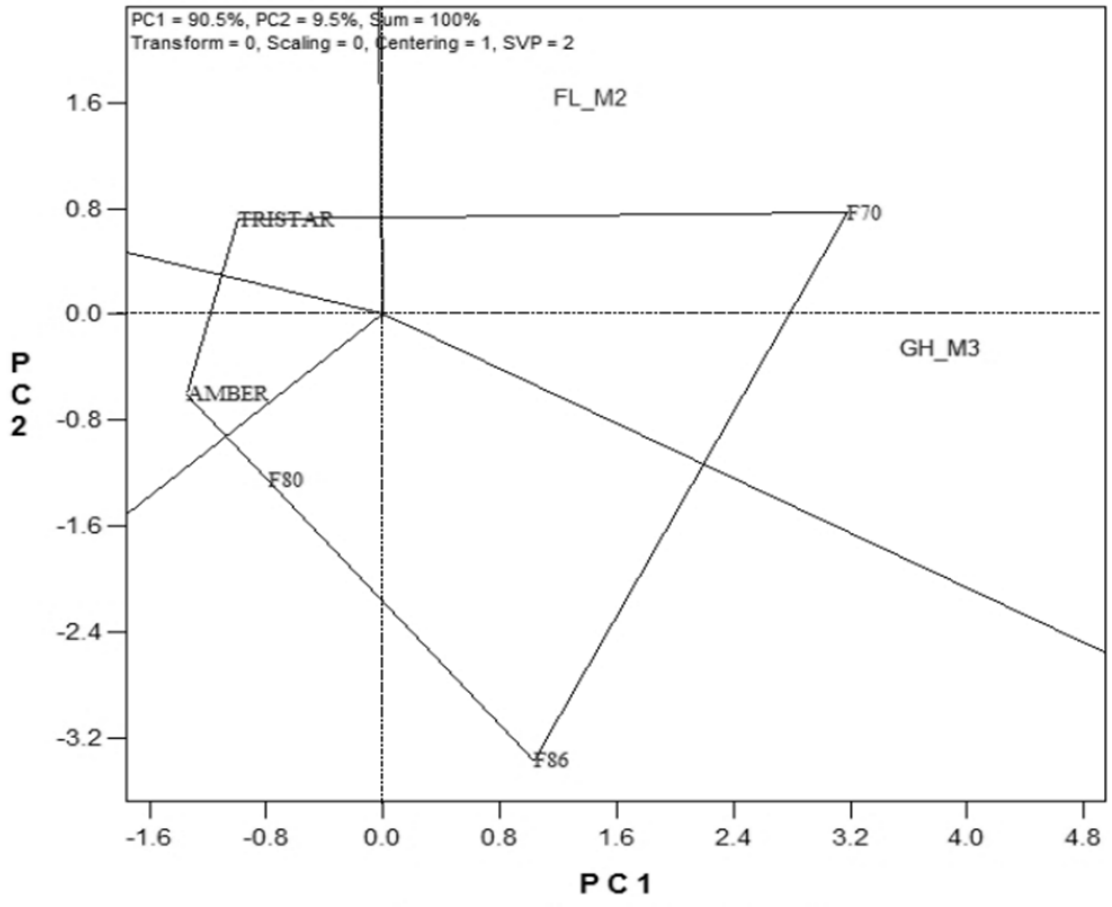

Figure 2.3. The "which wins where" view of GGE biplot for assessment of variation in pod number per plant of the mutant groups over two mutant generations (FL_M2 = field grown M2¬ generation, and GH_M3 = greenhouse grown M3 generation). Variation in pod number per plant was assessed using a Principle Component Analysis of mutated plants from five different genotypes (Tristar, Amber, F80, F70, and F86) over two generations (FL_M2 and GH_M3). Highly positive PC 1 scores indicate superior performance, whereas PC 2 scores close to 0.0 indicate trait stability.

It is encouraging to note that plants with some beneficial traits (such as apical flowers, double pods, and multiple basal stalks) were found in the mutant treatment groups in every generation (Figure 2.4). Presence of an apical flower is considered a sign of the determinate growth habit since presence of apical flowers is a sign of arrested apical growth, and it is expected that these plants will help the lower pods to fill and mature early (Petropoulos 2002). Presence of double/twin pods is considered an indication of higher diosgenin content in fenugreek seeds (Lee 2009; Petropoulos 2002), whereas presence of multiple basal stalks is reported to have positive correlation with higher seed yield (Gangopadhyay et al. 2009; Singh and Pramila 2009; Chandra et al. 2000; Sharma et al. 1990). However, these traits were not present in the base populations and if present, they only are marginally expressed.

The mutant groups produced a good proportion of double or twin pods in the M_2 and M_3 generations. The proportion of plants with double pods was improved as the number of mutant generations increased (Table 2.3). Very few double pods were produced in the M_2 generation (Fig. 2.5). In the M_2 generation highest mean for double pod number per plant was obtained in the F70 group (1.0±0.49), while the lowest mean was observed in the Tristar treatment group (0.4±0.22) (Table 2.3). In the M_3 generation, the highest mean for double pod numbers per plant (11.06±1.04) was also observed in the F70 group, and the lowest mean (1.11±0.37) was found in the Tristar group (Table 2.3.). The frequency of plant producing double pods was highest (25.4%) in the Amber treatment group and lowest (14.3%) in the Tristar group for the M_2 generation, while 96.9% of the F70 group had double pods when only 29.6% plants of the Tristar group showed double pods in M_3 generation (Table 2.3). For the M_2 generation, the plant

Table 2.4. Range of different morphometric parameters of field grown M_2 plants and greenhouse grown M_3 plants.

Genotype used as base populations	M_2 generation					M_3 generation				
	Height (cm)	Pod number	Double pod number	Seed weight (g)	Dry biomass weight (g)	Height (cm)	Pod number	Double pod number	Seed weight (g)	Dry biomass weight (g)
Amber	18.3 – 59.4	1 - 38	2 - 6	0.1 – 2.8	4.1 – 19.3	15.5 – 51.5	0 – 24	2 – 18	0 – 3	2.2 – 12.1
F70	25.4 – 56.6	4 – 40	2 – 10	0.1 – 6.2	5.8 – 16.6	28.2 – 63.7	9 – 43	4 – 24	1 – 5.8	6.8 – 19.4
F80	20.0 – 55.9	1 – 55	2 – 6	0 – 4.7	4.6 – 17.2	15.2 – 62.0	0 – 26	4 – 14	0 – 3.7	0.7 – 13.4
F86	25.9 – 66.8	1 – 40	2 – 6	0 – 2.9	4.1 – 16.1	37.8 – 64.0	0 – 27	4 – 20	0 – 3.2	4.4 – 16.9
Tristar	18.3 – 59.4	1 - 38	2 - 6	0.1 – 2.8	4.1 – 19.3	19.8 – 73.4	2 - 18	2 - 6	0 – 2.7	2.1 – 16.3

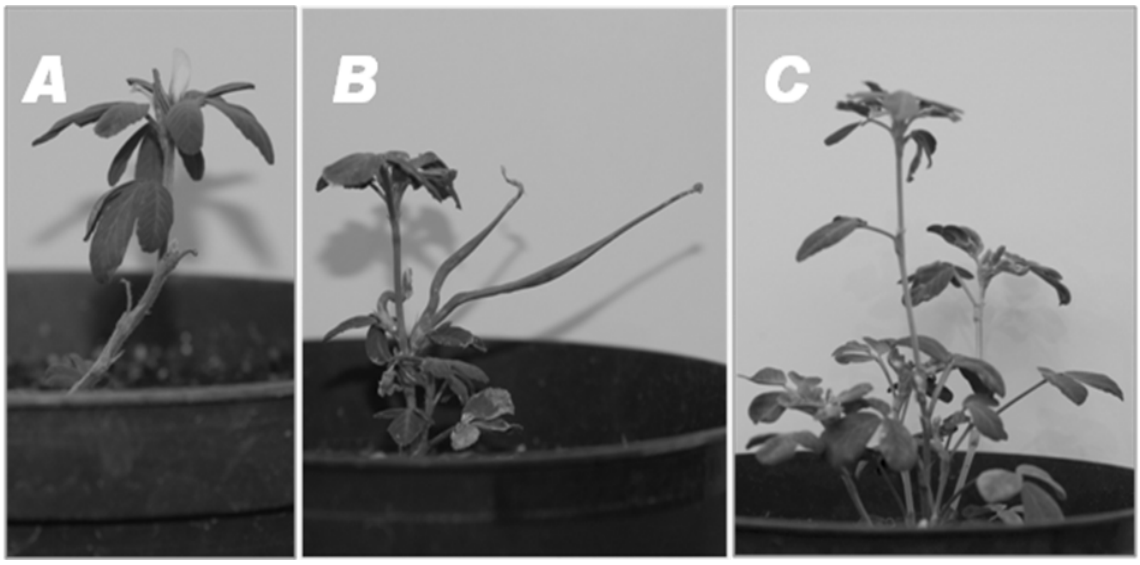

Figure 2.4. Mutant plants showing (A) apical flowers, (B) double pods, and (C) multiple basal stalks.

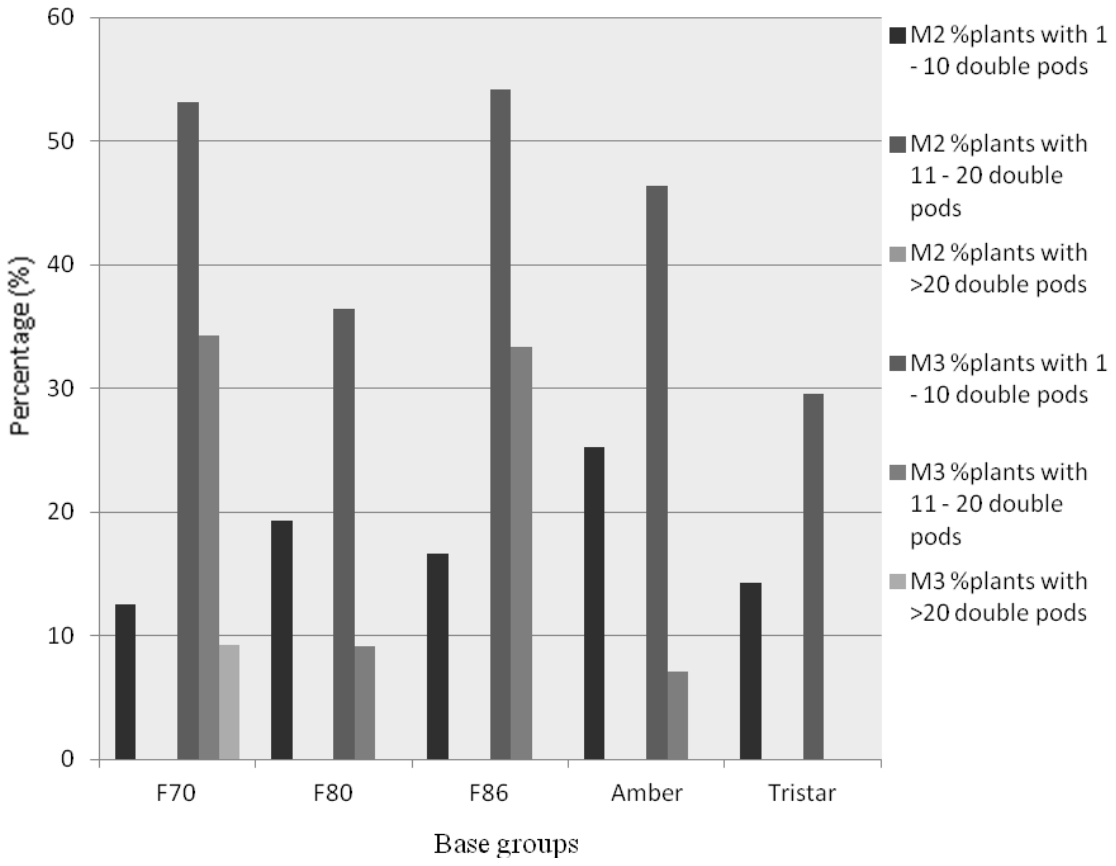

Figure 2.5. Percentage of plant producing different number of double pods for M2 and M3 mutant generations for each base population.

producing maximum number of double pods (10) was found in the F70 group, while the plant producing maximum number of double pods (24) was found in the F70 group for the M_3 generation (Table 2.4).

The highest mean for seed weight per plant (1.96±0.31g) and the lowest mean (0.54±0.1 g) were found in the F70 and F80 groups, respectively, for the M_2 plants. For the M_3 generation, the highest mean for seed weight per plant (2.73±0.19 g) and the lowest (1.18±0.18 g) were found in the F70 and the Amber groups, respectively (Table 2.3). For the M_2 generation, the plant producing highest amount of seed (6.2 g) was found in the F70 group. For the M_3 generation, the plant producing highest amount of seed (5.8 g) was also found in the F70 group (Table 2.4). Every mutant groups produced plants with different amount of seed in different frequencies in the M_2 and M_3 generations (Figure 2.7). The overall mean seed weight per plant in the M_3 generation for all the groups increased in comparison to that of the M_2 generation (Table 2.3). The GGE biplot analysis on seed weight per plant showed that all the mutant groups were responsive (Figure 2.8). The analysis showed that mutant treatment group F70 (high PC 1 score) was superior for seed yield per plant. The mutant groups Tristar and F70 were found more stable (low PC 2 scores) for this trait. Although all the mutant groups performed well in the greenhouse grown M_3 generation in comparison to field grown M_2 generation (revealed by their close proximity to GH_M3 in the biplot), the mutant group F70 were found more suited in the M_3 generation for this trait. The given scores for PC 1 and PC 2 showed that the M_3 generation was superior and more stable than the M_2 generation for this trait.

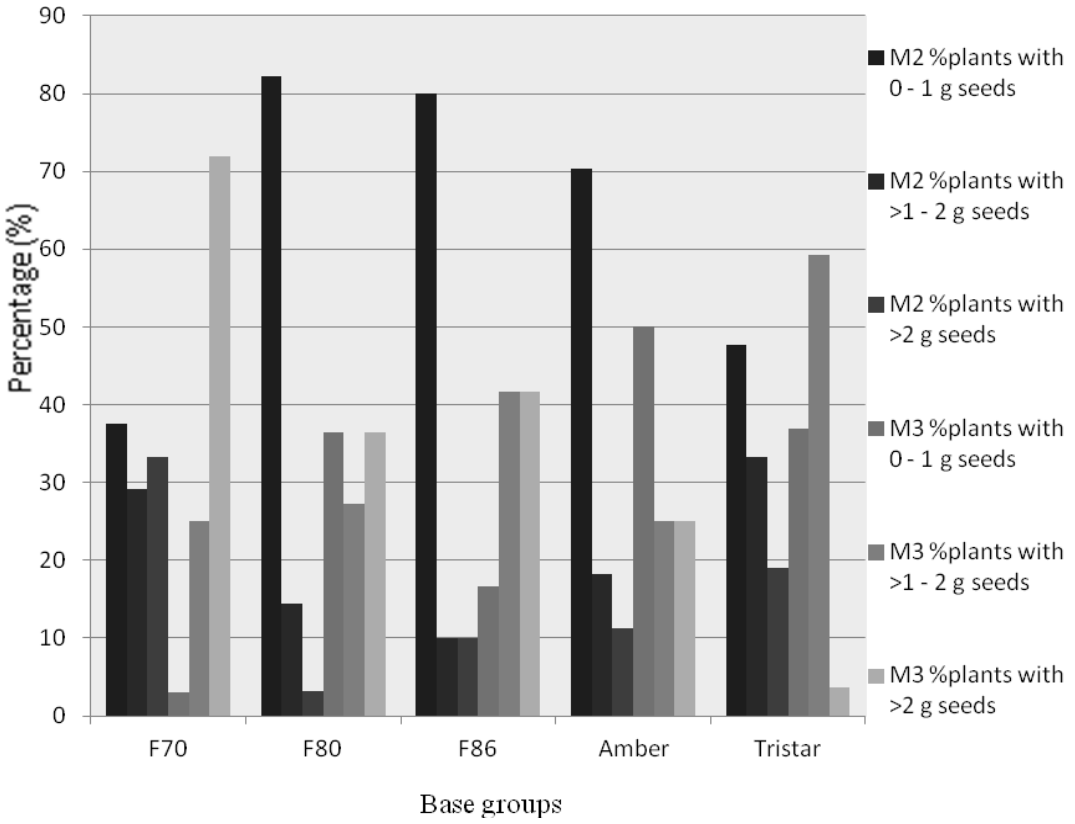

Figure 2.6. Percentage of plant producing different amount of seed for M_2 and M_3 mutant generations for each base population.

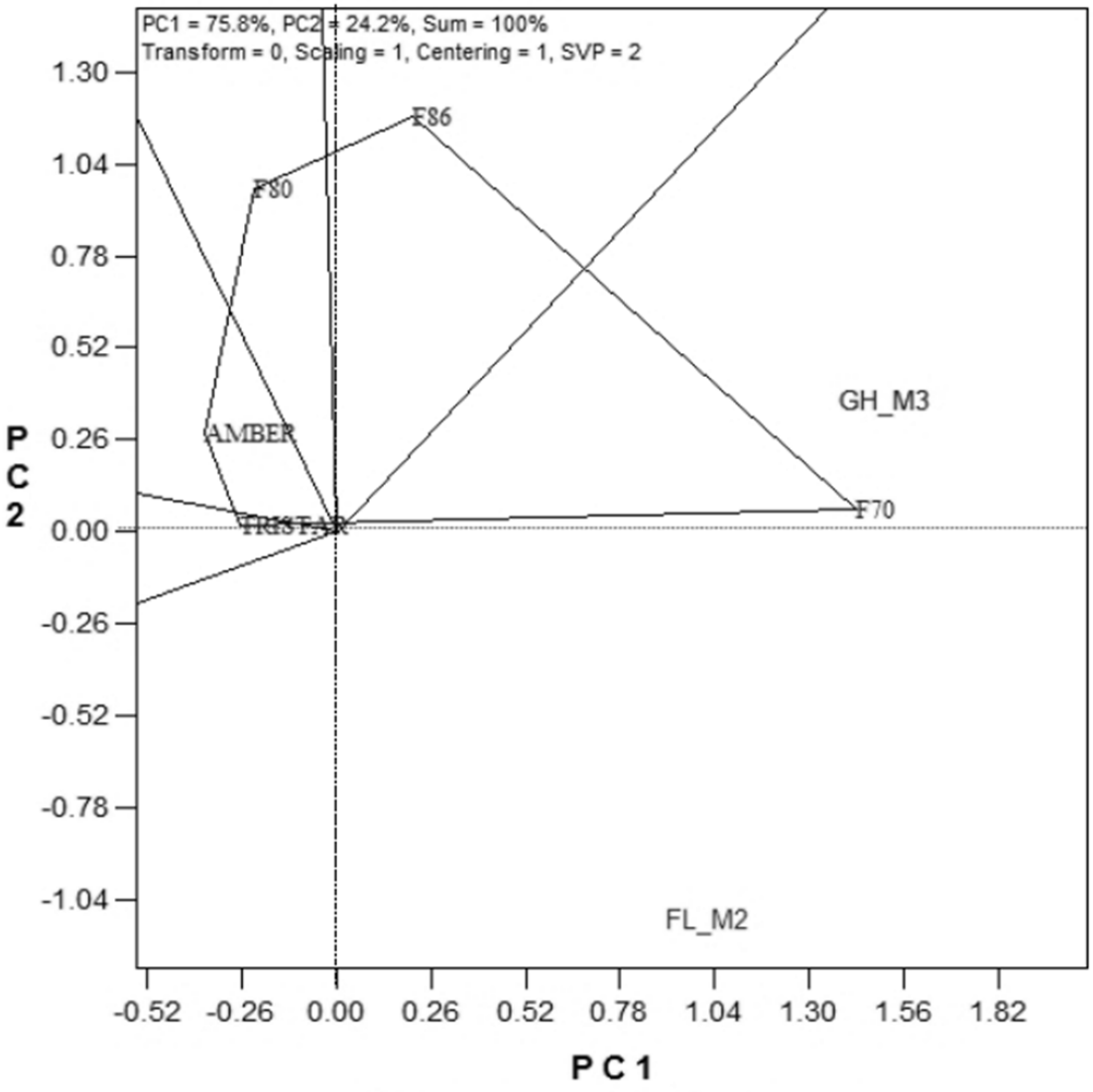

Figure 2.7. The "which wins where" view of GGE biplot for assessment of variation in seed weight per plant of the mutant groups over two mutant generations (FL_M2 = field grown M2¬ generation, and GH_M3 = greenhouse grown M3 generation). Variation in seed weight per plant was assessed using a Principle Component Analysis of mutated plants from five different genotypes (Tristar, Amber, F80, F70, and F86) over two generations (FL_M2 and GH_M3). Highly positive PC 1 scores indicate superior performance, whereas PC 2 scores close to 0.0 indicate trait stability.

The highest mean for dry biomass per plant (12.9±0.81 g) and the lowest mean (8.5±0.45 g) were found in the Tristar and F86 groups, respectively, for the M_2 plants. For the M_3 generation, the highest mean for dry biomass per plant (13.0±0.5 g) and the lowest mean for dry biomass per plant (5.3±0.44g) were found in the F70 and Amber groups, respectively (Table 2.3). For the M_2 generation, the plants producing highest amount of dry biomass (19.3 g) belonged to the Amber and Tristar groups. For the M_3 generation, the plant producing highest amount of dry biomass (19.4 g) was found in the F70 group (Table 2.4). The overall mean for dry biomass per plant in the M_3 generation for all the groups except for the F70 group and the F86 group decreased in comparison to that of the M_2 generation (Table 2.3). The GGE biplot analysis on dry biomass weight per plant (Figure 2.9) showed that all mutant groups were responsive for this trait. The location of the F70 group at the farthest right of the polygon (high PC 1 score) indicates superior performance for this trait. The mutant group Amber was found more stable groups (low PC 2 scores) for this trait. The mutant group F70 performed well in greenhouse grown M_3 generation, while the Tristar group performed well in field grown M_2 generation (revealed by its close proximity to GH_M3 and FL_M2 in the biplot). The generation M_3 (revealed by its furthest distance from the biplot origin) was found more discriminating than the M_2 generation. The given scores for PC 1 and PC 2 showed that the M_3 generation was superior and more stable than the M_2 generation for this trait.

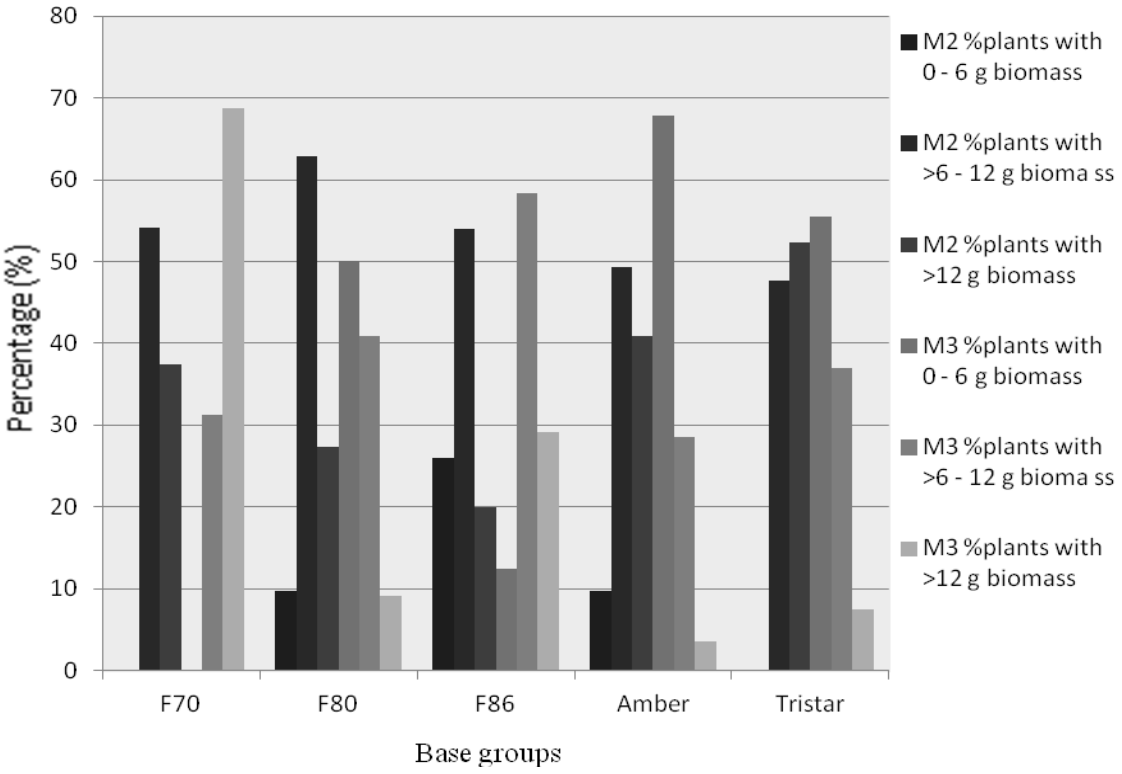

Figure 2.8. Percentage of plant producing different amount of dry biomass for M_2 and M_3 mutant generations for each base population.

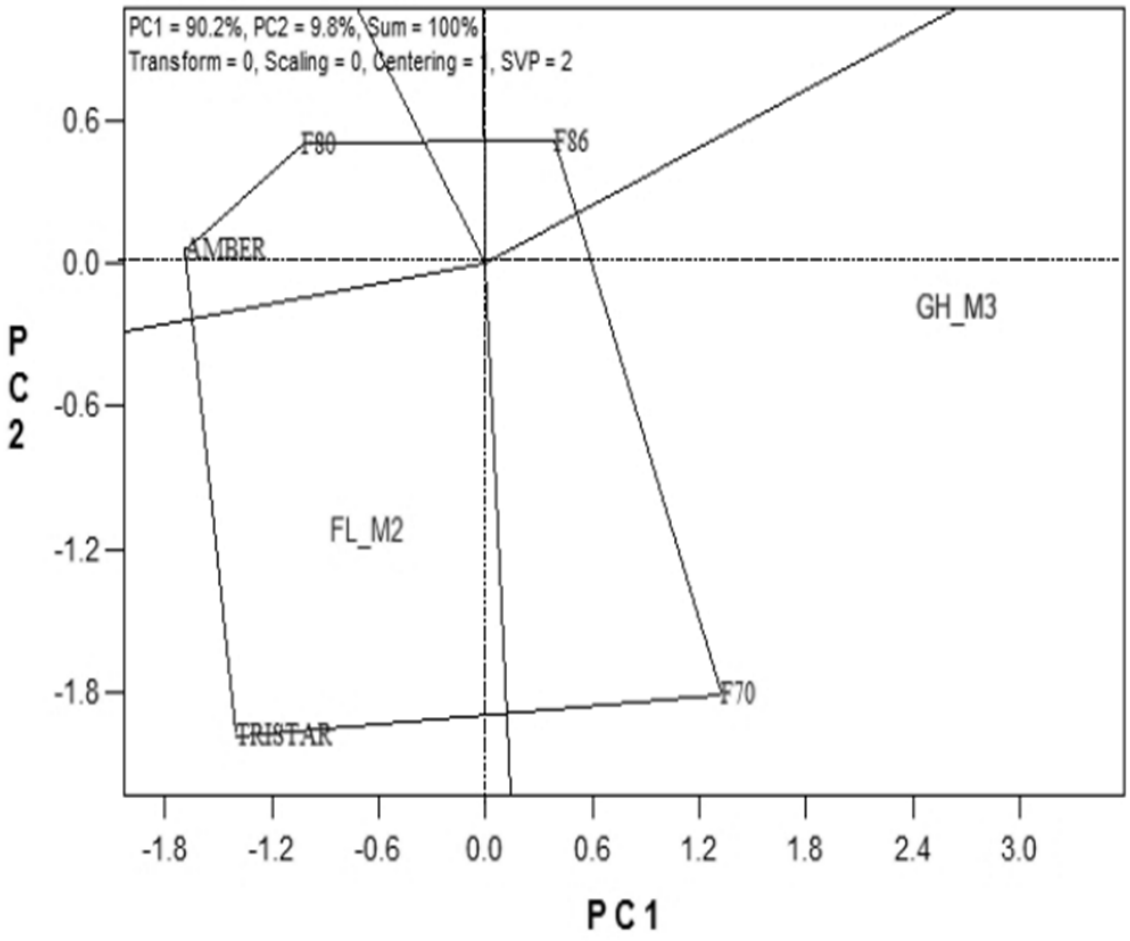

Figure 2.9. The "which wins where" view of GGE biplot for assessment of variation in dry biomass per plant of the mutant groups over two mutant generations (FL_M2 = field grown M2¬ generation, and GH_M3 = greenhouse grown M3 generation). Variation in dry biomass per plant was assessed using a Principle Component Analysis of mutated plants from five different genotypes (Tristar, Amber, F80, F70, and F86) over two generations (FL_M2 and GH_M3). Highly positive PC 1 scores indicate superior performance, whereas PC 2 scores close to 0.0 indicate trait stability.

In the mutant generations (M_1 to M_3), no distinct pattern in earliness (maturity) was detected. The mutant plants from different groups exhibited a random pattern of maturation; *i.e.*, some plants matured earlier while some matured late. Some plants in the mutant populations produced apical flower that is considered a sign of determinate growth habit since presence of apical flower refers the arresting of apical growth quickly thus making the seed pods mature early (Basu et al. 2009; Petropoulos 2002).

2.4 Discussion

In this study it was found that mutagenic treatment severely affected germinability of the mutant groups in M_1 generation confirming earlier mutation work done on fenugreek using chemical mutagens (Basu et al. 2008; Siddiqui et al. 2008). In the previous studies the authors concluded that EMS and sodium azide treatment result in reduction of fenugreek seed germination compared to control in a dose-dependent manner. In the present study dose was not considered and so comments in this regard cannot be made. However, the base populations differed in their response to mutagenic treatment confirming earlier work about use of multiple adapted cultivars for crop improvement using mutation breeding (Yadav et al. 2007).

Seeds of different base groups treated with EMS produced mutants of different kinds in the present study. Each group of mutants has shown high level of variability for all the traits evaluated across all the mutant generations, which was an important indication of mutation induction on multiple traits. Basu (2006) also observed modifications in more than one character in fenugreek treated with mutagenic agent. This may have resulted from a pleiotropic effect of mutated genes or mutations at different loci. Singh (2005) stated that mutations generally have pleiotropic effects due to closely linked gene(s). Singh and Singh (1974) also suggested that there is a high possibility that gene involved for a trait could have pleiotropic effects or that the trait(s) involved is governed by multiple genes with tight linkage that is being transmitted as a single unit. Saini et al. (1974) reported that for a sterile mutant of legume crop *Phaseolus aureus*, although many characters were affected, only single gene differences were detected. Sjodin (1971) observed a number of morphological mutations that exhibited

modifications in more than one character in legume plants (Sjodin 1971). It is also possible that a change in a single base pair in the DNA was involved for bringing changes in each trait separately. Such a phenomenon was observed by Singh and Singh (1974) in a spontaneous “green-trailing” fenugreek mutant.

The results of the present study indicate that multiple traits have been affected, but whether this is due to alteration in a single gene or multiple genes or tightly linked genes acting as one functional gene complex is not clear. The present study indicated that mutagenesis using EMS was able to generate a large amount of variability in the fenugreek population and many mutant lines showed important traits that were rare among world collections (Figure 2.5).

Mutation breeding is more adaptable for inducing recessive genes than dominant genes (Toker et al. 2007). Micke and Donini (1993) suggested that as mutations are mostly recessive, they cannot be selected for until the second generation, thus it is common practice to use only normal-looking M_2 plants to obtain the M_3 generation and to apply preliminary visual selection in M_3 (Begum and Dasgupta 2010). In this study, natural selection was applied in M_2 generation. The selection criteria resulted in a reduction in poor performing phenotypes or deleterious alleles for the traits being examined in M_3 generation. Strong selection pressure was not used in this study as the generation was an early generation. The plants in early generations generally segregate and may not be phenotypically highly desirable, but some of them can give rise to highly desirable plant(s) with superior combination of desirable traits in successive generations. Therefore strong selection pressure was not applied in early generations in this study as

was suggested by Fehr (1987). In later generations multiple homozygote formation is likely to take place leading to expression of traits controlled by recessive gene(s).

The natural selection in M_2 generation brought changes in mean values for the traits in every mutant group. The natural selection in M_2 generation decreased percentage of poor performing plants and increased percentage of better performing plants for traits in M_3 generation (Figure 2.3, 2.6, 2.7 and 2.9). Selection has been reported in fenugreek (Basu 2006), *Vigna radiata*, *Vigna mungo* (Kozgar et al. 2010), and soybean (Pavadai et al. 2010) to have effect to bring increase in mean values for yield and other agronomic traits in successive mutant generations. Mean seed weight per plant, mean number of double pod per plant was higher in M_3 groups than M_2 groups (Table 2.3 and Figure 1). Mean height and mean pod number per plant also increased in M_3 generation except for Amber and Tristar groups. Mean dry biomass per plant increased for only F70 and F86 groups in M_3 generation. These improvement for the traits evaluated must have been acquired through mutation induction and effectiveness of natural selection on mutant generation M_2 (Chatterjee et al. 2011). Basu (2006) also reported reduction in mean biomass weight per plant in M_3 generation. Increase in mean for the traits evaluated along with advancement of generations also indicate increase in stability level for the traits. Continued selection in successive generations for desirable characters is expected to further stabilize important characters in later generations.

Broadening the genetic base through induced mutation is a supplementary tool by creating genetic variability for specific traits in a crop when there is lack of variability. This genetic variability can then be used for crop improvement through conventional breeding techniques (Chatterjee et al. 2011). Therefore, mutation breeding is an

appropriate and valuable method for fenugreek improvement in Canada. The objectives of the present study were to generate variability in locally adapted genotypes, the results indicate that this was achieved. Advancement of generations of the generated mutant groups and appropriate selection for desired trait(s) among the mutant groups can result in development of superior cultivars in this species. If for some reason all the desirable traits are not available in any mutant then the selected genotypes can be used as important germplasm in fenugreek breeding program. More studies should be done in future to determine if there is any change in the chemical constitution and oil content due to pleiotropic effects of the mutations.

Chapter Three: Mutant Evaluation

3.1 Introduction

As a cultivated crop, fenugreek is gaining popularity in countries that are not traditionally large producers of this plant species, such as America, Australia and Canada (Acharya et al. 2008; McCormick et al. 2006; Berti et al. 1993). In Europe and North America, fenugreek is more admired as a component in artificial flavorings such as maple and butterscotch, and mostly as a health-food or nutraceutical product (Slinkard et al. 2006). Although fenugreek cultivar development activities have been focused mainly on development of genotypes for the spice market, interest in fenugreek has also extended to development of forage-type genotypes in Western Canada (Acharya et al. 2010b, 2007). "Tristar" is the first forage fenugreek cultivar developed by Agriculture and Agri-Food Canada (AAFC); it was developed at the Lethbridge Research Centre (LRC) in Alberta, Canada for its ability to produce a high biomass yield consistently (Acharya et al. 2010b).

Although fenugreek is fairly new to the Canadian agriculture, some genotypes of this species are found to be adapted for growth under rain-fed conditions of western Canada (Acharya et al. 2008, 2007). Most of these fenugreek genotypes including "Tristar", capable of producing good amount of biomass in western Canada exhibit an indeterminate growth habit and take more than 120 days to produce a good proportion of high quality seed (Basu et al. 2006). Although for forage production indeterminate growth habit helps high biomass production, seed production is adversely affected as the growing season in western Canada is short (~ 100 days). Previous work has made use of mutation breeding to generate new breeding material for fenugreek improvement in western Canada. In that work, seeds from "Tristar" were treated with different levels of

EMS and plants generated from the treated seeds that exhibited a determinate growth habit, high seed yield and relatively early maturity were selected (Basu 2006). This mutation breeding approach produced new breeding materials exhibiting variation for different agronomical characteristics (Acharya et al. 2008). The seeds from the mutated plants were increased to produce a base for plant selection and eventual production of new early maturing fenugreek cultivar(s) for western Canada.

Objectives of the present study were to:

i. evaluate and advance the mutant generations in multi-location trials.

ii. develop stability indices for the agronomic traits.

iii. identify correlations among morphological traits to facilitate indirect selection for desirable agronomic traits.

3.2 Materials and Methods

3.2.1 Seed material

Fenugreek (*Trigonella foenum-graecum* L.) seeds from a previous mutation breeding project were obtained from the Lethbridge Research Centre, Lethbridge, Alberta. The seeds were results of a mutation breeding experiment that used Tristar as base population and EMS as mutagenic agent. Bulk seeds derived from M_3, M_4, M_5 and M_6 harvest in 2007, 2009 and 2010 were used for the purpose. In the year 2009, M_7 seeds from a M_6 generation were collected and included in 2010 test. Seeds of "Tristar" was used as a control in all field tests.

3.2.2 Growing environments

The multi-environment study was conducted over three cropping years (2007, 2009 and 2010) at Lethbridge, Alberta and at Creston, British Colombia. In 2007, the trials were planted under two growing conditions, rain-fed and irrigation, in Lethbridge. In 2009, the trials were conducted under rain-fed growing conditions in both Creston and Lethbridge. For the year 2010, there were two growing conditions, rain-fed and irrigation, in Lethbridge, and one growing condition (rain-fed) in Creston. For statistical purposes, each year × growing condition × location was considered as a growing environment (Lin and Binns 1991). The growing conditions, locations and years produced a multi-environment trial with a total of seven growing environments.

3.2.3 Location parameters

Lethbridge: Lethbridge (49º 45' N and 112º 45' W) is located in southern Alberta (AB), Canada with an average elevation of 900 m MSL (Harsh 1985) and is about 216 km south-east of Calgary. It is situated in a semi-arid zone, with a moderate continental type climate characterized by mild summers and warm winters (Harsh 1987). The soil type of the area is Orthic Dark Brown Chernozem (Wyatt et al. 1939). The average annual maximum and minimum temperatures are 12.1 ºC and - 1.0 ºC respectively (Environment Canada). The annual average snowfall is around 1600 mm and the average annual precipitation is about 262 mm. The GPS coordinates for the 2007 LRC irrigated field was 49º 42' 24.98" N and 112º 45' 47.77" W and for the 2007, 2009 and 2010 LRC rain-fed field was 49º 42' 16.74" N and 112º 45' 55.41" W, while the GPS coordinates for the 2010 LRC irrigated field was 49º 42' 1.73" N and 112º 45' 55.36" W. The average monthly temperature, maximum temperature, minimum temperature and monthly average precipitation for the growing seasons for this location are presented in Table 3.1.

Creston: Creston (49º 10' N and 116º 31' W) is in British Columbia (BC), Canada, 760 km east of Vancouver. The elevation of Creston is 762 m MSL. It represents a temperate climate with a relatively long growing season. Creston provides good crop growing weather without the high or low temperature extremes. The summers in Creston are warm and sunny, though the winters are mild as a result of Pacific systems crossing British Columbia and the influence of Kootenay Lake (Harsh 1985). The soil type at Creston is a stone-free alluvial deposit of Carbonated Rego Gleysol (composed of silt loam and silty clay loam) with poor to moderately poor drainage (Wittneben and Sprout 1971). The average annual maximum and minimum temperatures are 12.9 ºC and 3.1 ºC respectively

(Environment Canada). The average annual snowfall is 1406 mm and the average annual rainfall is 454 mm (Wittneben and Sprout 1971). The GPS coordinates for the 2009 and 2010 Creston rain-fed field was 49º 06' 28.43" N and 116º 34' 06.66" W.

3.2.4 Experimental design

The mutant generations M_3, M_4, M_5 and M_6 including Tristar were used every year, while the mutant generation M_7 was added to the trials for the year 2010. The fenugreek mutant generations and Tristar were seeded into 3×2 m^2 plots with 10 rows spaced 18 cm apart. The plots were arranged in a Randomized Complete Block Design with four replicates at each growing environment. The seeding rate for each mutant generation and Tristar in all of the environments was 15 kg ha^{-1}. The seeding was done on May 18th for 2007 LRC irrigation and rain-fed trials; on May 26 and May 19, respectively for LRC rain-fed and Creston rain-fed fields in 2009; and in 2010 LRC irrigation and rain-fed trials were seeded on June 11th while Creston rain-fed trial was seeded on May 19th.

For weed control Edge (Dow AgroScience Canada Inc.) and Odyssey (BASF Canada) were used in the field experiments and Reglone (Syngenta Crop Protection Canada Inc.) desiccant was used for the seed yield trials. Edge was applied about two weeks before the seeding of fenugreek seeds, while Odyssey was applied on the field after two weeks of fenugreek seed germination. The plants were desiccated two weeks before harvesting the seeds using Reglone.

For seed yield trials, at maturity individual plots at each test site were mechanically harvested using a small plot Wintersteiger combine harvester. The seed

harvesting was done on September 5th in 2007 at LRC sites; and on September 23 and October 7th in 2009 at Creston and LRC sites, respectively. In 2010 the seed harvesting was done on October 14th and November 4th at Creston and LRC sites, respectively. For forage yield, fenugreek plants from an 0.25 m^2 area were harvested from each plot with in 10th to 12th week after seeding.

In this study, for seed yield and forage yield whole plots were visually evaluated. This whole plot evaluation provided a general idea about the performance of the fenugreek mutant generations (M_3 to M_6) over different environments, but did not provide a quantitative estimation of individual performance of the mutant generations. For segregating populations, this is a common criterion when the plot harvest data is used for evaluation (Fehr 1987). To get a specific idea about the mutant generations (M_3 to M_7), randomly picked plants were used to gather data for selected agronomic traits. As these plants were picked without any biases, the plants represented the generation. The data from randomly picked plants was often discriminative for different segregating populations (Begum and Daasgupta 2010; Khan and Wani 2005; Njunie et al. 1996)

For the year 2010, when plants were cut to determine forage yield, five plants were randomly picked and tagged from each plot. These tagged plants were manually harvested before two weeks of harvesting for seed yield. These plants were used to determine generation and environment effects on plant height, pod number, double pod number, seed yield, biomass yield and basal stalk number. After harvesting, the plants and the seeds were dried in a ventilated dryer for several days to remove moisture from the plants and the seeds. Plant height was measured from the ground to the top most point of the plant. Plants were cut at about 2.54 cm above ground, and then kept for drying.

Individual plants was weighed for dry biomass after drying. Seeds were extracted manually from each plant and then weighed.

3.2.5 Statistical analysis

Treatment (mutant generation and growing environments) effects on seed yield, forage yield, plant height, pod number, double pod number, seed yield (individual plant), biomass yield (individual plant) and basal stalk number were subjected to Analysis of Variance (ANOVA) using PROC MIXED (SAS Institute, Cary, NC) software. The data were subjected to Square Root Transformation as the CVs were high indicating that some of the ANOVA assumptions were not met. Whenever the main effects of mutant generations and environments were found significant at $p \leq 0.05$, treatment mean comparisons were made using Tukey's Honestly Significant Difference test. Microsoft Excel 2007 program was used to calculate the correlation coefficient among different agronomical traits.

Table 3.1. The monthly average temperature, maximum average temperature, minimum average temperature and monthly average precipitation for each growing in this study.

Location year		Month						
		May	**June**	**July**	**Aug.**	**Sept.**	**Oct.**	**Nov.**
2007 Lethbridge	Average temperature (^{o}C)	12.4	16.7	22.8	17.9	12.5	8.6	0.1
	Maximum temperature (^{o}C)	18.6	23.2	31.1	26.3	19.6	14.8	5.9
	Minimum temperature (^{o}C)	5.9	10.2	14.6	9.6	5.0	2.3	-5.0
	Average precipitation (mm)	85.6	23.0	0.0	12.3	46.3	12.1	10.1
2009 Lethbridge	Average temperature (^{o}C)	11.0	14.3	17.6	17.4	16.8	3.6	3.8
	Maximum temperature (^{o}C)	18.0	21.2	24.3	24.8	26.0	8.4	9.7
	Minimum temperature (^{o}C)	4.4	7.4	10.9	10.1	8.2	-0.7	-1.4
	Average precipitation (mm)	34.8	65.7	53.0	80.4	7.4	46.4	16.4
2010 Lethbridge	Average temperature (^{o}C)	8.7	15.0	17.9	17.0	11.6	9.8	-3.0
	Maximum temperature (^{o}C)	14.4	20.9	24.7	23.6	17.0	16.3	2.8
	Minimum temperature (^{o}C)	2.9	9.2	11.2	10.5	6.5	3.2	-8.7
	Average precipitation (mm)	121.2	109.8	59.4	56.4	46.9	4.8	44.7
2009 Creston	Average temperature (^{o}C)	13.2	17.4	21.4	21.3	16.7	5.7	3
	Maximum temperature (^{o}C)	31.2	30.3	35.2	35.5	34	16.6	10.32
	Minimum temperature (^{o}C)	2	4.9	7.4	7.5	3.3	-6.6	-2.5
	Average precipitation (mm)	38	19.6	52.6	38.6	18.2	66.6	46.6
2010 Creston	Average temperature (^{o}C)	11.7	15.5	20.1	19.5	14	9.5	0.3
	Maximum temperature (^{o}C)	26	30	33.2	33.4	27.5	25.8	15
	Minimum temperature (^{o}C)	0	6	7.6	6.4	4.7	-1.4	-18.6
	Average precipitation (mm)	4	0.4	19	33	60.8	30.2	54.6

Source: Environment Canada

3.3 Results

Seed yield among growing environments varied significantly ($p \leq 0.01$) with a range of 229.5±19.7 kg/ha for Lethbridge irrigation in 2010 to 2290.0±121.1 kg/ha for Lethbridge irrigation in 2007 (Table 3.2 and 3.3). The growing environment for Lethbridge irrigation, 2010 produced the lowest seed yield, but it was statistically similar to the seed yield observed at Lethbridge rain-fed (Leth RF) and Creston, 2010. Lethbridge irrigation (Leth IR) 2007 produced the highest seed yield followed by Leth RF 2009. Seed yield observed at Leth RF 2007 was statistically similar to that of Creston 2009. Although the generation effect was not statistically significant ($p \geq 0.05$) for seed yield, the mutant generation M_5 (783.5±134.5 kg/ha) and the mutant generation M_6 (776.4±116.5 kg/ha) produced relatively good amount of seed (Figure 3.1). The check cultivar Tristar produced the higher seed yield (813.7±140.0 kg/ha) compared to the mutant generations. The generation M_3 was the lowest in seed yield (741.3±117.3 kg/ha), whereas the generation M_4 was the second lowest (758.0±146.5 kg/ha).

Table 3.2. Results of the ANOVA for seed and forage yield of fenugreek generations.

Seed yield (kg ha^{-1})		
Source of variation	**DF**	**Mean Square**
Generation	4	14632
Environment	6	11143012**
Generation × Environment	24	125937
Replication	3	402716*
Coefficient of variation (%)	20.23	
Forage yield (kg ha^{-1})		
Source of variation	**DF**	**Mean Square**
Generation	4	1361862
Environment	5	184514478**
Generation × Environment	20	1933605
Replication	3	578817
Coefficient of variation (%)	14.66	

** Denotes significance at $p \leq 0.01$. * Denotes significance at $p \leq 0.05$.

The mean forage yield expressed as kg ha^{-1} significantly ($p \leq 0.01$) varied among growing environments with a range of 1869.4±71.5 kg ha^{-1} to 8468.8±375.0 kg ha^{-1} (Table 3.2 and 3.3). Leth IR 2007 produced the highest forage yield among all the environments, but it was statistically similar with RF 2010, Creston 2010 and Leth IR 2010. Leth RF 2007 and 2009 produced the lowest forage yield among the growing environments. Like seed yield, generation effect was not statistically significant ($p \geq 0.05$) for forage yield. The mutant generation M_6 produced the highest forage yield (6091.2±637.6 kg ha^{-1}) followed by the mutant generation M_3 (5677.4± 577.5 kg ha^{-1}) (Figure 3.2). The generation M_5 was the lowest in forage yield (5390.6± 507.6 kg ha^{-1}), whereas the generation M_4 was the second lowest (5556.0±599.2 kg/ha). The control cultivar Tristar produced 5620.9±638.7 kg ha^{-1} forage yield.

Table 3.3. Mean seed and forage yield in the seven environments of fenugreek mutant generations.

Environment	Seed yield (kg/ha)	Forage yield (kg/ha)
Creston 2009	714.6 ± 97.4^{c}	
Creston 2010	240.0 ± 41.1^{d}	7495.2 ± 571.2^{a}
Lethbridge Irrigation 2007	2290.0 ± 121.1^{a}	8468.8 ± 375.0^{a}
Lethbridge Irrigation 2010	229.5 ± 19.7^{d}	7175.0 ± 281.7^{a}
Lethbridge dry 2007	869.6 ± 61.1^{c}	1869.4 ± 71.6^{b}
Lethbridge dry 2010	248.2 ± 22.0^{d}	7632.0 ± 210.5^{a}
Lethbridge dry2009	1216.2 ± 93.2^{b}	1908.0 ± 71.5^{b}

Means sharing similar superscripts within the same column were not significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$).

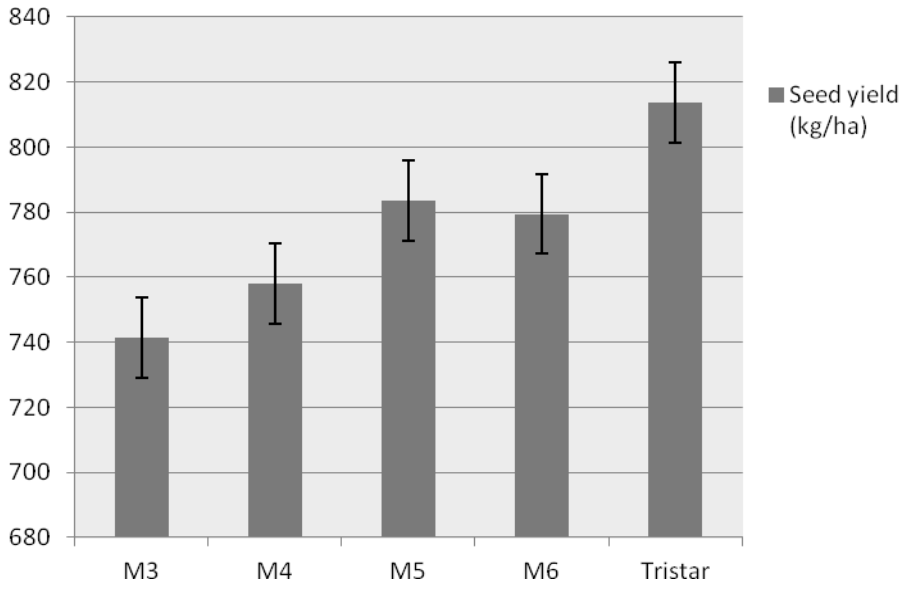

Figure 3.1. Mean seed yield (kg ha^{-1}**) based on plot harvest data of M_3 to M_6 generation along with control Tristar.**

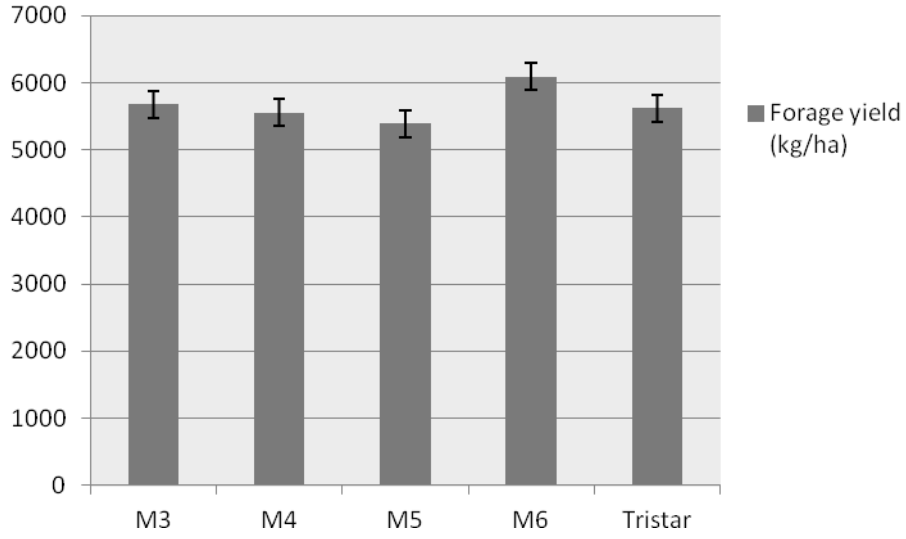

Figure 3.2. Mean forage yield (kg ha^{-1}**) based on plot harvest data of M_3 to M_6 generation along with control Tristar.**

The mutant generations M_3, M_4, M_5, M_6, and M_7, and the control cultivar Tristar were grown in three growing environments in 2010. Each generation was replicated four times at every environment. For every generation and for Tristar, five plants were randomly picked from every replicated plot for the analysis of some agronomic traits.

The plant height of fenugreek mutant generations varied significantly ($p \leq 0.01$) with a range of 52.8 cm for M_7 to 58.8 cm for M_4 (Table 3.4 and 3.5). The highest height was obtained by the M_4 generation, but it was not statistically different from M_3, M_5 and Tristar. The mean plant height of M_6 was statistically comparable to that of M_7, but was significantly lower than that of M_3, M_5 and Tristar. The plant height among growing environments significantly varied with a range of 47.6 cm to 59.8 cm. The mean plant height at Leth RF 2010 was the highest among the growing environments, and was statistically similar to Leth IR 2010. The plant height at Creston 2010 was the lowest. The Generation × environment interaction was found significant for plant height, although the interaction effect contributed only 16.6% of the total variation.

Pod number plant^{-1} of fenugreek generations tested varied significantly with a range of 19 for M_4 to 32 for M_7 (Table 3.4 and 3.5). The pod number plant^{-1} of M_7 was statistically similar to M_6 and M_5. The effect of growing environments on pod number plant^{-1} was not significant.

Table 3.4. Mean squares taken from ANOVA tables for plant height, pod number per plant, double pod number per plant, seed weight per plant, dry biomass weight per plant and basal stalk number per plant of fenugreek mutant generations grown in 2010.

Source of variation	DF	Height (cm)	Pod number	Double pod number	Seed weight (g)	Dry biomass weight (g)	Basal stalk number
Genarations	5	113.91**	1489.19**	41.75	18.56**	16.26*	8.17**
Environments	2	2228.43**	192.23	9.10	0.40	3.80	24.43**
Generation × Environment	10	100.27**	140.77	28.47	0.76	18.55**	0.87
Replication	19	116.41**	117.08*	34.23	1.53	9.65	2.31
Coefficient of variation (%)		8.1	19.9	175.2	26.6	9.0	28.5

** Denotes significance at $p \leq 0.01$. * Denotes significance at $p \leq 0.05$.

The effect of mutant generation on double pod number plant^{-1} was not found statistically significant. The number of double pods plant^{-1} among growing environment was not statistically significant as well. Moreover, the calculated value of coefficient of variation was very high (175.22) for this character.

Seed weight plant^{-1} among mutant generations varied significantly with a range of 1.75 g for M_4 to 3.13 g for M_7 (Table 3.4 and 3.5). The highest seed weight plant^{-1} was obtained by M_7 generation, and this was statistically similar to M_6 and M_5. The mutant generation M_4 produced the lowest seed weight plant^{-1}, but this was statistically similar to M_3 and Tristar. The effect of growing environments on seed weight plant^{-1} was not significant.

The dry biomass weight plant^{-1} of fenugreek mutant generations varied significantly with a range of 14.11 g for M_3 to 15.54 g for M_4 (Table 3.4 and 3.5). The highest dry biomass weight plant^{-1} was obtained by the M_4 generation, but it was statistically comparable to that of M_5, M_6, M_7 and Tristar. The mutant generation M_3 produced the lowest dry biomass weight plant^{-1} among the generations. The effect of growing environments on dry biomass weight plant^{-1} was non-significant. The Generation × environment interaction was found highly significant for dry biomass weight plant^{-1}. The Generation × environment interaction effect contributed only 67.60% of the total variation.

Table 3.5. Main effect of mutant generations and environments on the mean performance of plant height, pod number per plant, double pod number per plant, seed weight per plant, dry biomass weight per plant and basal stalk number per plant of fenugreek.

Generation	Height (cm)	Pod number	Double pod number	Seed weight (g)	Dry biomass weight (g)	Basal stalk number
M_3	55.14±1.54 ^{ab}	21.10±1.09 ^{cd}	1.37±0.44 ^{a}	1.83±0.12^{c}	14.11±0.35 ^{b}	1.70±0.10 ^{b}
M_4	58.85±1.67 ^{a}	18.81±1.12 ^{d}	1.00±0.34 ^{a}	1.75±0.12 ^{c}	15.54±0.38 ^{a}	1.85±0.13 ^{b}
M_5	55.80±0.99 ^{ab}	27.60±1.31 ^{ab}	2.13±0.54 ^{a}	2.65±0.16 ^{ab}	14.86±0.25 ^{ab}	2.10±0.15 ^{ab}
M_6	53.61±0.91^{b}	28.95±1.18 ^{ab}	3.13±0.66 ^{a}	2.84±0.14 ^{ab}	14.44±0.26 ^{ab}	2.37±0.19 ^{ab}
M_7	52.88±0.88 ^{b}	32.13±1.20 ^{a}	2.87±0.50 ^{a}	3.13±0.16 ^{a}	15.21±0.25 ^{ab}	2.70±0.17 ^{a}
Tristar	56.97±1.42 ^{ab}	25.70±0.97 ^{bc}	1.80±0.49 ^{a}	2.37±0.11 ^{bc}	14.60±0.31 ^{ab}	1.93±0.13 ^{b}
Environment						
Creston 2010	47.62±0.76 ^{b}	26.60±0.93 ^{a}	2.32±0.42 ^{a}	2.42±0.12 ^{a}	15.00±0.25 ^{a}	2.31± 0.11^{a}
Lethbridge irrigation 2010	59.25±0.81^{a}	24.26±0.86 ^{a}	1.77±0.31 ^{a}	2.37±0.09 ^{a}	14.67±0.20 ^{a}	1.59±0.07 ^{b}
Lethbridge dry 2010	59.76±0.81^{b}	26.28±0.82 ^{a}	2.07±0.35 ^{a}	2.49±0.09 ^{a}	14.71±0.19 ^{a}	2.43±0.12 ^{a}

Means that share similar superscripts within the same column under generation main effect and environment main effect are not significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$). The sample nimber (N) for each generation for each trait evaluated was 60.

The basal stalk number plant^{-1} varied significantly among mutant generations of fenugreek (Table 3.4 and 3.5). The highest basal stalk number plant^{-1} was obtained by the M_7 generation, and was statistically comparable to that of M_5 and M_6. The least basal stalk number plant^{-1} was found in M_3 which was statistically similar to that of M_4 and Tristar. The basal stalk number plant^{-1} also varied significantly among growing environments. The highest basal stalk number plant^{-1} was obtained at Lethbridge dry, 2010 and was statistically similar to Creston, 2010. The least basal stalk number plant-1 was found at Lethbridge irrigation, 2010.

Randomly selected plants from the M_3, M_4, M_5, M_6 and M_7 generations were subjected for calculation of means for selected agronomic characters (plant height, number of pods plant^{-1}, number of double pods plant^{-1}, seed weight plant^{-1}, biomass weight plant^{-1} and number of basal stalk plant^{-1}) for the above mentioned mutant generations. These means were plotted in a graph to show the trend of these agronomic characters over the mutant generations (Figure 3.3 and 3.4).

The mean plant height (Figure 3.4) increased in M_4 generation, and then it dropped steadily up to the M_6 generation. The mean plant height then again ascended slightly from M_6 to M_7 generation. The lowest mean difference between two immediate mutant generations for plant height was observed between M_5 and M_6. The mean number of pods plant $^{-1}$ dropped from M_3 to M_4 generation, and then the mean increased sharply from M_4 to M_5 generation and continued to increase steadily up to the M_7 generation (Figure 3.4). The lowest mean difference between two immediate mutant generations for this character was also observed between M_5 and M_6. The mean for the character,

number of double pods plant^{-1} dropped from generation M_3 to M_4, and then it increased steadily

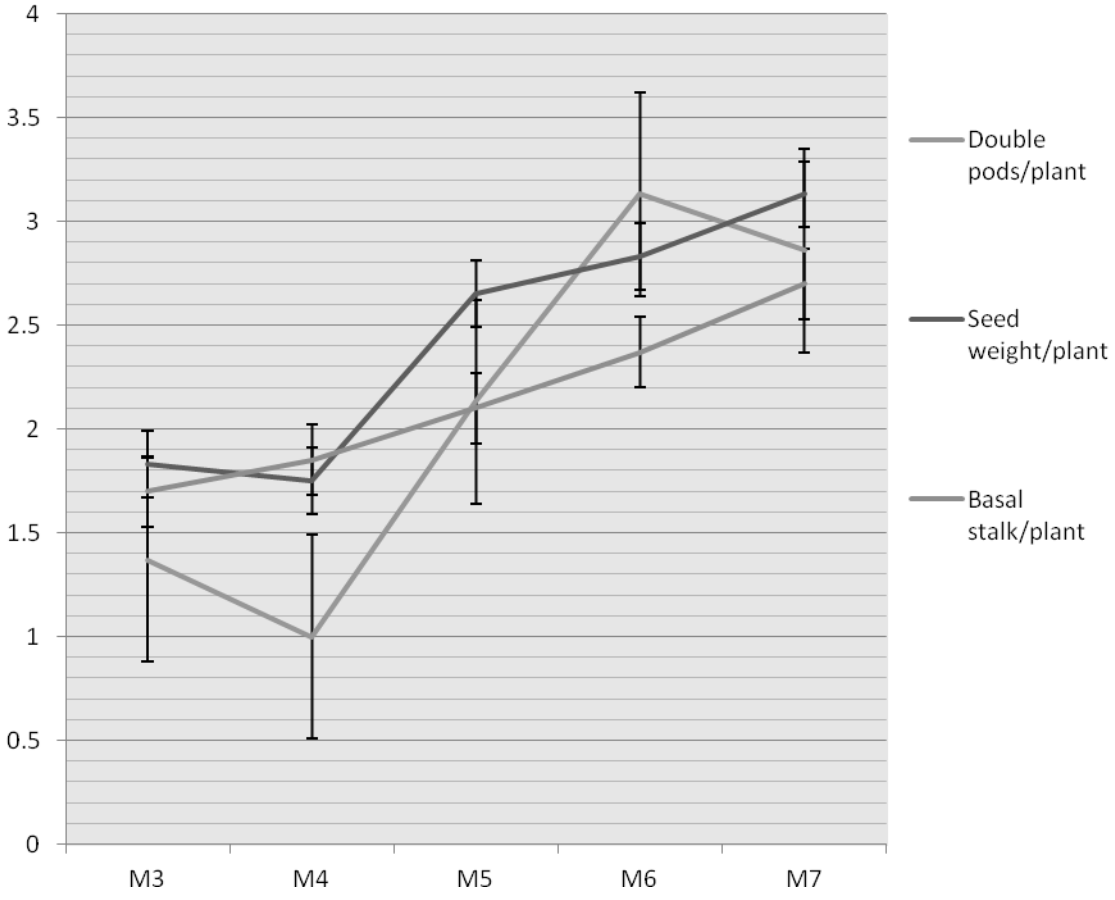

Figure 3.3. Trend of seed weight/plant, double pods/plant and basal stalk/plant over the mutant generations (M_3 to M_7) over all the growing environments (Creston 2010, Lethbridge Irrigation 2010, and Lethbridge Dry 2010).

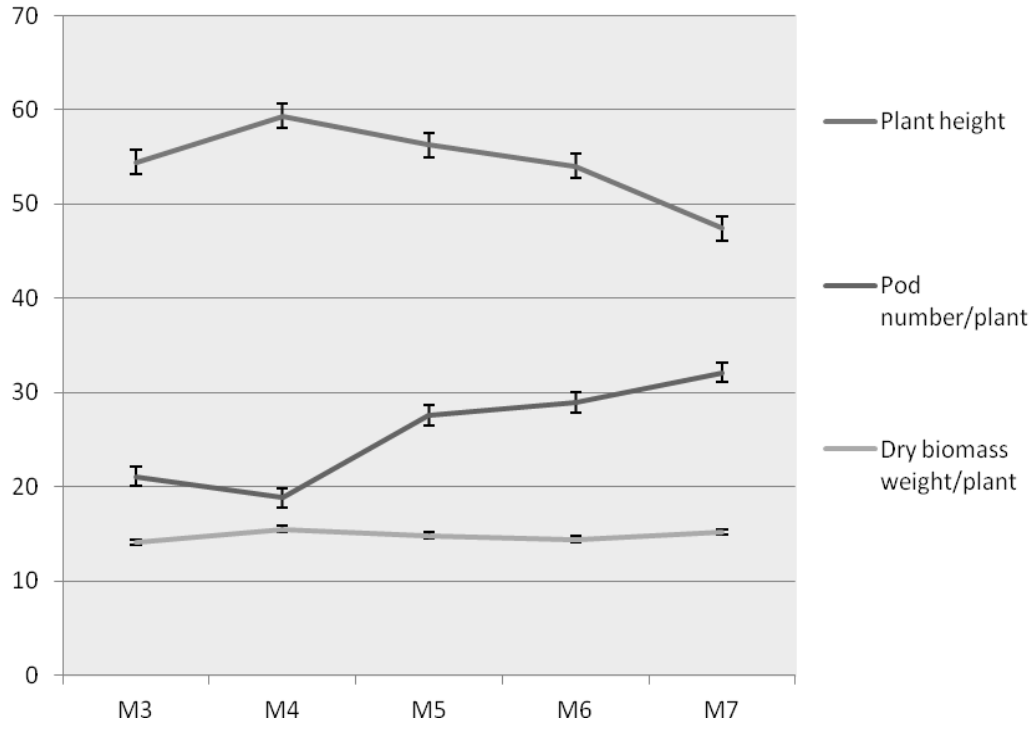

Figure 3.4. Trend of height/plant, pods/plant and biomass weight/plant over the mutant generations (M_3 to M_7) over all the growing environments (Creston 2010, Lethbridge Irrigation 2010, and Lethbridge Dry 2010).

up to generation M_6, and slightly dropped in generation M_7 (Figure 3.3). For the characters seed weight plant^{-1} and number of basal stalk plant^{-1}, the mean reduced from M_3 to M_4, and then increased steadily up to the M_7 (Figure 3.3). The mean for the character biomass weight plant^{-1} rose from generation M_3 to M_4, and then it dropped steadily up to generation M_6, and rose again in generation M_7 (Figure 3.4).

The lowest mean differences between most subsequent generations in advanced mutant generation for the character number of double pods plant^{-1}, seed weight plant^{-1}, biomass weight plant^{-1} and number of basal stalk plant^{-1} were found between M_6 and M_7.

The data for the selected traits gathered for the mutant generations and the control cultivar Tristar were analyzed to identify a correlation coefficient (*r*) for these traits. Pearson correlation coefficient values (*r*-values) representing associations among the six traits examined are presented in Table 3.6. Results indicated a strong significant positive correlation between seed yield and number of pods. Seed yield was also found positively correlated with number of twin (double) pods and number of basal stalks. Plant height was found to respond positively with biomass yield, and negatively with number of pods, seed yield and number of basal stalks. The number of basal stalks was positively correlated to number of pods and number of double pods as indicated by their *r*-values. Significant positive correlation was also found between number of pods and number of twin pods.

Table 3.6. Correlation coefficients (*r*) among traits measured on fenugreek mutant generation and check variety grown across 3 environments.

	Height	Number of pods	Number of twin pods	Seed yield	Biomass yield
Number of pods	**-0.369**				
Number of twin pods	-0.076	**0.416**			
Seed yield	**-0.321**	**0.834**	**0.379**		
Biomass yield	**0.575**	-0.056	0.034	-0.035	
Number of basal stalks	**-0.293**	**0.631**	**0.317**	**0.586**	0.061

Values in bold are significant at 1% level of probability.

3.4 Discussion

The genotype, environment, and genotype by environment interaction effects are commonly considered as the major factors influencing crop performance and so are important for plant breeding and crop production studies. Genotype, environment, and genotype × environment interaction effects for various traits have previously been studied in various legume crops including fenugreek (Acharya et al. 2010a; Sadeghzadeh-Ahari 2010, 2009; Basu et al. 2009; Berger et al. 2004; Arshad et al. 2003). However, information on impact of these sources of variation on seed and forage yield and yield components observed for different mutant generations of fenugreek are extremely limited.

In this study, the whole plot harvest data for seed and forage yield showed a significant environment effect for both of these characters. Lee (2009) evaluated ten adapted fenugreek genotypes in different environments of western Canada, and found that environment had a significant influence on seed yield. In another study with 83 world accessions of fenugreek, environmental effects were found significant for seed and forage yield (Basu et al. 2009). In this multi-environment trial, the highest seed yield was obtained for plots in Lethbridge that were irrigated in 2007. On average, weather conditions in 2007 were dry during the growing season. Fenugreek is known to be adapted to rain-fed growing conditions, but its yield can be increased considerably by application of minimal irrigation in dry areas such as those found in southern Alberta (Basu et al. 2009; Acharya et al. 2008; Petropoulos 2002). This study also is in agreement with earlier observations in this regard (Huang and Liang 2000; McCormick et al. 1998; Mir et al. 1993). The lowest seed yield was obtained for the environments examined in

2010. This observation may be due to high levels of precipitation that were available in Lethbridge during this growing season (Table 3.1). The prolonged rainfall and moisture availability observed might have delayed shift of the plant to a reproductive phase from a vegetative phase, and subsequent delay in flower initiation. In legumes flowering is mainly induced by water stress after the end of a maximum rainfall event (Lascano et al. 2002) and so the delay in flower initiation in 2010 may have been the reason for low seed yield. Andersson et al. (2006) also reported presence of a similar phenomenon in the legume crop *Cratylia argentea*. The opposite condition was observed for the environment observed at Creston in 2010. In Creston, the weather in 2010 was extremely dry during the first two months of the growing period (May and June) (Table 3.1). This extreme weather conditions might have had a negative effect on flower initiation and pod set, and might have eventually resulted in poor seed yield. Statistically, the highest forage yield in this study was obtained for environments with a high moisture availability (Leth IR 2007, Leth IR 2010, Leth RF 2010). This data supports the results of Basu et al. (2009) who noticed a higher forage yield for fenugreek in environments with high moisture availability in southern Alberta. Njunie et al. (1996) investigated 18 herbaceous legume species grown under semi-humid and semi-arid conditions. Their study also found that higher forage yields for legume crops were obtained at semi-humid sites in comparison to semi-arid sites. Although the environment at Creston in 2010 was dry, it also produced the highest forage yield that was significantly different from others. This may be due to the fact that as the plants produced the lowest amount of seed yield in this environment, the major portion of plant metabolites might have been utilized for vegetative growth. Lee (2009) reported significant genotype effects for fenugreek seed yield, whereas Basu

et al. (2009) reported significant genotype effects on seed and forage yield of fenugreek. Effect of mutant generations on seed and forage yield was not statistically significant for whole plots harvest in this study. Fehr (1987) stated that in segregating populations that are advanced by the bulk method, all types (high, moderate and low types) of plant productivity will be present for a certain plant trait when the trait is affected by hybridization and/or mutation. In this study, early segregating mutant generations were advanced from the bulked seeds; thus, the mutant populations were a mixture of high yielding, moderate yielding and low yielding plants for traits such as seed and forage yield during each mutant generation. This may be the reason why the mutant generations were found to be statistically similar to each other for these traits. In this study, no mutant generation out-produced the control Tristar for either seed yield or forage yield. This result was expected as the mutant generations were segregating generations and had received little or no active selection for improved seed or forage yield. A similar interpretation of the data was suggested by Robinson et al. (2007) in a study of perennial legumes native to Australia which were compared with the growth of other exotic legumes, *Medicago sativa* and *Lotus corniculatus*, that had been subjected to significant breeding and selection pressures.

There were differences in the results for seed and dry matter yield when data from randomly sampled individual plants (Table 3.4 and 3.5) rather than whole plot harvest data were considered. Comparable observations were made in another study focused on mutant generations of the legume crop, *Sesamum indicum* L. (Begum and Daasgupta 2010). In this present study, mutant generations were found to have a significant effect on plant height, pod/plant, seed weight/plant, dry biomass/plant and basal stalk number/plant

when data gathered from randomly selected plants were considered. For all of the selected characters except for double pods/plant and dry biomass/plant, different mutant generations represented different mean groups from the base population Tristar. This suggests presence of sufficient variability for the traits examined in the mutant generations. Similar observations were also made by Chatterjee et al. (2011) when they examined mutant generations of opium poppy. The presence of sufficient variability for the selected agronomic traits in the mutant generations suggests a scope for effective selection for these traits. A significant shift in mean values in a positive direction was observed for quantitative traits like pods/plant, seed weight/plant and basal stalks/plant. Furthermore, the highest means for these traits were obtained in the most advanced generations, which indicates that the most advanced generations harbored plants with these desired characters in higher frequencies than earlier generations. Earlier studies involving different fenugreek genotypes have shown that plant height, pods/plant, seed yield/plant, and basal stalks/plant, all show variation in response to growth in different environments (Gangopadhyay et al. 2009; Singh and Pramila 2009). Although quantitative traits are controlled by a polygenic system and are influenced by the environment, only plant height and basal stalk number/plant traits were influenced by environmental differences in the present study. As expected, statistically more basal stalks/plant was obtained under rain-fed growing conditions than under irrigated growing condition since fenugreek normally produces more basal stalks in response to water stress (Petropoulos 2002).

The results of the present study showed that mean plant height and biomass weight/plant increased in the M_4 generation, whereas mean number of pods/plant and

seed yield/plant decreased in the M_4 generation (Fig. 2 & 3). Basu (2006) selected high seed yielding plants with shorter height and a lower biomass yield during growth of the M_2 generation to produce an M_3 generation. This might be the reason why an immediate benefit of selection was observed in the M_3 generation in this study, and why segregation for these traits resulted in increased mean plant height and biomass weight/plant, and decreased number of pods/plant and seed yield/plant in the M_4 generation. If seeds of the segregating generations are bulked and a portion of the bulked seeds are used to grow the next generation, it is likely that the proportion of seeds from high yielding plants will increase in bulk since plants that produce more seeds will provide a higher ratio of seeds in the bulk in comparison to plants that produce few seeds (Fehr 1987). The statement is supported by the results of this study as a steady increase in number of pods/plant and seed yield/plant was observed for the most advanced generations. This result is consistent with earlier studies with fenugreek (Singh et al. 1991), *Vigna radiata*, *Vigna mungo* (Kozgar et al. 2010; Wani and Khan 2006; Khan and Wani 2005), and soybean (Paqvadai et al. 2010). The lowest mean differences between two successive mutant generations for the traits examined were noticed in the most advanced generations, indicating an increase in trait stability. The increase in stability in the most advanced generations in comparison to the earlier generations, may be due to increased homozygosity of the genes involved (Khan and Wani 2005).

Previous studies on fenugreek have identified several traits associated with seed yield. In the present study seed yield showed a significant positive correlation between the number of pods and the number of basal stalks/plant. This result is in agreement with earlier studies with fenugreek (Gangopadhyay et al. 2009; Singh and Pramila 2009;

Chandra et al. 2000; Sharma et al. 1990). Contrary to most previous studies (Zandi et al. 2011; Gangopadhyay et al. 2009; Singh and Pramila 2009; Sharma et al. 1990), in the present study a negative correlation was found between seed yield and plant height. The deviation of the present result from the previous studies in this regard, may be due to differences in agro-climatic conditions of western Canada. The studies that reported a positive association between seed yield and plant height in fenugreek were conducted in tropical regions where fenugreek is a native species and have much longer growing period compared to western Canada, thus getting sufficient time for vegetative and reproductive growth including seed maturation (Sadeghzadeh-Ahari et al. 2010; McCormick et al. 2009). In contrast, fenugreek grown in temperate regions like western Canada appear to remain in a vegetative state for a long time and, do not get sufficient time for pod formation and seed maturation.

Successful breeding of a crop species to meet the requirements of prevailing growing conditions depends on the selection criteria used in the breeding program, together with realistic evaluation systems. This study showed that the mutant generations are variable enough for generation of important agronomic traits to facilitate effective selection for genetic improvement of the crop. Moreover, this study also has found a significant association among plant characteristics such as seed yield and other agronomic characters. Pods/plant, basal stalks/plant and seed yield/plant have been reported to be highly heritable in fenugreek (Gangopadhyay et al. 2009; Singh and Pramila 2009; Yadav and Raje 2008). Yadav and Raje (2008) reported that seed yield was significantly correlated with pods/plant and basal stalks/plant. These characters can be used to isolate high and stable seed yielding plants from the current mutant population

to generate potential lines which can be evaluated in different environments to develop new cultivars or used in further breeding work as important germplasm.

Chapter Four: Mutant Lines

4.1 Introduction

Fenugreek is fairly new in Canada as a cultivated crop species. It has been grown commercially in western Canada since 1992. Currently available fenugreek cultivars in Canada were developed through selection among the world accession and the successive introduction of these genotypes to this region. Earlier fenugreek cultivar development activities in Canada were mainly focused on development of genotypes for the spice market (Lee 2009). Tristar is the first forage fenugreek cultivar developed by Agriculture and Agri-Food Canada (AAFC) at the Lethbridge Research Centre (LRC), Alberta, for its ability to produce high biomass yield consistently in western Canada (Acharya et al. 2006 b, 2008). Although Tristar produces high quality and quantity of biomass, it is indeterminate in nature and slow to mature under prairie environment, making consistent high quality seed production difficult. Previous work has made use of mutation breeding to develop new variability for early maturity and determinate growth habit to address this problem. In that work, seeds of Tristar were treated with different levels of EMS (Basu 2006). The mutant plants generated from the treated seeds were grown to advance the mutant generations. In the present study, seeds from different mutant generations were used to select individual plants with desirable agronomic traits such as high seed yield, early maturity etc.

Another approach for generating variability in the population is through increasing the ploidy level of this deploid species. The utilization of induced polyploidy is not so rare for cultivar development in this crop species (Fehr 1987). Increased ploidy

levels often result in vigorous plant with bigger seeds. The somatic chromosome number of a plant can be doubled by a number of physical and chemical agents. Colchicine has been the chemical most commonly utilized for this purpose (Fehr 1987). Natural and chemically induced polyploidy has been reported in fenugreek. Singh and Singh (1976) identified five fenugreek lines with double trisomics (2n+1+1) along with primary trisomics (2n+1) from the progeny of natural autotetraploids. Singh and Raghuvanshi (1980) and Roy and Singh (1968) produced tetreaploid fenugreek plants using colchicine with the intent to develop vigorous plants. In a earlier study, Tristar fenugreek plants were treated with colchicine to double the chromosome number (Basu 2006). Plants possessing tetraploid genotypes were selected from the survivors and their seeds were collected for further studies. Basu (2006) has reported that the seeds produced by the tetraploid lines are bigger than that of Tristar. As fenugreek seed contains beneficial chemicals such as diosgenin, 4-hydroxy leucine, and galactomannan, as well as is a good source extractable aromatic oil. The aroma and flavor of fenugreek are attributed to volatile oil. Being strongly scented, the oil is used as an insect repellent for grains, wooden furniture and fabrics (Ciftci et al. 2011; Duke 1986). Fazil and Hardman (1968) referred to the use of fenugreek oil in perfumes. Petropoulos (2002) mentioned the presence of fenugreek oil as a secret ingredient in a very famous perfume of France. In Europe and North America, fenugreek is used for artificial flavoring such as maple and butterscotch, which is mostly attributed to fenugreek oil (Slinkard et al. 2006). It is hypothesized that the cholchicine treated tetraploid fenugreek line with larger seeds than diploid Tristar may contain more extractable oil than Tristar seeds.

The objective of this study was:

i) To grow and characterize lines from selected individual mutant (diploid) plants

ii) To assess seed yield and seed oil content of the tetraploid line.

4.2 Materials and Methods

4.2.1 Selected mutant diploid lines

The seeds of Fenugreek (*Trigonella foenum-graecum* L.) were collected from Lethbridge Research Centre, Lethbridge, Alberta. The seeds were of different mutant generations developed using Tristar as base population and EMS as mutagenic agent. Bulk seeds from M_2, M_3, M_4 and M_5 were seeded in an irrigated field at Lethbridge in 2008. The test was observed at different growing stages, and plants were selected according to phenotypic appearance (early maturity, number of pod and biomass production) and tagged. At maturity, the tagged plants were harvested individually. The seeds from each selected plants were kept separately.

Ten seeds from each selected plant were planted individually in the greenhouse in 6 inch plastic pots containing a non-sterile, soil-free mix (LRC Soiless mix/Cornell mix) to increase the seed number. The soil free mix was composed of a 3.8 cubic feet bale of sphagnum peat moss, 18.6 kg bag of medium horticultural grade vermiculite, 1000 g of calcium carbonate flour, 1500 g of 18-6-12 Osmocote (Southern Agricultural Insecticides, Inc.), 1200 g 0-21-0, 20 g of “Fritted” trace elements, 15 g of 13.2 % (W/W) chelated iron, 7 g of 14 % (W/W) chelated zinc and 30 L of washed mortar sand (The Scotts Co.). After maturation seeds were collected from individual plants and the seeds originated from a single mother plant were bulked and kept separate from others such mother plants.

On 7th June 2010, 50 seeds from each plant group were seeded in a field at LRC along with the check line Tristar. The seeds from each group were planted in 2 meter

long lines, and line to line spacing were maintained at 40cm. The GPS coordinates for the field was 49º 41' 56.45" N and 112º 45' 24.93" W. The field was manually weeded every two-weeks. At maturity five plants from each line plus the Tristar control were randomly picked. After harvesting, the plants and the seeds were dried in a ventilated dryer for several days to remove moisture from the plants and the seeds. Plant height was measured from the ground to the top most point of the plant. Plants were cut at about 2.54 cm above ground, and then kept for drying. Individual plants was weighed for dry biomass after drying. Seeds were extracted manually from each plant and then weighed.

4.2.2 Tetraploid line

The tetraploid fenugreek line used in this study was generated by Basu (2006) using Tristar seeds and colchicine to double the chromosome number. Basu examined chromosome numbers of the plants generated from the colchicine treated seeds and only took seeds from plant possessing 4n=32 chromosomes, bulked the seeds and considered it as the tetraploid fenugreek line.

In the present study, the tetraploid line and Tristar (a diploid) were grown in three environments. The multi-environment study was conducted over two cropping years (2008 and 2009) at Lethbridge, Alberta, and at Creston, British Colombia. In 2008, the trials were planted under one irrigated growing condition in Lethbridge. In 2009, the trials were conducted under rain-fed growing conditions in both Creston and Lethbridge. The GPS coordinates for the 2008 LRC irrigated field was 49º 42' 24.98" N and 112º 45' 47.77" W, and in 2009 and the Lethbridge rain-fed field was situated at 49º 42' 16.74" N

and 112º 45' 55.41" W. The GPS coordinates for the 2009 Creston rain-fed field was 49º 06' 28.43" N and 116º 34' 06.66" W.

The fenugreek tetraploid line and Tristar diploid control were seeded in 3×2 m^2 plots with 10 rows spaced 18 cm apart. The plots were arranged in a Randomized Complete Block Design with five replicates at each growing environment. The seeding rate for each mutant generation and the Tristar control in all of the environments was 15 kg ha^{-1}.

For weed control, Edge (Dow AgroScience Canada Inc.) and Odyssey (BASF Canada) were used in the field experiments and Reglone (Syngenta Crop Protection Canada Inc.) desiccant was used for the seed yield trials Edge was applied about two weeks before the seeding of fenugreek seeds, while Odyssey was applied after two weeks of fenugreek seed germination. The plants were desiccated two weeks before seed harvest. For seed yield trials, at maturity individual plots at each test site were mechanically harvested. After harvesting, the seeds were dried in a ventilated dryer for several days to remove moisture from the material.

Total lipid of pre-dried fenugreek seed was analyzed by LR-NMR. Approximately 6 g of seed was added to a flat-bottomed 16 x 150 mm test tube (to a fill height of 4 cm) and the seed oil content was measured in a Minispec mq20 LR-NMR instrument (Bruker Optics Canada, Milton, ON). Before measurement of test samples the instrument was calibrated using a set of six fenugreek seed samples of known oil content, and the gain was tuned using the reference sample with the highest oil content. The oil content of the reference samples was determined gravimetrically following lipid

extraction by the hexane:isopropanol method adapted from Hara and Radin (1978). All analyses were performed in triplicate.

4.2.3 Statistical analysis

Treatment effects were subjected to ANOVA using PROC MIXED (SAS Institute, Cary, NC) model. The analysis was subjected to Square Root Transformed as ANOVA assumptions were not met with the raw data. Whenever the main effects of treatment were found significant at $p \leq 0.05$, treatment mean comparisons were made using Tukey's Honestly Significant Difference test.

4.3 Results

4.3.1 Selected mutant diploid lines

In the present study, the selected mutant lines showed difference from the check variety Tristar for all of the quantitative traits evaluated except for double pod number per plant (Table 4.1).

Plant height of the fenugreek mutant lines varied significantly ($p \leq 0.01$) with a range of 44.45 cm for LRCF0805 to 57.09 cm for LRCF0811 (Table 4.1, 4.2 and Figure 4.1). The check line Tristar was among the highest height group along with eight other mutant lines (LRCF0804, LRCF0806, LRCF0809, LRCF0811, LRCF0815, LRCF0816, LRCF0819 and LRCF0820). The mean plant height of LRCF0805 was statistically shorter than Tristar.

Pod number plant^{-1} of fenugreek mutant lines tested varied significantly (Table 4.1, 4.2 and Figure 4.1). The highest (53.6) number of pods plant^{-1} was obtained for mutant line LRCF0809, whereas LRCF0811 produced the lowest (20.2) number of pods plant^{-1}. The pod number plant^{-1} of LRCF0809 was statistically comparable to that of LRCF0804, LRCF0805 and LRCF0821. The check variety Tristar was statistically similar to those mutant lines that produced the lowest number of pods plant^{-1}. Although double pod number plant^{-1} varied among mutant lines the effect of mutant lines for double pod number was not significant (Table 4.1).

Table 4.1. Mean square results of the ANOVA for plant height, pod number per plant, double pod number per plant, seed weight per plant, dry biomass weight per plant and basal stalk number per plant of fenugreek selected lines.

Source of variation	DF	Plant height (cm)	Pod number plant^{-1}	Double pod number plant^{-1}	Seed weight (g)	Dry biomass weight (g)	Basal stalk number
Lines	15	27.915087**	389.97**	22.396667	6.115421**	10.822777**	3.899167**
Residual	64	3.962877	34.09375	16.525000	0.422916	0.704767	0.4125
Coefficient of variation (%)		3.13	9.17	193.9	11.45	3.52	16.21

** Denotes significance at $p \leq 0.01$.

Seed yield plant^{-1} among the mutant lines varied significantly with a range of 1.77g for LRCF0811 to 5.38g for LRCF0809 (Table 4.1, 4.2 and Figure 4.2). The highest seed yielding line LRCF0809 was statistically comparable to that of LRCF0804 and LRCF0805 and these three produced significantly higher seed yield compared to check cultivar Tristar. In fact, seed yield for Tristar was statistically similar to the lowest seed yielding mutant LRCF0811.

Some selected lines showed early maturity relative to control Tristar. The fenugreek mutant line LRCF0804, LRCF0809, LRCF0811 and LRCF0813 flowered 7 to 12 days earlier than Tristar, and also matured earlier than Tristar. The seed size and color were also variable among the selected mutant lines (Figure 4.3). The seed size of Tristar was considered large as most mutant lines produced smaller seed except for LRCF0806 and LRCF0815. The later two lines produced seeds as large as Tristar. Although the mutant lines LRCF0805 and LRCF0816 produced high seed yield the seed was green in color whereas the rest of the lines produced yellow seeds. The LRCF0813 produced a mixture of yellow and green seeds.

The dry biomass yield plant^{-1} of the fenugreek mutant lines also varied significantly ($p \leq 0.01$) with a range of 9.81g for LRCF0814 to 15.09g for LRCF0811 (Table 4.1, 4.2 and Figure 4.1). The highest dry biomass yielding line LRCF0811 was statistically comparable to that of LRCF0802, LRCF0806 and LRCF0809. The mean dry

Table 4.2. Main effect of selected lines on the mean performance of plant height, pod number per plant, double pod number per plant, seed weight per plant, dry biomass weight per plant and basal stalk number per plant of fenugreek.

Lines	Height (cm)	Pod number	Double pod number	Seed yield (g)	Dry biomass weight (g)	Basal stalk number
LRCF0802	46.93±1.41^{bc}	30.80±1.85^{cde}	0.0^{a}	3.42±0.32^{cde}	13.73±0.41^{abc}	2.80±0.20^{abc}
LRCF0803	45.66±1.14^{bc}	28.80±2.35^{cde}	1.20±1.20^{a}	1.78±0.17^{g}	11.40±0.34^{efg}	1.20±0.20^{de}
LRCF0804	51.51±1.44^{abc}	41.80±2.69^{abc}	6.80±3.82^{a}	4.41±0.26^{abc}	12.78±0.18^{bcde}	2.20±0.480^{bcde}
LRCF0805	44.45±0.71^{c}	47.80±1.68^{a}	0.0^{a}	4.97±0.20^{ab}	10.84±0.16^{fg}	4.00±0.0^{a}
LRCF0806	56.74±1.47^{a}	32.20±4.72^{bcde}	0.80±0.80^{a}	2.87±0.54^{defg}	13.41±0.59^{abcd}	1.80±0.20^{cde}
LRCF0808	49.12±1.87^{bc}	29.00±2.21^{cde}	4.00±2.60^{a}	3.44±0.52^{cde}	10.73±0.54^{fg}	1.20±0.20^{de}
LRCF0809	50.95±1.57^{abc}	53.60±2.94^{a}	4.80±3.20^{a}	5.37±0.18^{a}	14.33±0.33^{ab}	3.40±0.40^{ab}
LRCF0811	57.09±1.93^{a}	20.20±2.72^{e}	0.0^{a}	1.77±0.39^{g}	15.08±0.35^{a}	2.20±0.58^{bcde}
LRCF0813	46.73±1.29^{bc}	28.20±2.10^{de}	0.0^{a}	2.60±0.21^{defg}	10.87±0.43^{fg}	2.00±0.31^{bcde}
LRCF0814	46.22±0.55^{bc}	27.60±2.42^{de}	1.60±1.60^{a}	2.21±0.22^{defg}	9.80±0.37^{g}	1.20±0.20^{de}
LRCF0815	51.10±1.62^{abc}	27.80±2.03^{de}	0.0^{a}	1.91±0.19^{fg}	12.18±0.26^{cdef}	1.00±0.00^{e}
LRCF0816	52.42±1.54^{ab}	28.40±2.80^{de}	1.20±0.80^{a}	3.15±0.27^{cdefg}	11.40±0.36^{efg}	1.00±0.0^{e}
LRCF0819	52.57±1.47^{ab}	33.20±1.31^{bcde}	2.40±1.93^{a}	3.25±0.20^{cdef}	10.72±0.16^{fg}	2.00±0.0^{bcde}
LRCF0820	52.07±1.57^{ab}	33.40±3.37^{bcd}	0.0^{a}	2.04±0.06^{efg}	11.88±0.41^{cdef}	1.40±0.24^{cde}
LRCF0821	47.59±0.63^{bc}	44.80±2.35^{ab}	4.40±2.85^{a}	3.65±0.20^{bcd}	11.83±0.39^{def}	2.60±0.40^{abcd}
Tristar	52.47±1.29^{ab}	29.60±2.40^{cde}	2.00±2.00^{a}	2.55±0.19^{defg}	12.78±0.35^{bcde}	1.40±0.22^{cde}

Means that share similar superscripts within the same column under generation main effect and environment main effect are not significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$).

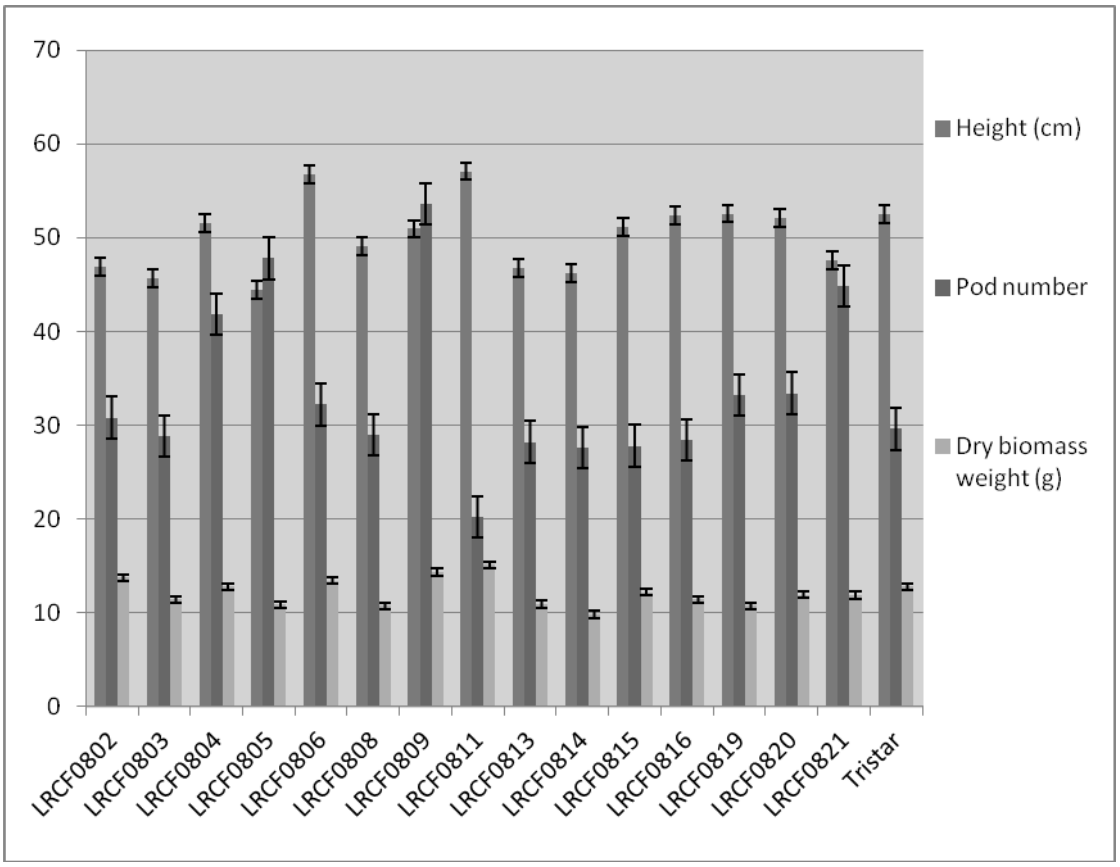

Figure 4.1. Mean plant height/plant, pod number/plant and dry biomass weight/plant of selected mutant lines and Tristar fenugreek.

Means were based on data from 5 plants.

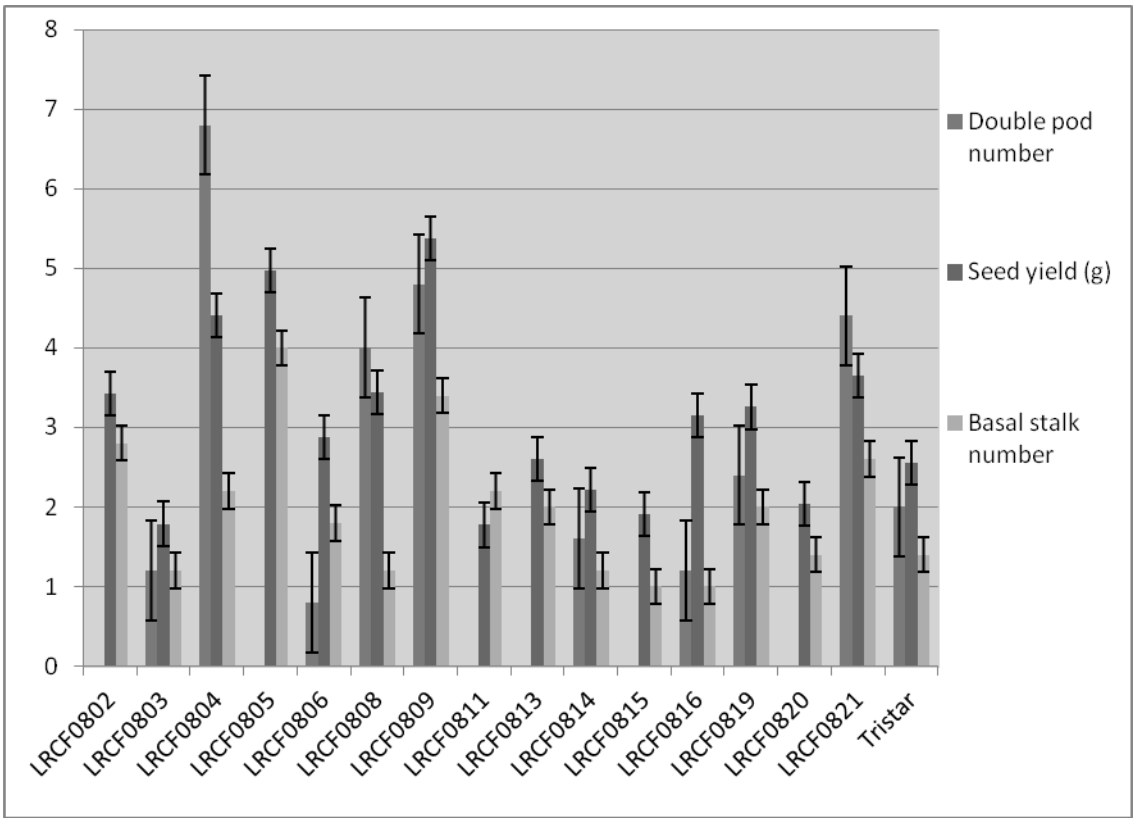

Figure 4.2. Mean seed weight/plant, double pod number/plant and basal stalk number/plant of selected mutant lines and Tristar fenugreek.

Means were based on data from 5 plants.

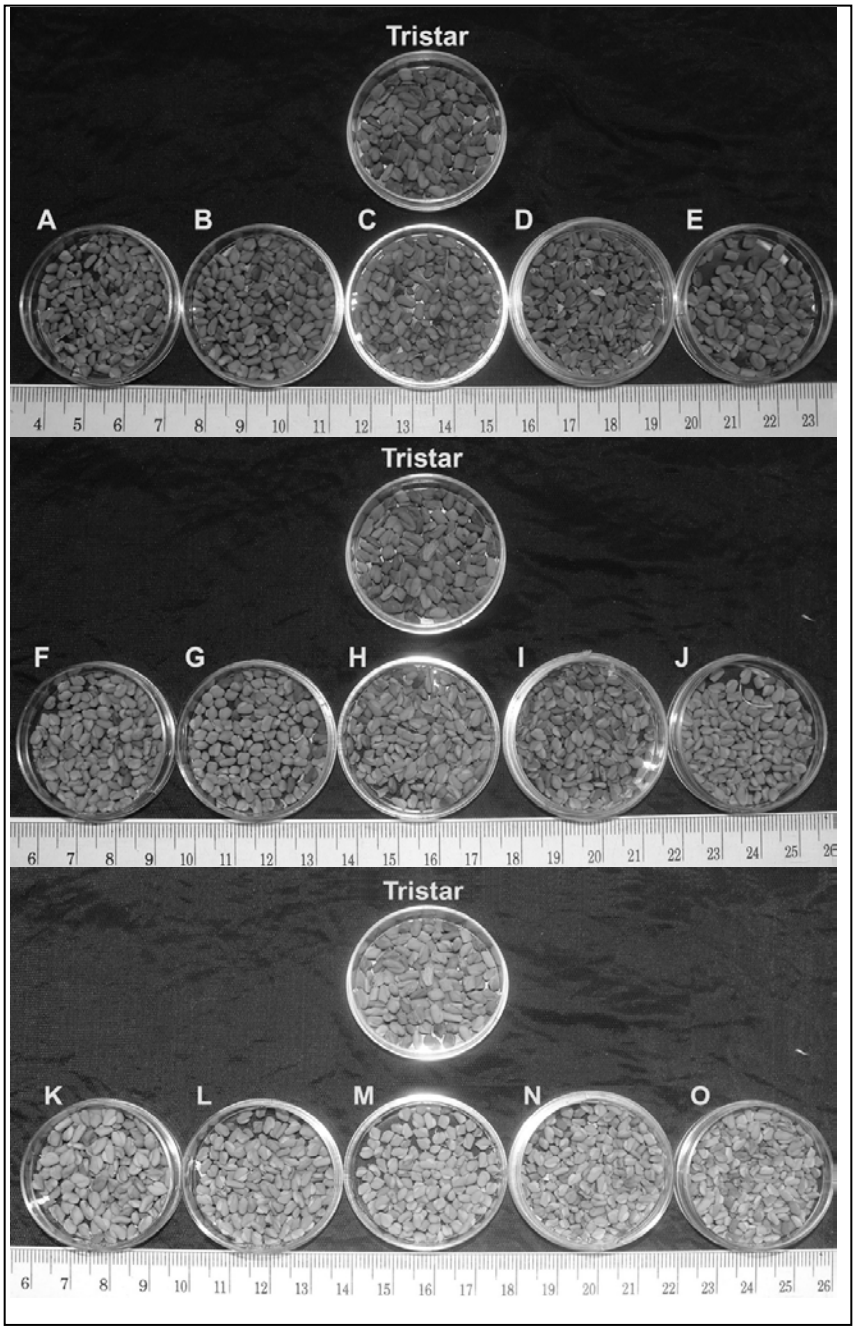

Figure 4.3. Seeds of selected fenugreek lines and Tristar grown in the field of LRC (Summer, 2010).

Here, A = LRCF0802, B = LRCF0803, C = LRCF0804, D = LRCF0805, E = LRCF0806, F = LRCF0808, G = LRCF0809, H = LRCF0811, I = LRCF0813, J = LRCF0814, K =LRCF0815, L = LRCF0816, M = LRCF0819, N = LRCF0820, and O = LRCF0821.

biomass yield plant^{-1} of LRCF0814 was statistically comparable to that of LRCF0803, LRCF0805, LRCF0808, LRCF0813, LRCF0816 and LRCF0819, but was significantly lower than that of LRCF0815, LRCF0820 and LRCF0821. The Tristar control was positioned in the top second group for this trait, and produced statistically similar amount of dry biomass plant^{-1} to that seen for the lines LRCF0804, LRCF0806 and LRCF0809.

Basal stalk number plant^{-1} for the fenugreek mutant lines tested varied significantly (Table 4.1, 4.2 and Figure 4.2). The highest (4.0) number of basal stalks plant^{-1} was obtained for the mutant line LRCF0805, whereas LRCF0815 and LRCF0816 produced the lowest (1.0) number of basal stalks plant^{-1}. The basal stalk number plant^{-1} of LRCF0805 was statistically comparable to that of LRCF0802, LRCF0809 and LRCF0821. The control Tristar produced statistically similar number of basal stalk plant^{-1} to those mutant lines that produced the lowest number of basal stalk plant^{-1}.

4.3.2 Tetraploid line

In the experiment using the tetraploid fenugreek line and Tristar grown in three environments, the effect of the genotype was not significant for seed oil content and seed yield (Table 4.3 and 4.4). Seed oil content among growing environments varied significantly with a range of 7.8 (%) for Lethbridge irrigation 2008 to 6.7 (%) for Creston 2009. Genotype × Environment interaction was found to have significant effect on seed oil content, and it contributed ~20% of the total variation observed. Seed yield among growing environments varied significantly with a range of 570.3 kg ha^{-1} for Creston 2009 to 1208.0 kg ha^{-1} for Lethbridge rain-fed 2009. The growing environment Creston 2009

Table 4.3. Results of the Analysis of Variance (ANOVA) for seed oil content (%) and seed yield (kg/ha) of tetraploid fenugreek line and Tristar fenugreek.

Seed oil content		
Source of variation	**DF**	**Mean Square**
Generation	1	0.001470
Environment	2	3.081213**
Generation × Environment	2	0.756520**
Replication	4	0.058037
CV (%)	2.2	
Seed yield		
Source of variation	**DF**	**Mean Square**
Generation	1	105759
Environment	2	1097824**
Generation × Environment	2	289977
Replication	4	48726
CV (%)	25.01	

** Denotes significance at $p \leq 0.01$.

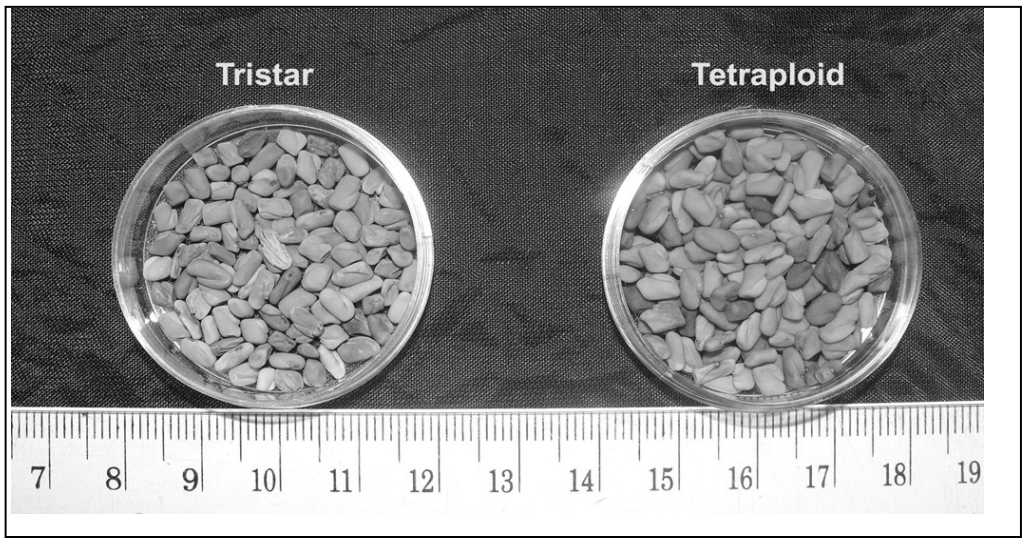

Figure 4.4. Seeds of tetraploid fenugreek line and Tristar.

Table 4.4. Main effect of environments and genotypes on the mean performance of seed oil content (%) and seed yield (kg/ha) of tetraploid fenugreek line and Tristar fenugreek.

Environment	Seed oil content (%)	Seed yield (kg/ha)
Creston 2009	6.673[c]	570.32[b]
Lethbridge rain fed 2009	7.245[b]	1208.04[a]
Lethbridge Irrigation 2008	7.783[a]	733.20[b]
Genotype		
Tetraploid line	7.24[a]	777.81[a]
Tristar	7.22[a]	896.56[a]

Means that share similar superscripts within the same column under generation main effect and environment main effect are not significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$).

produced the lowest seed yield, but it was statistically similar to the seed yield obtained at Lethbridge irrigation 2008.

4.4 Discussion

A successful breeding of a crop species depends on the selection criteria used in the breeding program along with realistic evaluation systems to meet the market demand for the crop (Evans 1996). In the present study, different mutant generations (M_2, M_3, M_4 and M_5) were used to assess variability among mutant generations and for identifying individual plants with high seed yield and quality. The mutant generations were advanced by following bulk method plant breeding protocols in which the M_1 plants were grown and their M_2 seeds were harvested together as one bulk population, and then a sample of the M_2 seeds were planted to raise an M_2 generation from which the M_3 seeds were harvested, bulked and eventually used to raise an M_3 generation. The same method was used to produce the M_4 and M_5 generations. This scheme was suggested by Fehr (1987) to maintain mutant populations and identify suitable mutant lines.

Mutation breeding is considered an effective tool to generate variability in the existing adapted plant varieties (Pavadai et al. 2010; Khan and Goyal 2009). The mutant lines used in this study was generated in an earlier mutation study where EMS was used to induce mutations in Tristar fenugreek (Basu 2006). In the present study, sufficient amount of variability was found within each mutant generation for all the quantitative traits evaluated expect for double pod number plant^{-1}. A number of lines were identified that were superior for seed yield and yield attributing traits than the check variety Tristar. The increase in mean values could be due to the alteration in genes involved in polygenic system governing the traits by cumulative effects. Similar changes in mean values in mutants over the base cultivar used for mutation has been reported in many legume crops

including mungbean (Kozgar et al. 2010; Tah 2006; Wani et al. 2005), urdbean (Kozgar et al. 2010; Deepalakshmi and Kumar 2003) and lentil (Singh et al. 2006).

Reports on successful selection in mutant populations in fenugreek are not available but, in another legume crop lentil improved lines were generated through mutation breeding (Ali et al. 2010; Ali and Shaikh 2007; Singh et al. 2007). Among the mutant lines evaluated in this study, four showed early maturity trait. Mutation breeding was reported effective to generate early maturing lines in mungbean by Khan and Goyal (2009). Fortunately, two of them (LRCF0809 and LRCF0804) were also the top most high yielding lines (Table 4.2). Identification of these two lines may be useful to create early maturing stable and high seed yielding fenugreek cultivar in western Canada where the growing period is short. Another early maturing mutant line LRCF0811 was among the top biomass yielding lines. This line may be also useful for forage type cultivar development. For seed yield trait, the selected lines LRCF0809, LRCF0805, LRCF0804 and LRCF0821 were superior over others, since these lines ranked top for the traits seed yield plant^{-1}, pod number plant^{-1}, basal stalk number plant^{-1} (Table 4.2). In the fenugreek crop, it has been shown that pod number plant^{-1} and basal stalk number plant^{-1} are highly heritable and have a direct positive effect on seed yield plant^{-1} (Yadav and Raje 2008; Koli 2002; Arora and Lodhi 1993; Mehta et al. 1992; Sharma et al. 1990).

The mutant lines LRCF0811, LRCF0809 and LRCF0806 were found superior over other lines for biomass production as these lines ranked at the top for the characters, dry biomass yield plant^{-1} and plant height. Ahari et al. (2010) and Sharma et al. (1990) reported that plant height is positively associated with biomass yield in fenugreek. These lines may be utilized in development of forage type fenugreek genotypes. The lines that

were generated and evaluated in the present study showed potential for improvement of different agronomic traits in fenugreek. For successful utilization of these lines, progenies of these lines must be evaluated in multi-environment trails for stability and uniformity of the characteristics desired. If the progeny shows that it expresses the desired phenotype but is segregating for the character(s), individual plants may be selected and harvested separately. Then the plants may be progeny tested in successive seasons and additional selection among and within lines may be conducted in subsequent generations. Then the performance of uniform lines may be evaluated again in multi-environment trails, and desirable lines may be tested for release as fenugreek cultivars or for use as parents in other fenugreek breeding studies.

Tetraploid fenugreek has been reported to produce larger seeds than its diploid parent Tristar fenugreek (Basu 2006). Seeds harvested from these tetraploid plants grown in three environments also showed that the seed size for the tetraploid line was larger than Tristar (Figure 4.4). however, the seed yield for the tetraploid line was not statistically different from that of Tristar. The seed yield for the tetraploid line was not significantly higher than Tristar. This may have been due to the fact that in the tetraploid line there was no change in gene level, only the chromosome number was doubled. The seed yield of the tetraploid line may be increased by hybridizing with other tetraploid fenugreek lines. Singh et al. (1990) obtained higher seed yield in F_6 than one of the high yielding tetraploid parents. The effect of environment on seed yield was found to be significant. Similar results have been reported in other studies of fenugreek conducted in western Canada (Basu et al. 2009; Lee 2009). Surprisingly, the result for the seed oil content of the tetraploid line was contrasting with the hypothesis made for the study.

Seeds of the tetraploid line were found to contain same amount of oil as Tristar. This is may be due to the fact that the level of expression of the genes involving lipid production in fenugreek seed was not changed though the chromosome number was increased by two fold in tetraploid fenugreek. The environment effect was significant for seed oil content in fenugreek. The seeds harvested from irrigated trial produced the highest amount of seed oil. From the result it may be assumed that lipid production in seeds is influenced by moisture availability during seed set. This is just one observation, further studies including a number of different environments with different level of moisture availability will be required to confirm this. Although seed oil content of the tetraploid line was same as diploid, other seed constituents such as protein, diosgenin, isoleucine and galactomannans might have changed along with the increase of seed size. Future studies should be done to assess these components in seeds of this tetraploid fenugreek line. The seeds of the tetraploid line were uniform in size and color and so it might be more appealing for the spice market than the variable diploid seed.

Chapter Five: Screening Resistant Fenugreek Genotypes Against Cercospora Leaf Spot Disease

5.1 Introduction

Fenugreek is affected by many pathogenic fungi. Among the fungal diseases of fenugreek, Cercospora leaf spot disease caused by *Cercospora traversiana* is considered one of the most serious, destructive and widespread diseases (Acharya et al. 2010a; Petropoulos 2002; Ryley 1989). This disease is capable of causing considerable economic loss (Ryley 1989; Zimmer 1984; Leppik 1960, 1959). The disease is prevalent in countries where fenugreek is cultivated extensively (Singh et al. 2011; Elwakil and Ghoneem 2002; Petropoulos 2002). It is also reported to infect fenugreek 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 introduced and is not grown widely. The pathogen *Cercospora traversiana* is a member of the Dothideomycetes, which is a seed-borne fungus (Acharya et al. 2010a; Elwakil and Ghoneem 2002; Ryley 1989). Several researchers have suggested that *Cercospora traversiana* is the only species of the *Cercospora* that can infect fenugreek (Ryley 1989; Cook 1978). Leppik (1960, 1959) noted that the centre of origin of *Cercospora traversiana* is southern Asia, where fenugreek is native. This author also stated that the occurrence of the pathogen in other countries is due to transport of infected seeds.

Management of plant pathogenic diseases is based on use of chemicals, genetic resistance or some combination of these approaches. Continuous use of chemicals to control plant pathogens is not environmentally sound as these chemicals can create a

hazardous impact on the surrounding environment (Iqbal et al. 2011; Nariani 1960). Use of disease resistant/tolerant crop cultivars is regarded as an economical and durable method of controlling fungal disease (Tivoli et al. 2006). Measures to control foliar diseases caused by fungus have relied on identification of resistant/tolerant germplasm and development of resistant/tolerant cultivars through effective screening (Tivoli et al. 2006). Disease resistance is currently a primary objective of many plant breeding programs. However, literature on *Cercospora traversiana* is scarce, and there is no literature on screening of fenugreek germplasm to identify resistant/tolerant genotype against the fungal pathogen. In this study, 73 fenugreek world accessions were screened for *Cercospora traversiana* resistance/tolerance. To the best of our knowledge, it is the first report on screening of fenugreek world accessions for tolerance to Cercospora leaf spot disease. Although this disease is not a yield limiting factor in Canada, the disease has been reported in Canada in a previous study (Zimmer 1984). Zimmer (1984) reported that the disease occurred on fenugreek in Morden, Manitoba and other locations in prairie provinces during 1983. The author also reported that the pathogen was able to reduce yields up to 80% in that year. As fenugreek is gaining more recognition in Canada and other countries where it has been introduced, the acreage of fenugreek will eventually increase in these countries. With these increases in acreage the pathogen has increased potential to become a problem in these areas. So, identification of tolerant genotypes may be useful to develop resistant cultivars in the future. Moreover, the identified tolerant genotypes have potential for use in countries where Cercospora leaf spot disease is prevalent.

The objectives of this study ware:

i) To screen for resistance/tolerance against *Cercospora traversiana* among the fenugreek world accessions,

ii) To re-characterize the disease as it presents itselfs in western Canada.

5.2 Materials and Methods

5.2.1 Plant genotypes

Screening of available varieties and available germplasm constitutes the basis of identifying resistant sources to plant pathogenic diseases (Ashfaq et al. 2007; Tivoli et al. 2006; Buchwaldt et al. 2003). In this study, a total of 53 fenugreek world accessions (Appendix I.) were evaluated for cercospora leaf spot development after artificial inoculation with C*ercospora traversiana* spores in a growth chamber. Among the 53 germplasms examined, two were Canadian cultivars (Amber and Tristar), and three genotypes (F70, F80 and F86) were selected at the Crop Diversification Centre South (CDCS) Brooks, Alberta, Canada for adaptability and seed yield traits when grown in Canadian conditions.

5.2.2 Culture of pathogenic fungi

A live pure culture of *Cercospora traversiana* on agar was obtained from CAB International (CABI), United Kingdom. In the strain information sheet provided by CABI, it was mentioned that the microorganism, *Cercospora traversiana*, was isolated from *Trigonella foenum-graecum* L. The microorganism was sub-cultured on plates of potato-dextrose agar (PDA) from a live pure culture obtained from CABI. PDA plates containing the cultures were incubated at $25^{o}\pm2^{o}$C in darkness for 30 days to promote sporulation (Ryley 1989).

5.2.3 Growth chamber experiment

A screening experiment for resistant fenugreek was done in a growth chamber at the Lethbridge Research Centre, Lethbridge, Alberta. The temperature in the growth chamber was maintained at $23^{o}\pm2^{o}$C with a 16/8 h day/night photoperiod cycle (Sillero et al. 2006). To find an effective spore concentration for use in a spray solution, one Indian origin and one Iranian origin fenugreek genotype were inoculated separately with 0.5×10^{4} spores/ml, 1×10^{4} spores/ml and 1.5×10^{4} spores/ml. The number of conidia in the spray suspension was estimated using a hemocytometer (Reddy and Singh 1987). A conidial concentration in the spray suspension of 1×10^{4} spores/ml was effective at producing symptoms of infection in developing fenugreek plants.

A total of 53 fenugreek accessions were evaluated for cercospora leaf spot development. Among the 53 genotypes, 5 genotypes showed very poor germination ability repeatedly, and for this reason they were removed from the experiment. For each genotype, four plants were inoculated with *Cercospora traversiana* and four plants were kept as controls. The disease severity in each fenugreek genotype was measured by comparing treated plants with control plants of the same genotype. Inoculations with the microorganism, *Cercospora traversiana*, were done on seedlings to monitor symptom development. The inoculation was done by spraying a conidial suspension on 21-day old fenugreek seedlings. Inoculums were prepared from 30-day old fungal colonies growing on PDA plates kept at $25^{o}\pm2^{o}$C in darkness. Sterile distilled water (5 ml) was added to each plate and conidia were removed by brushing the colony's surface with a fine camel hair brush. The suspension was filtered through double layers of cheese cloth and then adjusted to 1×10^{4} spores/ml (Ryley 1989).

Temperature and relative humidity were critical factors in controlled inoculations (Tivoli et al. 2006). For cercospora leaf spot of fenugreek, it has been reported that high temperature and a high level of relative humidity is critical for development of the disease (Acharya et al. 2010a, Petropoulos 2002, Ryley 1989). To maintain a high relative humidity for successful infection, a "mini-dome technique" developed by Chen and Muehlbauer (2003) was applied. After inoculating 21-day old seedlings by spraying with a conidia suspension until run-off from the sprayed plants was observed, the seedlings were immediately covered with inverted translucent polythene bags to form mini-domes. The plastic bags were removed after 5 days.

Disease development was evaluated after 10 days post inoculation with *Cercospora traversiana*. Infection by *Cercospora traversiana* were scored in these artificially infected trials as the % of affected leaves on the inoculated plants. *Cercospora traversiana* infection is able to generate high levels of leaf infection on susceptible fenugreek plants and so it is relatively easy to see. Objective scores based on % infected leaves can be done rapidly and are reproducible (Sillero et al. 2006). The percentage of leaves affected by cercospora leaf spot were assessed visually on a 0 (highly resistant) to 5 (highly susceptible) scale (Iqbal et al. 2011). The scale was 0 = 0% plant leaves affected (highly resistant), 1 = 1-15% plant leaves affected (resistant), 2 = 16-40% plant leaves affected (moderately resistant), 3 = 41-65% plant leaves affected (moderately susceptible), 4 = 66-90% plant leaves affected (susceptible) and 5 = 91-100% plant leaves affected (susceptible). Visual assessment of disease severity on a given plant can be different depending on the evaluator, as each evaluator has a subjective perception of the percent plant leaves affected by the disease. To overcome this, two persons

individually rated disease severity and the average of these two ratings were used as a final disease score for use in the study. This 0-5 point scale has been used for evaluation of mungbean germplasm against Mungbean yellow Mosaic Virus (Iqbal et al. 2011; Khattak et al. 2008), chickpea genotypes against Ascochyta blight (Reddy et al. 1984), resistance of pea genotypes against powdery mildew and downy mildew (Falloon et al. 1995), resistance of mulberry germplasm against powdery mildew (Chattopadhyay et al. 2010) and resistance of lentil germplasm against anthracnose (Buchwaldt et al. 2003).

The 12 top genotypes exhibiting cercospora leaf spot resistance and 3 susceptible genotypes revealed by primary disease trials were taken for the final disease trials. The five Canadian adapted genotypes (Amber, Tristar, F70, F80 and F86) were also added in the final disease trials. However, variability in disease expression occurred from plant to plant of the same genotype in the primary disease trials, even though great care was taken to reproduce all procedures. Thus, in the final disease trial sufficient replication was used to distinguish levels of resistance among genotypes. In the final trial, eight plants were inoculated with *Cercospora traversiana* and eight plants were kept as controls for each genotype. The inoculation and disease severity rating process were the same as those used in the primary disease trial. Analysis of variance was performed on the disease scores gathered from the eight treated plants from each genotype using PROC MIXED (SAS Institute, Cary, NC). The data were subjected to Square Root Transformation before ANOVA was done on the data. Treatment mean comparisons were made using Tukey's Honestly Significant Difference test.

The plants in both categories (treated and control) were allowed to grow and mature after a disease severity rating was assigned and used to evaluate disease reaction

for selected agronomic traits. Data on plant height, number of pods/plant, seed weight/plant and biomass weight/plant were observed from treated and control plants for each genotype. These data were subjected to ANOVA using PROC MIXED (SAS Institute, Cary, NC).

5.2.4 Evaluation of seed-borne nature of *Cercospora traversiana*

Fenugreek seeds from diseased pods of diseased plants were collected. The disease affected seeds were taken to evaluate the seed-borne nature of the microorganism. Five seeds were dipped separately in 95% ethanol for 40 seconds and then washed in two changes of sterile distilled water (Ryley 1989). These seeds were then partly submerged in separate PDA plates; the PDA plates were sealed with parafilm and kept at $25^{o}\pm2^{o}$C in darkness for 10 days. The same procedure was repeated three times during three consecutive weeks.

5.2.5 Morphology of *Cercospora traversiana*

The pathogen *Cercospora traversiana* that grew on infected fenugreek leaves was checked with a compound microscope. Then, the shape and structure of the microorganism was evaluated under an electron microscope (HITACHI S-3400N). Scanning Electron Microscopy (SEM) images were taken to measure the size of the conidia, conidiophores and mycelia of *Cercospora traversiana*. Fifteen randomly selected SEM images were used to measure these structures.

5.3 Results

Spraying of different fenugreek genotypes with a concentration of 1×10^4 spores/ml of *Cercospora traversiana* was effective to develop disease symptoms of the fungus on the fenugreek plants. To confirm that the microorganism extracted from diseased fenugreek leaves were *Cercospora traversiana*, spores were cultured on PDA plates, then used to inoculate healthy fenugreek plants, and the disease symptoms developed on those plants were compared with the disease symptoms developed on plants that the microorganism was extracted from previously. Disease symptoms were similar in these two groups of plants, and the microorganism culture was also same as the microorganism culture of *Cercospora traversiana* that was obtained from CAB International (CABI), United Kingdom, confirming infection by *Cercospora traversiana*.

In general, disease symptoms appeared within 10 days on fenugreek plants inoculated with conidial suspensions. Cercospora leaf lesions initially presented as circular, sunken spots that were bleached in color, with narrow (1–2 mm) chlorotic halos on the surface of the leaves. These lesions tended to elongate rapidly as the infection progressed, producing gray necrotic areas on the leaves. The necrotic areas were sharply defined, often surrounded by a characteristic yellowish halo. Lesion size was increased significantly on mature leaves, where sporulations were frequently evident with the appearance of a whitish, velvet-like layer. The development of more than one spot was followed rapidly by yellowing and withering of the leaves. Severely infected plants were found to have only a few leaves situated towards the apex of the plant, or no leaves at all. Stem and seed pod infections were also observed. Sunken and bleached lesions were observed on stems and petioles. In severely infected plants, the main stem became yellow

and secondary branches were found to dry up. Disease symptoms on pods included discoloration of infected areas, and severely infected areas on pods were shrunken and twisted.

Evaluation of the fenugreek accessions were done under controlled growth chamber conditions using a 0 to 5 point scoring scale based on visual judgment of the disease severity. Scoring scales based on visual judgment of disease severity have been successfully used in many plant species including leguminous plants for evaluation of resistance against many pathogenic organisms. The results of this study revealed that there was great variation among genotypes. All of the genotypes were categorized into six classes based upon disease severity. None of the genotypes tested was found to be highly resistant against *Cercospora traversiana*. Among the fenugreek accessions evaluated in this study, 4.16% genotypes were found to be resistant, 14.58% were moderately resistant, 41.66% were moderately susceptible, 37.50% were susceptible, and 2.08% genotypes were highly susceptible to *Cercospora traversiana* (Appendix II.).

After primary screening, 20 genotypes from the 53 initial genotypes tested were subjected to a final disease resistance screening against *Cercospora traversiana* in a growth chamber. These 20 genotypes included the most resistant genotypes from the priliminary disease screening test, a few susceptible genotypes from the priliminary disease screening test, and a few locally adapted genotypes. The statistical analysis showed that the genotypes differed significantly (at $p \leq 0.01$) from each other for disease severity to *Cercospora traversiana*. The final disease screening result showed that the accessions L3717 and PI138687 were less affected by the pathogen and were categorized as resistant among the accessions tested (Table 5.2). The accession L3721 (categorized as

highly susceptible) was found to be severely affected by *Cercospora traversiana*, followed by the susceptible accessions L3699, L3704,L3312, L3697, L3700, and L3705 (Table 5.2). Among the locally adapted genotypes, F86 performed better against *Cercospora traversiana* and was placed in a moderately resistant category with the L3698 fenugreek accession.

Table 5.1. Results of the ANOVA for disease severity of twenty fenugreek accessions used in final disease screening test.

Disease Severity		
Source of variation	**DF**	**Mean Square**
Accession	19	6.018421**
Residual	140	0.405357
Coefficient of variation (%)	10.05	

** Denotes significance at $p \leq 0.01$.

Table 5.2. Mean disease severity of 20 fenugreek genotypes used in final disease screening trial using *Cercospora traversiana* inoculum.

Accession	Disease severity	Resistant category
Amber	2.625^{def}	Moderately susceptible
F70	2.625^{def}	Moderately susceptible
F80	3.250^{bcdef}	Moderately susceptible
F86	2.250^{fgh}	Moderately resistant
L3312	3.875^{abc}	Susceptible
L3693	3.250^{bcdef}	Moderately susceptible
L3697	3.875^{abc}	Susceptible
L3698	2.500^{efg}	Moderately resistant
L3699	4.000^{ab}	Susceptible
L3700	3.875^{abc}	Susceptible
L3704	4.000^{ab}	Susceptible
L3705	3.750^{abcd}	Susceptible
L3707	3.250^{bcdef}	Moderately susceptible
L3713	3.500^{abcde}	Moderately susceptible
L3716	3.500^{abcde}	Moderately susceptible
L3717	1.250^{h}	Resistant
L3720	3.500^{abced}	Moderately susceptible
L3721	4.500^{a}	Highly susceptible
PI138687	1.375^{gh}	Resistant
Tristar	2.750^{cdef}	Moderately susceptible

Means sharing different superscripts are significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$).

To assess disease response for the plants, plant height, pod number per plant, seed weight per plant, and dry biomass weight per plant was measured for the 20 fenugreek accessions included in the second test. For the purpose each accession treated with *Cercospora traversiana* and their non-infected counterparts were utilized to find out the percent loss for the traits due to pathogenic infestation. Statistical analysis showed that the accessions were significantly ($p \leq 0.01$) different from each other (Table 5.3). The effect of treatment with *Cercospora traversiana* was significantly ($p \leq 0.01$) different from the control. The interaction effect of accession and treatment was also found statistically significant (at $p \leq 0.01$).

Table 5.3. Effect of *Cercospora traversiana* treatment, accession and their interaction on plant height, pod number per plant, seed weight per plant and dry biomass weight per plant observed on 20 fenugreek genotypes as determined by ANOVA.

Source of variation	DF	Mean Square			
		Height (cm)	Pod number	Seed weight (g)	Dry biomass weight (g)
Accession	19	52.65**	445.42**	3.26**	31.33**
Treatment	1	3165.76**	33636.00**	338.62**	2328.14**
Accession×Treatment	19	37.78**	221.80**	1.62**	2.53**
Replication	7	5.18	35.62	0.62	1.85
CV (%)		7.49	15.51	19.25	10.72

** Denotes significance at $p \leq 0.01$.

The results for the disease effects on plant height, pod number per plant, seed weight per plant, and biomass weight per plant revealed that these agronomic traits were influenced by *Cercospora traversiana* infestation. For plant height, a reduction of 0.8% to 63.8% occured as a result of cercospora leaf spot disease (Table 5.4). The accessions L3717 and PI138687 were found to be less affected by the pathogen for this trait, whereas L3700 was found to be the most affected genotype followed by L3697 and L3721. A range of 22.1% to 100.0% pod loss was observed among the fenugreek accessions due to cercospora disease (Table 5.4). The accession L3717 and PI138687 performed well for this trait under disease pressure, followed by accession L3698. The accessions L3697 and L3721 were affected most by the pathogen and produced no pods at all. For seed weight per plant, a 30.3% to 100.0% loss was observed due to cercospora leaf spot disease (Table 5.4). The accessions PI138687, L3698 and L3717 were found to be the least affected genotypes, whereas L3697 and L3721 were most affected genotypes followed by L3700. For biomass weight per plant, a 8.9% to 90.5% loss was observed due to cercospora leaf spot disease (Table 4). The accessions PI138687 and L3717 performed well for this trait, whereas L3697 and L3700 were found to be the most affected genotypes followed by L3721.

Table 5.4. Mean performance of 20 fenugreek genotypes used in the second disease trial and percentage loss due to *Cercospora traversiana* treatment as compared to untreated control for each genotype (in parenthesis).

Genotyp	Height (cm)		Pod number		Seed yield (g)		Biomass weight (g)	
Amber	33.35^{abcd}	(73.9)	12.50^{bcd}	(44.1)	0.91^{bc}	(34.4)	5.13^{abcd}	(64.3)
F70	32.63^{abcd}	(73.9)	14.88^{bc}	(52.6)	0.73^{bcde}	(23.3)	5.51^{abcde}	(64.4)
F80	24.76^{def}	(59.8)	7.00^{cde}	(22.1)	0.34^{cdef}	(11.6)	3.39^{cdef}	(40.7)
F86	34.62^{abcd}	(73.6)	12.75^{bcd}	(25.5)	0.81^{bcd}	(29.3)	5.63^{abcd}	(62.1)
L3312	29.94^{bcde}	(66.7)	1.50^{e}	(5.5)	0.12^{f}	(4.2)	1.74^{f}	(17.9)
L3693	25.19^{def}	(55.6)	3.50^{e}	(19.6)	0.27^{def}	(10.4)	3.03^{cdef}	(32.8)
L3697	15.64^{f}	(37.5)	0.0^{e}	(0.0)	0.0^{f}	(0.0)	0.79^{f}	(9.5)
L3698	41.50^{abc}	(87.4)	18.86^{ab}	(70.9)	1.97^{a}	(68.8)	8.18^{a}	(65.4)
L3699	23.39^{def}	(51.8)	0.50^{e}	(1.0)	0.03^{f}	(0.8)	1.24^{f}	(12.4)
L3700	15.84^{f}	(36.2)	0.88^{e}	(3.4)	0.11^{f}	(5.5)	0.90^{f}	(9.5)
L3704	27.76^{def}	(61.2)	2.50^{e}	(8.5)	0.09^{f}	(3.2)	2.53^{ef}	(24.1)
L3705	28.80^{cde}	(74.6)	4.13^{e}	(16.0)	0.30^{def}	(1.3)	3.23^{cdef}	(36.4)
L3707	33.55^{abcd}	(65.7)	7.13^{cde}	(22.7)	0.47^{cdef}	(17.7)	4.99^{bcde}	(48.8)
L3713	26.06^{def}	(57.4)	4.88^{de}	(16.6)	0.23^{ef}	(9.7)	3.41^{cdef}	(34.7)
L3716	27.55^{def}	(57.9)	1.34^{e}	(6.1)	0.19^{ef}	(8.2)	2.76^{def}	(31.4)
L3717	44.93^{a}	(99.2)	24.36^{a}	(77.9)	1.88^{a}	(68.1)	8.01^{ab}	(85.7)
L3720	28.29^{def}	(54.7)	3.63^{e}	(11.4)	0.29^{def}	(11.1)	3.12^{cdef}	(31.9)
L3721	18.69^{ef}	(40.6)	0.0^{e}	(0.0)	0.0^{f}	(0.0)	0.81^{f}	(10.2)
PI138687	42.74^{ab}	(96.0)	24.63^{a}	(71.6)	1.83^{a}	(69.7)	7.53^{ab}	(91.1)
Tristar	31.85^{bcd}	(71.1)	17.50^{ab}	(52.1)	1.14^{b}	(31.5)	5.94^{a}	(63.8)

Means with different superscripts within the same column were significantly different from each other (Tukey's Honestly Significant Difference (HSD) at $p < 0.05$).

Fenugreek seeds collected from diseased plants were used to evaluate the seed-borne nature of the microorganism. *Cercospora traversiana* colonies were recovered from all contaminated seeds, confirming the seed-borne nature of the pathogen. In this study, the *Cercospora traversiana* colonies were seen as cottony white and slightly raised on the upper side of the colony, and the underside of the colonies was olivaceous black with narrow sectors of pale olivaceous grey. The colonies were circular, 46±4 mm in diameter, with irregular margins. The fungal growth on the diseased leaves is shown in Figure 5.1. Conidiophores of *Cercospora traversiana* were dark, paler towards the tip, unbranched, and rarely geniculate or septate. These conidiophores developed in fascicles of 3 to 12 conidiophores per fascicle, with a length ranging from 17.6 to 28.8 µm and a width ranging from 1.78 to 3.01 µm (Figure 5.2). The conidia were hyaline, acicular, straight or slightly curved, with a rounded apex, a truncate base and multicellular, with a length ranging from 2.3 to 2.8 µm and a width ranging from 1.2 to 1.9 µm.

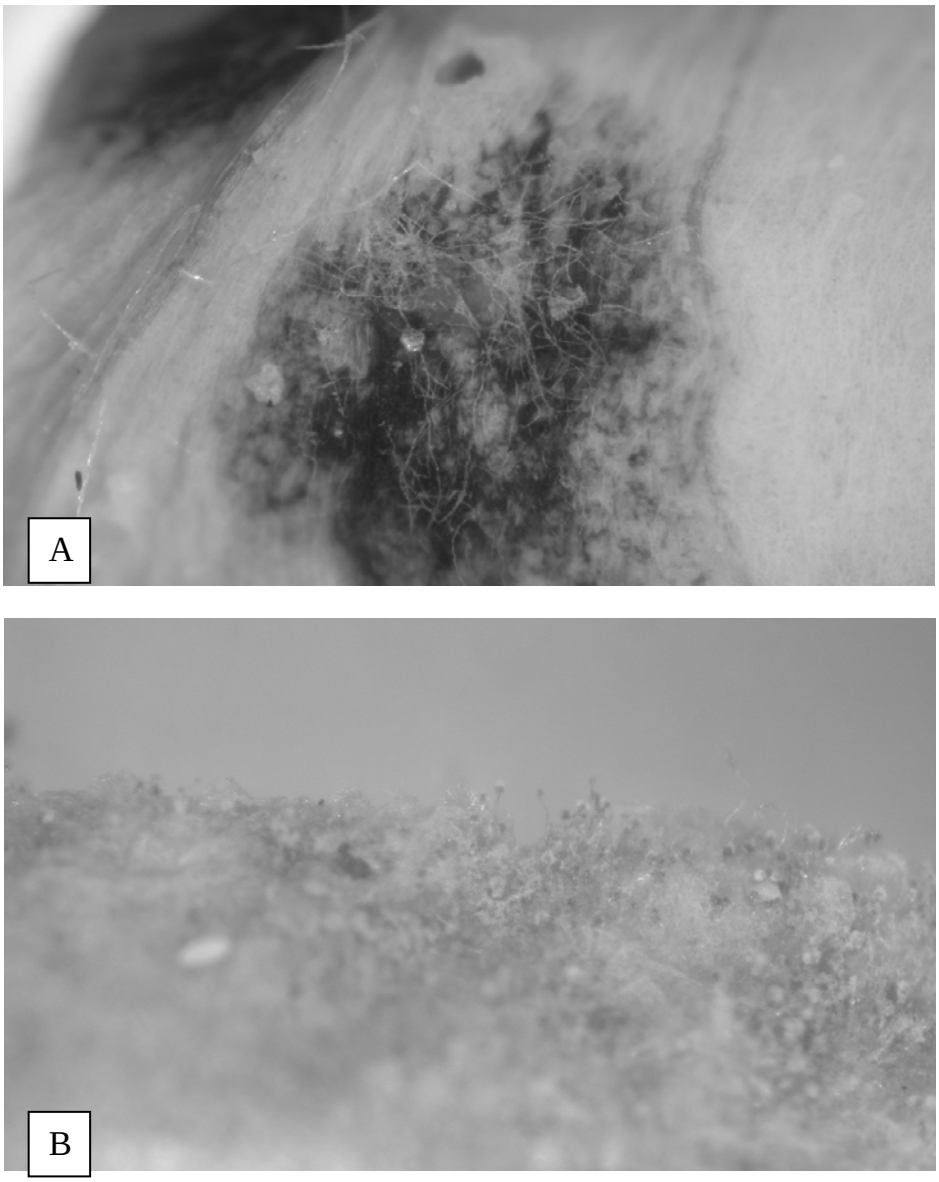

Figure 5.1. *Cercospora traversiana* fungal structures on diseased fenugreek leaves (A and B) under a compound microscope.

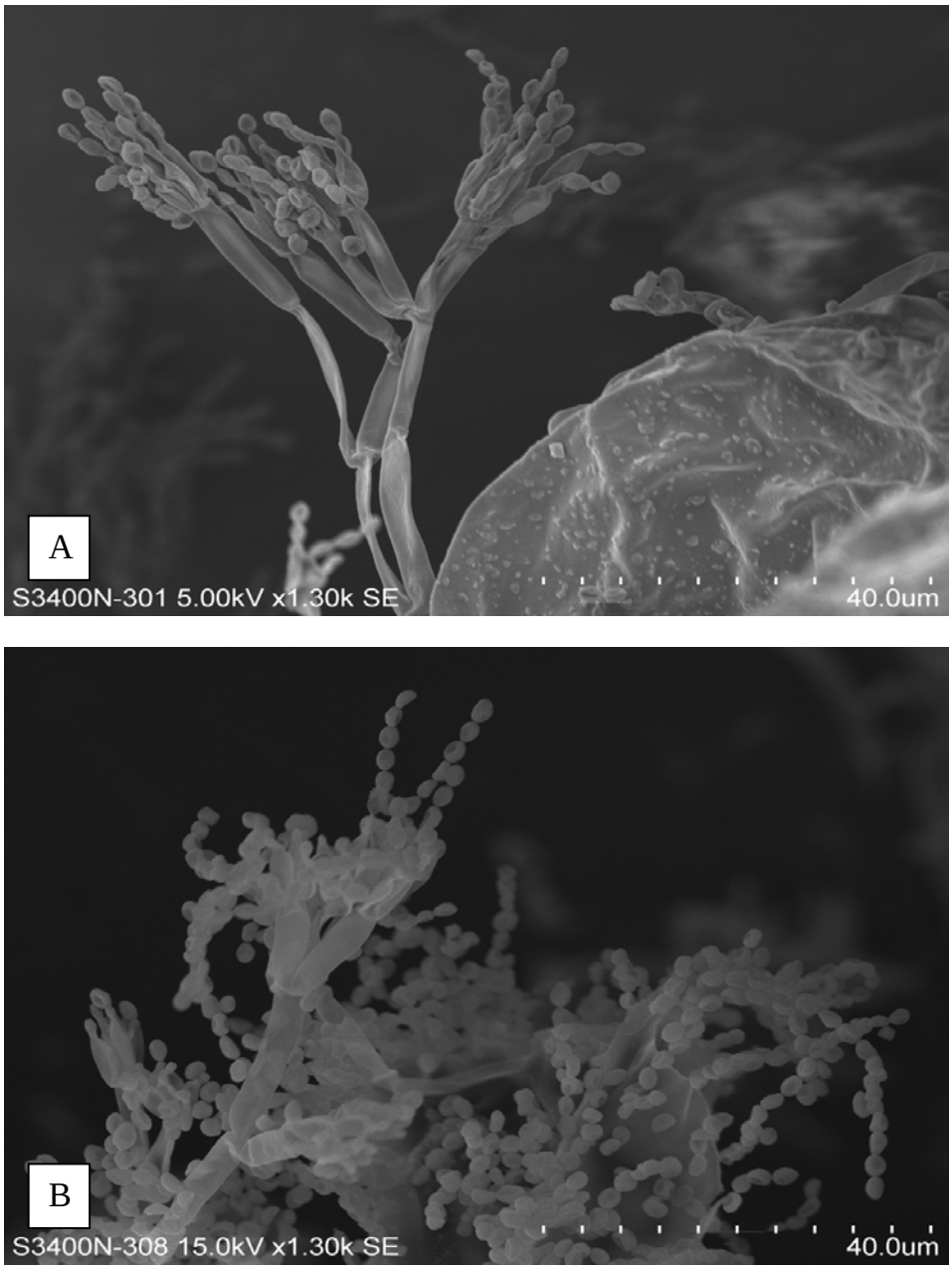

Figure 5.2. Scanning Electron Micrograph (SEM) of *Cercospora traversiana* fungal structures on diseased fenugreek leaves showing conidiophores and conidia (A and B) under an electron microscope (magnification at 1.3×10^3x).

5.4 Discussion

Screening of fenugreek genotypes for *Cercospora traversiana* resistance was carried out under controlled conditions in a growth chamber. The pathogen *Cercospora traversiana* is considered as an exotic microorganism in Canada; consequently, the study could not be carried out under field conditions. However, screening of plant genotypes for disease resistance within controlled environment has certain advantages over screening in the field. Screening within a controlled environment allowed effective control of environmental conditions and let large scale screening, especially during periods when environmental conditions in the field were not conducive for disease development and during an early stage of breeding program (Sillero et al. 2006). Testing under controlled conditions allowed epidemiological factors to be observed in a detailed manner that may otherwise be affected by other biotic or abiotic stresses under field conditions. Moreover, growth chamber experiments were more suitable than field assessments to measure inherent resistance levels, that were highly correlated to genetic resistance alleles, whereas field assessments measure these genetic effects along with strong interactions of these effects with environmental conditions (Tivoli et al. 2006).

Disease symptoms were successfully produced on fenugreek plants by contaminating the plants with *Cercospora traversiana* spore solutions. It was confirmed that the disease symptoms were due to infection by only one pathogen, *Cercospora traversiana*. Moreover, the disease symptoms that were observed on fenugreek plants in this study were similar to the disease symptoms caused by *Cercospora traversiana* on fenugreek reported in other studies (Acharya et al. 2010a; Bobev et al. 1999; Ryley 1989; Zimmer 1984).

Screening of fenugreek genotypes for *Cercospora traversiana* resistance was carried out under controlled conditions in a growth chamber. The pathogen *Cercospora traversiana* is considered as an exotic microorganism in Canada; consequently, the study could not be carried out under field conditions. However, screening of plant genotypes for disease resistance within controlled environment has certain advantages over screening in the field. Screening within a controlled environment allowed effective control of environmental conditions and let large scale screening, especially during periods when environmental conditions in the field were not conducive for disease development and during an early stage of breeding program (Tivoli 2006). Screening of fenugreek genotypes for *Cercospora traversiana* resistance was carried out under controlled conditions in a growth chamber. The pathogen *Cercospora traversiana* is considered as an exotic microorganism in Canada; consequently, the study could not be carried out under field conditions. However, screening of plant genotypes for disease resistance within controlled environment has certain advantages over screening in the field. Screening within a controlled environment allowed effective control of environmental conditions and let large scale screening, especially during periods when environmental conditions in the field were not conducive for disease development and during an early stage of breeding program (Parlevliet, 1979). In legume crops, partial resistance rather than complete resistance against fungal disease is a common phenomenon. In lentil and lupin only partial resistance to anthracnose was reported (Tivoli et al. 2006; Yang et al. 2004; Buchwaldt et al. 2003; Bernier et al. 1992), whereas in pea and chickpea partial resistance against ascochyta blight was reported (Tivoli et al. 2006; Yang et al. 2004; Buchwaldt et al. 2003; Bernier et al. 1992), whereas in pea and

chickpea only partial resistance against ascochyta blight have been reported (Kraft et al. 1998; Tivoli and Onfroy 1997; Malhotra et al. 1996; Knappe and Hoppe 1995). Qualitative resistance is usually monogenic in nature, typically inherited in a simple Mendelian fashion in the host plants, whereas quantitative resistance is based on the assumption that there is multiple gene control that collectively confers divergent levels of resistance (Chattopadhyay et al. 2010). The resistance levels found in the fenugreek accessions were either due to a qualitative resistance response or due to a quantitative resistance response, and is yet to be determined.

The results show that the fenugreek world accessions tested varied greatly in their reaction to *Cercospora traversiana* for the agronomic traits tested. Both resistant and susceptible genotypes to the pathogen were identified; the resistant genotypes were affected at a minimal level. Among the locally adapted genotypes, some interesting observations were made for Amber, F70 and Tristar. Although they showed moderate susceptibility to *Cercospora traversiana*, in general, they overcame the disease stress towards maturity, and performed relatively well in comparison to other genotypes as was observed earlier (Ryley 1989).

This study confirmed the seed-borne nature of the *Cercospora traversiana* that has been reported in other studies (Elwakil and Ghoneem 2002; Ryley 1989; Zimmer 1984). The morphology and shapes of different structures of the fungus reported in this study also was found to be in agreement with former studies (Ryley 1989; Zimmer 1984; Leppik 1960), although the size of the conidia and conidiophore differed from that reported in the earlier studies. The size of these structures was variable among the previous studies. Ryley (1989) suggested that the culture environment and nature of the

substrate used to grow the pathogen on might influence these characteristics. although the size of the conidia and conidiophore differed from that reported in the earlier studies. The size of these structures was variable among the previous studies.

This study was carried out to identify resistant fenugreek genotypes against cercospora leaf spot disease. The study identified some resistant genotypes along with some moderately resistant genotypes, which may be useful in breeding programs aiming to develop *Cercospora traversiana* resistant cultivars. Management through chemicals (pesticides) can reduce disease incidence to some extent, but continuous use of these chemicals also can create a hazardous impact on the surrounding environment and on animal health when grown as a forage crop. So, identification of plant genotypes having disease resistance can serve as an economical and practical approach to reduce disease severity and can take a prominent place in fenugreek improvement programs. In this study, disease symptoms, disease effect on some important plant traits, and morphology of the pathogen are characterized which may serve as a reference for future studies regarding fenugreek and its reaction to *Cercospora traversiana*.

Chapter Six: Thesis Summary

Fenugreek (*Trigonella foenum-graecum* L.) is traditionally used as a spice and forage crop in parts of Asia, Europe, Africa, North and South America and Australia. This crop is now being cultivated as an annual forage legume crop and spice crop in western Canada. Currently five fenugreek cultivars are being cultivated in Canada. "Tristar", developed by Agriculture and Agri-Food Canada, Lethbridge, is the most recent cultivar developed among the five and, was the first fenugreek cultivar developed for forage use. It is well adapted to semi-arid prairie regions of western Canada and can produce a high biomass yield. However, the released cultivars and adapted fenugreek germplasm are unable to support consistent high quality seed production in temperate climate of this region. As a crop originating from tropical regions, fenugreek is reported to mature in 130-140 days under tropical climate conditions. Although "Tristar" is a good cultivar for its use as forage, it does not produce a high quality and quantity of seeds every year due to an indeterminate growth habit and/or long maturity duration. "Tristar" takes about 120 days to produce high quality mature seed in western Canada where only about 100 frost free days are normally available for crop production. Extensive use of fenugreek requires a solution to the seed production problem in this crop which we believe can be achieved through development of early maturing cultivars. The germplasm at hand does not have the necessary variability for selecting out early maturing types and so creation of variability in the fenugreek population for seed yield and other seed yield contributing traits including early maturity and a determinate growth habit has become the primary objective of the fenugreek improvement program. Without enough variability and successive judicial selection, development of suitable cultivar(s) with high seed yield

and quality under prairie conditions will not be possible.

For the present dissertation, research was conducted with the primary objective of improving seed yield and quality of fenugreek grown in western Canada using a mutation breeding approach that is known to work well with this diploid and self pollinated crop. A summary of the experiments performed is described in following pages, along with some suggestions for future research.

Mutation breeding using Tristar fenugreek was attempted in the past. The current study used five western Canada adapted genotypes (Amber, F70, F80, F86 and Tristar) as base populations which were treated with the mutagen, EMS to generate additional variability. These mutant populations could then be used for selection of high seed yield and quality fenugreek genotypes. The idea behind using more than one genotype was to ensure development of a much more diverse group of mutant plants with a range of genetic backgrounds. This approach should facilitate development of new fenugreek cultivars faster than an approach using only a single genotype as the base population. Mutant generations of the plants were advanced from an M_1 to an M_3 generation. The present study indicates that mutagenesis using EMS was able to generate a large amount of variability in each fenugreek group used as a base population and many mutant plants showed important traits such as presence of an apical flower (determinate growth habit), double pods and multiple basal stalks, that were rare among the original base populations. Performance of M_2 plants was observed under field conditions so that poor performing phenotypes or plants carrying deleterious alleles could be eliminated quickly. Use of natural selection under field conditions in the M_2 generation produced the changes in mean values observed for traits such as seed yield, biomass yield, plant height, number of

pods and number of double pods in every mutant group in the M_3 generation. The selection process resulted in a decrease in the percentage of poor performing plants and an increase in the percentage of better performing plants for traits observed in the M_3 generation. Positive changes in mean values for the traits evaluated, along with advancement of generations indicated an increase in stability levels for these traits. Although EMS treatment severely affected germinability of seed in the M_1 generation, the germination percentages for each group increased gradually with the advancement of the mutant generations. Therefore, mutation breeding was found to be an appropriate and valuable method for fenugreek improvement.

The mutant population generated in this study, needs to be advanced and tested in multi-location trials in coming years to determine its performance under a wide range of growing conditions. Continued selection in successive generations for desirable characters is expected to further stabilize the selected characters in later generations. Further studies should be undertaken in the future to determine if there are any changes in traits affecting chemical constitution and oil content of the treated plants due to pleiotropic effects of the mutations.

In a previous mutation study using Tristar as the base population mutant generations (M_3 to M_6) were evaluated. In the present study, a multi-environment trial was done to observe seed yield and biomass yield on a whole plot basis and mutant generation was advanced to an M_7 generation. The plots were harvested to get a general idea about performance of the fenugreek mutant generations (M_3 to M_6) relative to different environments. In the mutant generations M_3 to M_7, randomly picked plants (an

unbiased sample) were observed for a number of agronomic traits (seed yield, biomass yield, plant height, number of pods, number of double pods and number of basal stalks).

Analysis of whole plot harvest data indicated that the effect of environment was significant for seed yield and biomass yield. Although the generation effect was not significant for whole plot harvest, high estimated CV values for these traits indicated presence of ample variation within the mutant generation for selection of seed yield and biomass yield. Multi-environment trials conducted in southern Alberta and in interior British Colombia indicated that seed yield of fenugreek favours rain-fed growing conditions in western Canada, but that biomass production can be increased by application of minimal irrigation in dry areas.

Significant differences were observed for seed and dry matter yield when data from randomly sampled individual plants rather than whole plot harvest data were considered. In this study, mutant generations were found to have a significant effect on plant height, pod number, seed weight, dry biomass and basal stalk number when observations were made on randomly picked individual plants. For all of the traits studied except for double pods/plant and dry biomass/plant, different mutant generations represented different mean groups from the base population Tristar. This suggests presence of variability for the traits examined in the mutant generations and thus scope for effective selection for these traits in each mutant generation.

A significant shift in mean values in a positive direction was observed for quantitative traits such as pods/plant, seed weight/plant and basal stalks/plant. It was interesting to note that the highest means for these traits were obtained in the most

advanced generations. This indicates that the advanced generations harboured plants with desired traits in higher frequencies than earlier generations. The other important observation is that the lowest mean differences between two successive mutant generations for the traits examined were noticed among M_6 and M_7 plants, indicating an increase in trait stability in the advanced generations.

Correlation coefficients calculated among select agronomic traits indicated a strong significant positive correlation among seed yield, number of pods and number of basal stalks. Plant height was positively correlated with biomass yield, but was negatively correlated with seed yield and its components such as number of pods and number of basal stalks. This negative correlation confirms our contention that reduction in plant height (a measure of indeterminate growth habit) may have a positive impact on fenugreek seed yield.

The mutant generations should be advanced in future years to further stabilize the desired characters, and plants with desired traits should be selected simultaneously to generate advanced lines. These lines should then be evaluated in multiple environments to select suitable cultivars for western Canada. The data collected on individual plants in this study will be helpful in selecting high and stable seed yielding plants from the mutant population for evaluation in multiple environments, and for development of new cultivars for this region or for use as potential germplasm in breeding programs.

In an earlier study where genetic improvement of fenugreek was attempted by using Tristar as a base population and EMS as a mutagen, mutant generations were advanced to an M_5 generation. In the present study, individual plants were selected from

M_2 to M_5 generations. Seed from these selected plants were tested again under field conditions for a number of quantitative traits such as seed yield, biomass yield, plant height, number of pods, number of double pods and number of basal stalks. From this a number of lines were identified that were superior for the above agronomically important traits over the check variety Tristar. For seed yield, the lines LRCF0809, LRCF0805, LRCF0804 and LRCF0821 were superior relative to the other lines tested, and ranked top for seed yield plant^{-1}, pod number plant^{-1}, and basal stalk number plant^{-1}. The mutant lines LRCF0811, LRCF0809 and LRCF0806 were found superior over other lines for biomass production, and ranked top for the characters dry biomass yield plant^{-1} and plant height. Among all of the mutant lines evaluated, four showed early maturity. Fortunately, two of the four lines were also the top most seed yielding lines (LRCF0809 and LRCF0804). Such identification of high yielding early maturing lines may be useful in producing stable fenugreek cultivars for use in western Canada. Another early maturing mutant line LRCF0811 was among the top biomass yielding lines that may also be useful as a future forage type fenugreek cultivar for development.

For successful utilization of these lines, progenies of these lines must be evaluated in multi-environment trails to determine their adaptation. Selection among and within the lines should also be done to produce a uniform population with a desired level of trait expression. Performance of lines and their uniformity may be evaluated again in multi-environment trails for eventual release of new fenugreek cultivars with shorter maturity duration and improved seed yield without sacrificing total biomass yield.

A study was also carried out to evaluate seed oil content and seed yield of a tetraploid fenugreek line in comparison to diploid Tristar fenugreek in a multi-

environment trail. The seeds harvested from tetraploid plants grown over three environments showed that the seed size for the tetraploid line was larger (by 24%) than Tristar although seed yield for the tetraploid line was not statistically different from the diploid Tristar. Seed of the tetraploid line were expected to have a higher oil content than the diploid Tristar, but the oil content was not statistically different. In this case the environment effect was significant for seed oil content and seed yield.

In this study only seed oil content of the tetraploid and diploid Tristar was observed. It is possible that the other important seed constituents like protein, diosgenin, isoleucine, and galactomannans may have changed in the seed along with the increase in seed size. Future studies should be done to assess these components in seeds of this tetraploid fenugreek line.

Fenugreek crops can be affected by many pathogenic fungi. Among the fungal diseases of fenugreek, Cercospora leaf spot disease caused by *Cercospora traversiana* is considered one of the most serious, destructive and widespread diseases. Although this disease is not a yield limiting factor in Canada, it has the potential to inflict yield loss of up to 80% in a year. As fenugreek acreage increases in Canada and in other countries where this crop is introduced in recent years, the adverse effect of this disease will be noticed. So, identification of resistant genotypes was considered an worthwhile objective for the fenugreek improvement program. For this purpose, 53 fenugreek accessions were evaluated under controlled growth chamber conditions against a virulent isolate of *Cercospora traversiana*. None of the 53 accessions exhibited total immunity or complete resistance. In this study, plants with 1-15% leaf affected due to disease was considered resistant whereas plants with 16-40% leaf affected categorized as moderately resistant. It

was encouraging to note that some accessions exhibited partial resistance against this pathogen. Among the lines tested accessions L3717 and PI138687 were less affected by the pathogen and were categorized as resistant. Among the locally adapted lines, only F86 showed moderate resistance to *Cercospora traversiana*.

Disease reactions for plant height, pod number per plant, seed weight per plant, and dry biomass weight per plant were measured for 20 fenugreek accessions. The results of the disease on these agronomic traits revealed that the traits were influenced by *Cercospora traversiana* infestation. Although both resistant and susceptible genotypes were affected by the pathogen the resistant genotypes were affected at a much lower level compared to those considered susceptible. This study also confirmed the seed-borne nature of *Cercospora traversiana* reported in other studies by culturing the pathogen from surface sterilized infected seeds on PDA plates. This study has identified some resistant genotypes along with some moderately resistant genotypes, which may be useful in breeding programs aiming to develop *Cercospora traversiana* resistant cultivars.

In the future, more studies should be done to identify resistant fenugreek genotypes among other world accessions. The nature of resistance and the number of gene(s) conferring the resistance against *Cercospora traversiana* are yet to be identified and so future studies should aim at providing answers to these questions.

Fenugreek is a new crop to North America that is being recognized in western Canada as having positive commercial, agricultural and environmental potential. It has been commercially grown in Canada for only 19 years with the release of the first fenugreek cultivar "Amber". Fenugreek is mainly grown for its forage and spice use in

western Canada. It can benefit the producer in a number of ways. As a legume crop it can improve soil nutrient status by fixing atmospheric nitrogen and thus reduce the need for expensive nitrogen fertilizers, consequently reducing cost of production for this crop and the crop planted after this. This property and its annual nature makes fenugreek a useful legume crop for incorporation into short term rotations. As a dry land adapted crop, its water requirements are low; use of fenugreek will allow water saving which can be used for other crops or for increasing the area under irrigation with the same volume of water. Moreover, there is growing interest in marketing fenugreek as a natural health product and functional food product in Canada. With accumulation of more experimental evidence in support of the nutraceutical properties of fenugreek this can be achieved.

New fenugreek cultivars with stable seed yield and an early maturity trait adapted to the western Canadian climate could prove to be a big boost to forage, spice and functional food markets in Canada and elsewhere. New cultivars of fenugreek with improved seed yield and enhanced levels of chemical constituents can be developed for improving efficiency of its use both for cattle and humans. The variability that was produced in fenugreek through mutation breeding in this study and in earlier studies from this program may be used for production of new and improved cultivars or as a good germplasm source for improvement of fenugreek with desired trait(s) in Canada and other parts of the world.

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Appendices

Appendix I: The fenugreek world accessions, their corresponding sources, and origin.

Accessions	Source	Origin
AMBER	AAFC, Lethbridge	Morden, Manitoba
Tristar	AAFC, Lethbridge	Canada
F70	CDC South, Canada	Turkey
F80	CDC South, Canada	India
F86	CDC South, Canada	Afghanistan
L3068	AAFC, Lethbridge	India
L3172	India	India
L3177	India	India
L3308	Alberta Province	Unknown
L3312	Alberta Province	Unknown
L3375	China	China
L3690	Gujrat	India
L3691	Hyderabad	India
L3692	Chennai	India
L3693	Rajasthan	India
L3694	Lucknow	India
L3695	New Deli	India
L3696	Guwahati	India
L3697	Amritsar	India
L3698	Madhya Pradesh	India
L3699	Bangalore	India
L3700	Kidderpore	India
L3701	Mumbai	India
L3702	Bhubaneshwar	India
L3703	Rajasthan	India
L3704	Amritsar	India
L3705	New Deli	India
L3706	Kulkata	India
L3707	Gujarat	India
L3708	Hyderabad	India
L3709	Mumbai	India
L3710	Varanasi	India
L3711	Lucknow	India
L3712	Pushkar	India
L3713	Bhopal	India
L3714	Chennai	India
L3715	Imphal	India
L3716	Gauhati	India
L3717	Bangalore	India

L3718	Bhubaneshwar	India
L3719	Srinagar	India
L3720	Rajasthan	India
L3721	Rajasthan	India
NGC 2001	Grocery store, Edmonton, Canada	Unknown
PI138687	PGRC, Canada	Shiraz, Iran
PI143504	PGRC, Canada	Hamadan, Iran
PI195691	PGRC, Canada	Ethiopia
PI199264	PGRC, Canada	Greece
PI211636	PGRC, Canada	Afghanistan
PI269994	PGRC, Canada	Pakistan
PI577711	PGRC, Canada	Meknes, Morocco
PI577713	PGRC, Canada	Madrid, Spain
PI229626	CDC – North, Canada	Unknown
QUATRO	PGRC, Canada	CDC Saskatchewan
X92-23-3	PGRC, Canada	CDC Saskatchewan
ZT-5	PGRC, Canada	CDC Saskatchewan

Appendix II: Distribution of fenugreek world accessions in various disease severity categories of cercospora leaf spot according to primary and final disease screening.

Resistant category	Disease severity	Genotypes number	Accessions
Highly resistant	0		
Resistant	1	2	L3717, PI138687,
Moderately resistant	2	7	L3690, L3696, L3698, L3701, L3715, PI269994, F86
Moderately susceptible	3	20	L3172, L3177, L3691, L3693, L3694, L3695, L3706, L3707, L3708, L3709, L3713, L3714, L3720, PI195691, PI577711, PI57713, Amber, F70, F80, Tristar
Susceptible	4	18	L3308, L3312, L3697, L3699, L3700, L3703, L3704, L3705, L3711, L3716, L3718, L3719, NGC2001, PI199264, PI211636, Quatro, ZT-5, X92-23-3
Highly susceptible	5	1	L3721