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TECHNIQUES FOR SCREENING CERCOSPORA LEAF SPOT RESISTANT FENUGREEK GENOTYPES

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ABSTRACT

Cercospora leaf spot (CLS) caused by *Cercospora traversiana* is an important phyto-pathological problem of fenugreek (*Trigonella foenum graecum* L), a multiuse legume crop. Field screenings for resistant plants, although accurate and effective, demand significant time and a sizable workforce to accomplish the goal. Also, weather conditions in the field may not always be favourable for uniform disease spread which eventually may lead to failure of the overall experiment. Whole plant assays (WPA) and detached leaf assays (DLA) with artificial inoculation not only help in scaling up the number of plants screened but also reduce the space, time and the amount of inoculum needed for the experiment. The results from our two experiments indicate that both WPA and DLA methods can be used reliably to differentiate the resistant and susceptible genotypes of fenugreek. In addition, the correlation coefficient ($r = 0.875$, $p < 0.01$), derived from the mean disease score of each genotype, between WPA and DLA, shows that they can be used interchangeably while screening fenugreek for CLS. The DLA was found to be temperature sensitive for the development of CLS symptoms and wounded leaves developed symptoms faster than non-wounded leaves. These indoor methods can be used for development of CLS resistant fenugreek cultivars in areas where disease development is difficult under field conditions.

Key words: Fenugreek, Cercospora leaf spot, Whole plant assay, Detached leaf assay

INTRODUCTION

Cercospora leaf spot (CLS) on fenugreek (*Trigonella foenum graecum* L), caused by *Cercospora traversiana* Sacc., is an important constraint to the use of fenugreek as a crop. This fungal disease is prevalent globally and was found to severely infect fenugreek even in countries where the crop was newly introduced and not grown intensively such as Hungary (Voros and Nagy 1972), Bulgaria (Bobev et al. 1999), Australia (Ryley 1989) and Canada (Zimmer 1984). Yield losses up to 80% are attributed to CLS in susceptible fenugreek cultivars when environmental conditions are conducive to disease development (Zimmer 1984). Besides reducing yield, the disease also has an adverse effect on seed quality—affecting marketability (Ryley 1989) and forage quality which renders it unsuitable as animal feed. Though considered as one of the major limitations to fenugreek production, a critical study of this disease has not been done in the past. *Cercospora traversiana* is a seed borne fungus which falls under the division Ascomycota (Agrios 1997). Researchers believe that *C. traversiana* is the only species of the genus *Cercospora* infecting fenugreek (Cook 1978; Ryley 1989; Voros and Nagy 1972). The center of origin for this pathogen is southern Asia and occurrence of this pathogen in other countries is likely due to transport of infected seed (Leppik 1959, 1970). *Cercospora* have unbranched, sparingly septate, uniform but slightly bent conidiophores which are 17.6 to 28.8 µm long and 1.78 to 3.01 µm wide. Conidia are translucent, multicellular, solitary, acicular, slightly curved, with a truncate base and rounded apex, 2.3 to 2.8 µm long and 1.2 to 1.9 µm wide (Prasad et al. 2014; Ryley 1989). Spread of conidia located on different living plant parts and decaying debris can happen through splashing rain and wind (Acharya et al. 2010). The initial symptom of CLS is marked by circular sunken lesions with 1-2 mm chlorotic halos. These lesions coalesce as the

infection progresses and lead to necrosis. Severe infection is marked by defoliation along with infected stems and pods (Prasad et al. 2014).

Disease resistance is a sustainable solution to plant disease control due to economic and environmental factors (Kibria et al. 2010; Komarek et al. 2010). Among several methods used to assess the level of resistance among plants or genotypes, field screening through natural or artificial inoculation is preferred as it matches the situation that a producer confronts while growing crops. However, field screening demands significant time, area and a workforce to maintain the number of plants required for an experiment (Gurung et al. 2015; Twizeyimana et al. 2007a). In addition, weather conditions in the field may not always be favourable for disease propagation (Twizeyimana et al. 2007b), which may lead to failure of the overall experiment. Whole plant assay (WPA) is an alternative screening method where every plant is kept in a controlled environment which is very conducive to disease. This method helps in scaling up the number of plants to be screened (Evans et al. 1999) while reducing the space and time needed to assess the disease (Dong et al. 2017; Twizeyimana et al. 2007a). The environment around the plants can be maintained as required and plants get only the specified inocula thus, increasing the reliability of the study (Blechert and Debener 2005; Dong et al. 2017). Several previous experiments have been done to identify a suitable model for WPA of cultivated crops against diversified pathogens. Screening of fenugreek through WPA in a controlled environment to identify resistance was carried out in Lethbridge, Canada by Prasad et al. (2014).

Various living plant parts such as leaves, stems, roots and fruits have been used for differentiating genotypes for disease resistance. A detached leaf assay (DLA) can facilitate rapid screening of a large number of genotypes without the use of a large space and amount of inoculum (Felsenstein et al. 1998). It allows screening of a single plant against different

pathogen races at a time. Moreover, this method can be utilized while screening a segregating population (Dong et al. 2017; Vail and Vandenberg 2010) where each plant has unique genetics; therefore, seeds from those plants can be saved for future breeding purposes. DLA has already been employed to differentiate the level of resistance in several plants ranging from herbaceous crops to large tree species with a high success rate (Browne and Cooke 2004; Hansen et al. 2005; Huang et al. 2016; Jackson et al. 2008). However, the use of this method to screen fenugreek genotypes for CLS has not been reported so far.

Temperature plays a significant role in pathogen survival, dissemination, disease appearance and severity (Agrios 2005; Jacobsen et al. 2004; Terefe et al. 2015). Pathogens have an ideal temperature regime for optimum disease development. The effect of temperature on the ability of *Cercospora* to cause disease in diverse crops has been examined by numerous researchers (Alderman and Beute 1987; Carisse et al. 2000; Cooperman and Jenkins 1986; Khan et al. 2009; Paul and Munkvold 2005). After close scrutiny of those experiments, the optimum temperature for maximum disease severity of CLS was found to be around 25°C. At that temperature, conidial germination and mycelial growth occur at full potential and thus exhibit maximum symptoms (Cooperman and Jenkins 1986; Paul and Munkvold 2005). However, no such studies have been performed for *C. traversiana*. Either human or natural factors can cause physical damage or wounding in plants; leading to direct economic loss. It is hypothesized that physical damage facilitates easier entry of pathogen into the plants eventually increasing susceptibility of plants to severe disease (Savatin et al. 2014). Fenugreek grown under field conditions are not always free from damage which may arise due to natural causes such as hailstones, wind, insects, frost and snowfall, and mechanical damage while intercropping. Physical damage accompanied by favorable temperature around plants may lead to maximum possible damage. Thus, this study

is essential not only to develop suitable methods for screening germplasm and studying disease ecology and morphology but also to determine effective management strategies to minimize economic losses to farmers.

The objectives of this study are to: i) Develop rapid screening methods to evaluate host resistance, which are needed to accelerate the progress in developing disease resistance in cultivars of fenugreek; ii) Determine the reliability of a detached leaf assay in assessing CLS resistance response in comparison to a whole plant assay and; iii) Identify effects of temperature and wounding on CLS severity in fenugreek.

MATERIAL AND METHODS

Preparation of plant material

Whole plant assay

Tristar, a western Canadian fenugreek cultivar developed for forage use and identified as susceptible to CLS (Acharya et al. 2007); and lines L3717 and PI 138687, categorized as resistant to CLS (Prasad et al. 2014), were grown in the greenhouse. The susceptible and resistant types were hand crossed, with susceptible and resistant lines used as both male and female parents and 43 successful artificially crossed flowers were allowed to form pods and mature seeds. F_1 seed obtained from each pod formed a line and each line was named for easier identification. Forty-three lines along with three parents were tested for disease severity in the first experiment. A second experiment, as a repeat of the first experiment, was also conducted to validate the results obtained from the first experiment. However, because of a lack of sufficient F_1 seed to provide required replication, the second experiment consisted of 15 homozygous F_3 lines (identified from a separate study) along with three parents and two F_1 s in common with the first experiment. A single seed was sown in each pot (10cm $\times$ 10cm $\times$ 12 cm) filled with a non-

sterile soil mix composed of fine sand, soil and Cornell mix (Basu et al. 2008), in equal proportions. Re-sowing was done after 4 days if no germination was observed. The pots were kept in the greenhouse at the Lethbridge Research and Development Center (LRDC). Plants were exposed to temperatures of 25°C during the daytime and 14°C during the night-time. In the second experiment, a whole set of plants was also kept in 20/12°C day/night temperature regime in order to understand the effect of reducing day/night temperature on CLS severity. A photoperiod of 16 hours was maintained throughout the experiment. Plants were irrigated regularly and sprayed with the insecticides- Kontos (Bayer CropScience Canada Inc.) (2mL/L) and Avid (Syngenta Canada Inc.) (0.309mL/L) once a month alternatively to prevent plant damage by western flower thrips (*Frankliniella occidentalis* Pergande). Before inoculating, plants were arranged in a randomized complete block design (RCBD) with four replications. Each block consisted of pots arranged in 1 long row on the green house bench, with all genotypes or treatments arranged randomly within each row (block). Four non-inoculated plants of Tristar were also included in both experiments as controls. These controls were used as a reference to observe any natural infection in plants before they were inoculated with *C. traversiana*, and thus only Tristar was used as it was the most susceptible genotype.

Detached leaf assay

Leaves for the DLA were obtained from the same genotypes used for the whole plant assay (forty three genotypes for experiment 1 and fifteen genotypes for experiment 2). Each experiment was conducted in a split plot design with four replications using two different temperature regimes (day/night) 20/12°C and 25/14°C as main plot factors. The combination of genotypes and leaf state (wounded and non-wounded) formed sub-plots, which were arranged randomly in a RCBD design.

One replicate block was comprised of a tray which contained all genotypes or treatments arranged randomly within the tray. Fenugreek leaves, recently formed but fully open (3-4 days old), were excised from the apical region of branches and kept in a moist plastic bag until they were taken to the lab for the assay. These leaves were then washed gently twice with distilled water to remove physical impurities. Wounds on the leaves were made by puncturing the leaflets on both sides of the midribs using fine forceps. As a control, four non-inoculated Tristar leaves (both wounded and non-wounded) were included in both the experiments. Two different greenhouse rooms were used to maintain temperatures of 20/12°C and 25/14°C. The rest of the environmental conditions within the greenhouse rooms were similar. Four leaves from different genotypes were placed randomly in 6 cm diameter Petri plates, which were lined with moist filter paper.

Culture maintenance and inoculum preparation

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

Inoculum application

Whole plant assay

30 day old plants from the greenhouse were inoculated with a fine mist hand sprayer until runoff from the leaves was observed. The pots were then placed on a tray containing a moist paper towel and covered with a transparent lid (Fig. 1A). Lids were removed after 7 days and trays were placed in normal greenhouse conditions. Temperature (day/night) and photoperiod were maintained at 25/14°C and 16 hours respectively throughout the experiment.

Detached leaf assay

Inoculation was done right after placing the leaves in the Petri plates with inoculum suspension at a concentration of 1×10^7 conidia/mL. The leaves (adaxial side) were inoculated with a fine mist hand sprayer until runoff from the leaves was observed. The Petri plates were then covered with lids and kept in a tray containing a moistened paper towel. This helped in maintaining the required humidity within the system by preventing moisture loss from the Petri plates. Trays containing samples were then kept in two different greenhouse rooms with day/night temperatures of 20/12°C and 25/14°C. A 16 hour photoperiod was maintained in each room. Shade cloth was used to prevent direct sunlight on the inoculated samples.

Disease assessment

Whole plant assay

Disease for the WPA was visually scored three times at 10, 20 and 30 days after inoculation based on a scale of 0-5, where: 0 = 0-5% of total leaves infected (highly resistant); 1 = 6-15% of total leaves infected (Resistant); 2 = 16-30% of total leaves infected (moderately resistant); 3 = 31-50% of total leaves infected (moderately susceptible); 4 = 51-80% of total leaves infected (susceptible);

5 = 81-100% of total leaves infected (highly susceptible). Area under disease progress curve (AUDPC) for each genotype was calculated using the following formula;

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

Where,

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

T_i = time on which Y_i was recorded and

n = number of times observations were taken.

AUDPC was also computed for number of lesions and lesion diameter in the same manner.

Lesions from ten randomly selected leaves per plant were counted to determine the average number of lesions per leaf. To obtain lesion diameter, ten lesions from those randomly selected leaves were measured and averaged.

Detached leaf assay

Four weeks after inoculation, the symptoms were visually scored on the same scale of 0-5 as in the WPA; however, this time percentage of individual leaves infected was assessed instead of percentage total leaves infected (Fig. 1B). We were unable to calculate AUDPC for detached leaves as disease appeared late in detached leaves and infected leaves started dying a few days after disease appearance.

Data analysis

Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test with significance level set at 5% ($p < 0.05$) were performed separately on data from the two experiments using PROC MIXED in the SAS statistical software package (SAS Institute, Cary,

NC, USA). Pearson correlation coefficient between the assays and different components of disease resistance along with different graphs were generated using Sigma Plot (Systat Software, San Jose, CA).

RESULTS

Disease symptoms

Under the above-mentioned conditions of the experiment, the conidial suspension induced typical symptoms of CLS even in resistant genotypes, though the severity was low. Symptoms were initiated with the formation of sunken lesions after 7 to 8 days which later enlarged to form circular brown or black spots. The gradual outward progression of a lesion led to the formation of concentric rings around the central black spot. Coalescence of lesions occurred as the disease developed further and eventually the whole leaf was covered by blackish fungal growth. This also induced defoliation of affected leaves. However, defoliation was also observed when leaves started yellowing, even in the absence of lesions on the leaf surface. Branches and pods were also affected especially in susceptible plants. Fig. 1C shows what resistant and susceptible plants looked like 30 days post-inoculation. Mycelial growth along with conidia from the infected five leaves from each assay were removed with the help of a sterilized scalpel and slides were prepared for observation under a compound microscope. Conidia and mycelia were observed. Conidia were solitary, multicellular, slender, slightly curved, translucent and blackish in colour which confirmed the symptoms within the fenugreek plants were due to *C. traversiana*. Other types of spores that were not consistent with *C. traversiana* morphology were not observed. Inoculation of detached fenugreek leaves with a spore suspension (1×10^7 conidia/mL) of *C. traversiana* resulted in clearly defined lesions which varied significantly among different genotypes, temperatures applied and state of the leaves, when scored based on the percentage of

leaf area infected. Symptoms were similar to those observed in WPA. The only visible differences in symptoms among WPA and DLA within a leaf were: a) fully grown lesions looked darker in DLA than WPA which we presume was due to presence of moisture within the leaves and b) most of the leaves in DLA had single lesions while in the case of WPA, most of the leaves had more than one lesion which coalesced later. Lesions on leaves of susceptible plants had large, spreading lesions that were covered in black fungal growth that often covered the entire surface (Fig. 1D), while lesions on resistant plants were smaller, had greyish-black fungal growth surrounded by a yellow margin. Only conidia characteristic of *C. traversiana* were observed under the microscope in scrapings taken from these leaf lesions. Non-inoculated leaves showed some yellowing due to senescence but did not display any fungal growth.

Effects of temperature

Disease score, in the detached leaf assay, was affected by temperature, while there was no significant difference in disease score and incubation period of whole plants due to temperature difference (Table 1). Less than 5% of DLA samples kept at the temperature regime 20/12°C showed disease symptoms, therefore they were not subjected to analysis. Even leaves of the susceptible cultivar 'Tristar' did not show symptoms until 45 days after inoculation. However, 99% of the leaf samples kept at a 25/14°C temperature regime showed CLS symptoms and thus were considered for further analysis. In the second experiment, around 10% of the total leaf samples showed symptoms but still it was not sufficient for data analysis. This inability of *C. traversiana* to grow and exhibit symptoms in detached leaf samples kept at 20/12°C suggests these temperatures may not be sufficient to initiate CLS infection in detached leaves of fenugreek, however, day/night temperatures of 25/14°C can produce sufficient symptoms to characterize the genotypes into different categories. In contrast to the detached leaf assay, whole

plant samples subjected to both temperature regimes showed adequate symptoms, with no significant difference between them.

Effects of genotype

Most of the genotypes used for the experiments were progenies generated from the crossing of two resistant and one susceptible parent. Since one of our objective was to identify the susceptible genotypes, we proposed a resistant category where genotypes with a mean disease score (DS) higher than 2.5 were considered susceptible and genotypes with mean disease score equal to or lower than 2.5 were considered resistant and thus kept for further generation analysis. ANOVA (Table 1) shows that there is a significant difference ($p < 0.01$) in DS between the genotypes treated in both the experiments (detached leaf and whole plant assays). The susceptible parent Tristar, was one of the most affected genotype in all the experiments. In experiment 1, all of the F_1 plants showed resistant reaction in the whole plant assay while there were eight F_1 's which showed susceptible reaction in the detached leaf assay. However, in the second experiment, no discrepancy between the genotypes was observed, although detached leaves depicted slightly higher disease severity than whole plants in most of the genotypes. Five genotypes which were common in both experiments showed similar levels of disease severity in both assays. The disease reaction for genotypes (F_3 plants) in the second experiment was no different than in previous studies conducted at the LRDC (unpublished data).

In the first experiment, significant difference ($p \leq 0.01$) between the genotypes was obtained in terms of incubation period (Table 1) for both the detached leaf assay and whole plant assay when analysed separately. However in the second experiment, genotypes were significantly different only in the whole plant assay. Disease appeared earliest (8.3/8.5 days) in the susceptible parent

‘Tristar’ in the WPA, experiment 1 and experiment 2 respectively. In the DLA, the earliest was PiT-10 (16 days) in experiment 1. In the experiment 1, disease appeared latest in PiT-1 (12.5 days) and PiT-9 (24 days) in WPA and DLA respectively. RT-22 was the latest to exhibit the symptoms in WPA, experiment 2. The average time for first symptom appearance in WPA and DLA in experiment 1 was 10.1 days and 19.2 days and in experiment 2 it was 10.3 days and 16 days (Table 3).

Effects of wounding

ANOVA (Table 1) conducted for samples kept at a 25/14°C temperature regime indicated that wounding significantly ($p < 0.01$) affected disease severity irrespective of genotype. Wounded leaves were affected more severely, with a disease score of 2.04 and 3.13 in experiment 1 and 2 respectively while non-wounded leaves showed fewer symptoms with a disease score of 1.71 and 2.61 in experiments 1 and 2 respectively. Higher disease scores in the second experiment are due to the presence of a higher proportion of susceptible plants as compared to the first experiment which was dominated by resistant F_1 plants. Symptoms of CLS appeared earlier (at 18.8 and 15.4 days in experiment 1 and 2 respectively) in wounded detached leaves as compared to non-wounded detached leaves (at 19.7 and 16.8 days in experiment 1 and 2 respectively) (Table 4).

Components of disease resistance in WPA

AUDPC was calculated for disease score, number of lesions and lesion diameter in fenugreek genotypes in order to see how the disease progressed with time (Table 5). Significant difference between the genotypes were obtained for AUDPC (disease score) and AUDPC (number of lesions), however, there was no significant difference between the genotypes for AUDPC (lesion diameter). Tristar, with a mean AUDPC (disease score) of 61.3, showed the fastest disease progress. RT-12 showed the slowest disease progress with a mean AUDPC (disease score) value

of 20.0, which was even lower than the AUDPC value of its corresponding resistant parent L3717. The increment in number of lesions was lowest in PiT-6 (28.7) and highest in genotype PiT-9 (39.1) which was not significantly different from the susceptible cultivar Tristar. Results showed a similar increment in number of lesions in all other genotypes. AUDPC (lesion diameter), with no significant difference among genotypes, indicated a similar pattern of lesion growth in all genotypes.

A separate data analysis was carried out to determine the change in lesion diameter and number of lesions per leaf over time (at 10, 20 and 30 DAI). Irrespective of genotype, the average size of lesions 10, 20 and 30 days after inoculation was 2.83mm, 3.15mm and 4.35mm respectively, showing an increment in lesion diameter with time (Fig. 2). Fig.2 also shows diameter increment was more rapid in the period from 20 DAI to 30 DAI than from 10 DAI to 20 DAI. Similarly, the number of lesions increased from 10 DAI (1.15) to 20 DAI (1.41); however, the number of lesions at 30 DAI (1.32) was lower than that found at 20 DAI. This rapid increase in lesion diameter and decrease in number of lesions from 20 DAI to 30 DAI may be due to coalescing of lesions after 20DAI under favorable conditions.

Correlation between components of CLS resistance in WPA

A moderate and inverse correlation ($r = -0.557$; $p < 0.01$) between incubation period and AUDPC (disease score) (Table 6), indicated that the shorter the incubation period the higher the disease severity will be. A significant but low positive correlation ($r = 0.261$; $p < 0.01$) was obtained between AUDPC (number of lesions) and AUDPC (disease score). Number of lesions (AUDPC) and incubation period were found to be slightly and negatively correlated ($r = -0.196$; $p < 0.05$). No other significant correlations between the disease components were found.

Correlation between WPA and DLA

A correlation analysis conducted for the disease score obtained for all genotypes from the whole plant assay (kept at 25/14°C) and the detached leaf assay (non-wounded leaves kept at 25/14°C) from both experiments (Fig. 3) showed a linear relationship between the two methods with the equation $DLA = -0.166 + 1.117 \times WPA$ and correlation coefficient ($r = 0.875$ ($p < 0.001$)).

DISCUSSION

We found that whole plant assays and detached leaf assays can be used quickly, efficiently and reliably to screen fenugreek for resistance to *C. traversiana*. These techniques can be used to accelerate a fenugreek breeding program without using a large space and workforce (Gurung et al. 2015; Twizeyimana et al. 2007a, b). Furthermore, alteration of environment around plants as required, lower inoculum requirement, and easier disease assessment made these methods superior over the field screening trials (Dong et al. 2017; Foolad et al. 2000). Artificial inoculation results in our experiment indicated that both methods could be used to differentiate the performance of genotypes. In addition, the correlation coefficient ($r = 0.875$, $p < 0.001$) derived from the mean disease score for each genotype, between WPA and DLA (non-wounded leaves kept at 25/14°C) shows that they can be used interchangeably to screen fenugreek for CLS (Fig. 3). Several other studies have shown a good correlation between DLA and WPA and their usefulness towards differentiating plant genotypes based on disease severity (Hansen et al. 2005; Parke et al. 2002). Congruency of their results with field experiments in earlier studies shows reliability of these methods. However, this study did not allow such correlation between WPA, DLA and a study mimicking actual field situations due to the limited number of F_1 seeds. The three parental lines along with 43 different F_2 lines derived from the same F_1 lines used in this study were screened under field conditions at Lethbridge but did not show any disease symptoms. This was attributed to unfavorable climatic conditions for disease proliferation

especially reduced precipitation and higher temperature (Subedi 2018) . This also highlights the importance of indoor screenings where environmental conditions can be controlled to provide favourable conditions that may not always occur in the outdoor environment.

In the whole plant assay we observed no contrasting difference between the genotypes in terms of number of lesions per leaflet and diameter of lesions. A number of factors may have acted in the development of similar sized lesions during this experiment. Firstly, most of the genotypes treated were F_1 s developed from crosses of resistant and susceptible parents which themselves were not significantly different from each other (Table 5) in the case of AUDPC (lesion diameter). Furthermore, a single isolate of pathogen inoculum, with uniform epidemiological conditions around the plants may be the reason symptoms progressed in a similar fashion.

Inoculum (conidia suspension) concentrations of 2×10^5 and 1×10^7 conidia/mL were successfully able to produce disease symptoms in WPA and DLA respectively, with the exception of samples of the DLA kept at the 20/12°C temperature regime. Disease symptoms on fenugreek plants and leaves in this experiment were similar to those described in a previous study undertaken in a controlled environment (Prasad et al. 2014). Disease scores in the DLA for most of the genotypes were slightly higher than the WPA when quantified based on the same scoring chart (0-5). The difference presumably is the result of the higher concentration of conidial suspension used in the DLA and favorable environmental conditions (long wetness period and high humidity) available to individual detached leaves compared to plants in the WPA. In the case of the WPA, high humidity was maintained for 7 days as compared to 28 days in the DLA. Such an increase in the level of CLS severity with the increase in inoculum concentration and longer humidity period has been noticed in several experiments conducted before (Khan et al. 2009; Wallin and Loonan 1971). Furthermore, Lui et al. (2007) observed a decline in the defensive

power of detached leaves as compared to intact ones. Surprisingly, the number of days for first symptoms to appear in fenugreek plants was shorter when whole plants were treated rather than detached leaves. A physiological difference between attached and detached leaves may be the reason behind the disparity in incubation period within the assays. However, further in-depth research is required to confirm these results.

In the DLA, samples kept under a 20/12°C temperature regime were asymptomatic (more than 95% and 90% in experiment 1 and 2 respectively) while almost every leaf sample kept at 25/14°C readily exhibited visible symptoms of CLS. This result is different from the observation for the whole plant assay where plants kept at 20/12°C temperature regime also fully exhibited disease symptoms and were not significantly different from plant samples kept at 25/14°C temperature regime. The result thus suggests that there is a large impact of temperature on CLS severity when DLA is followed. No exact reason behind such discrepancy is proposed but several reports suggest physiological changes after the detachment of leaves may create differences in disease expression (Felsenstein et al. 1998; Liu et al. 2007).

As with the results of the WPA from this study, various experiments carried out on divergent plants have identified a temperature around 25°C to be optimum for *Cercospora* species producing maximum symptoms and not much difference was observed when plants were kept at 20°C. (Alderman and Beute 1987; Cooperman and Jenkins 1986; Paul and Munkvold 2005). Most of the reported experiments for CLS were carried at the same day and night temperatures. However, in our study, lower night temperatures (12°C and 14°C) had little effect on disease severity when whole plants were inoculated. The temperature regimes used in this study depict the summer environmental conditions of western Canadian regions with warmer days followed by cooler nights. Thus, it can be predicted that CLS can be a serious problem of fenugreek in

western Canada – provided factors such as relative humidity, leaf wetness and disease inoculum are present.

One pragmatic finding of this experiment is that wounding/physical damage helps increase disease severity. Disease scores of leaves with wounds were significantly higher than the disease score of the leaves with no wounds in both the experiments (Table 4). It has been stated that wounds facilitate easier attachment and penetration of fungal mycelia into plants (Adorada et al. 2000; Misaghi 1982). An increase in fungal disease severity due to physical damage (wounding) has been observed in different parts of a plant such as stem (Hansen et al. 2005; Sakamoto and Gordon 2006), root (Adorada et al. 2000; Stutz et al. 1985), tubers (Salas et al. 2000), fruits (Oliveira et al. 2014), and leaves (De Dobbelaere et al. 2010). A field experiment conducted by Bradley and Ames (2010) found increased gray leaf spot severity in corn leaves which were physically injured by simulated hail, and prevention of yield loss in damaged corn with foliar fungicide was not effective. Our result of higher CLS severity in wounded leaves can be directly related to plants in the field as physical damage to the plant, especially leaves, can happen through divergent means such as severe weather, insects, machines and animals; this may help farmers to undertake preventive measures against the disease.

CONCLUSION

Resistance and host response to *C. traversiana* in fenugreek were evaluated earlier in growth chambers, using whole plants (Prasad et al. 2014). In contrast, we conducted whole plant assays in greenhouses where it is more economical to perform experiments. Similarly, a DLA was developed to compare to the WPA. Both methods were successful and found to be efficient in terms of inoculum volume, plant samples and working space. In addition, they gave reproducible results and were found to be interchangeable. Both methods can be incorporated in

a breeding program for identifying CLS resistant plants and culling susceptible plants in earlier generations. Our first experiment consisted of three parental genotypes viz. Tristar (susceptible), L3717 (resistant) and PI138687 (resistant), and 43 different F_1 's developed by artificial hybridization of those three parents. Almost all of the F_1 plants had disease scores similar to the resistant parents which indicates the genetic dominance of resistance over susceptibility in the case of CLS in fenugreek. However, further generation analysis is necessary to determine the number of genes involved, nature of their inheritance and heritability estimates. Those F_1 's found resistant to CLS disease by DLA and WPA can be used as germplasms to broaden the genetic base of fenugreek, particularly in western Canada. It was also interesting to note that the two methods (WPA and DLA) could be used to separate resistant plants from susceptible, an important consideration for breeding programs.

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Figure legends:

Fig. 1. (A) High humidity was maintained by placing plants in a tray containing water and covering the tray with a transparent lid. (B) Disease assessment chart as a percent of leaf area infection. (C) Premature defoliation and discoloration of leaves in susceptible (left) compared to resistant (right) plant in the WPA 30 days after inoculation. (D) Difference between resistant (left) and susceptible (right) leaves in the DLA at the time of disease assessment (28 days after inoculation).

Fig. 2. Changes in *Cercospora* leaf spot lesion diameter and number of lesions over time in fenugreek leaves (experiment 1) of whole plants. Error bars represent standard error of the mean and means with different superscripts are significantly different ($P < 0.05$) from each other; a, b, c are for lesion diameter and A, B, C are for number of lesions.

Fig. 3. Regression line and correlation coefficients on mean disease score between the detached leaf assay (wounded leaf samples) and whole plant assay when inoculated plants were kept at 25/14°C (combined data from experiment 1 and 2).

Table 1: Analysis of variance for whole plants and detached leaves of various genotypes in two experiments in terms of disease score (DS) and incubation period (IP).

Whole plant assay						
Sources	Experiment	DF	DS		IP (days)	
			F value	P value	F value	P value
Genotype	1	45	3.46	<.0001	1.81	0.005
	2	19	5.98	<.0001	1.73	0.04
Temperature (20 and 25°C)	1	-	-	-	-	-
	2	1	0.03	0.867	0.12	0.735
Genotype*Temperature	1	-	-	-	-	-
	2	19	0.95	0.523	0.73	0.782
Detached leaf assay						
Genotype	1	45	14.81	<.0001	4.65	<.0001
	2	19	12.55	<.0001	1.50	0.0972
State of Leaves (wounded and non-wounded)	1	1	39.45	<.0001	17.37	<.0001
	2	1	41.68	<.0001	26.73	<.0001
Genotype*State of Leaves	1	45	1.02	0.4440	0.95	0.5678
	2	19	0.70	0.8094	1.16	0.3003

Note: More than 95% of the detached leaves kept at 20/12°C in both experiments did not show any symptoms, therefore analysis was done only for samples kept at 25/14°C.

DF = Degrees of freedom; IP = Incubation period (number of days for appearance of first symptom)

Table 2: Categorisation of genotypes into resistant and susceptible based on average disease score (DS) obtained after treating whole plants (WPA) (kept at 25/14°C) and detached leaves (DLA) irrespective of state of leaves (wounded and non-wounded).

Genotype	Assay	Experiment ^a	DS ^b	Resistance Category ^c	Genotype	Assay	Experiment ^a	DS ^b	Resistance Category ^c
Tristar	WPA	1	3.75	S	RT-4	WPA	1	2.25	R
		2	3.75	S			1	2.75	S
PI138687	DLA	1	3.88	S	RT-5	WPA	1	1.75	R
		2	3.75	S			1	2.13	R
	WPA	1	1.75	R	RT-6	WPA	1	2.00	R
		2	2.25	R			1	1.50	R
L3717	DLA	1	2.00	R	RT-7	WPA	1	1.75	R
		2	2.00	R			1	1.13	R
	WPA	1	1.75	R	RT-8	WPA	1	1.50	R
		2	1.75	R			1	1.13	R
RT-15	DLA	1	1.50	R	RT-9	WPA	1	1.75	R
		2	1.88	R			1	1.88	R
	WPA	1	1.75	R	RT-10	WPA	1	2.25	R
		2	2.00	R			1	2.38	R
RT-22	DLA	1	2.13	R	RT-11	WPA	1	1.75	R
		2	2.25	R			1	1.38	R
	WPA	1	2.00	R	RT-12	WPA	1	1.50	R
		2	1.75	R			1	1.25	R
TPi-1	DLA	1	2.00	R	RT-13	WPA	1	1.00	R
		2	2.13	R			1	0.63	R
	WPA	1	1.75	R	RT-14	WPA	1	1.75	R
		1	1.50	R			1	1.25	R
TPi-2	DLA	1	2.00	R	RT-16	WPA	1	1.75	R
		1	1.13	R			1	1.75	R
	WPA	1	1.50	R	RT-17	WPA	1	2.25	R
		1	1.86	R			1	3.00	S
TPi-4	DLA	1	1.75	R	RT-18	WPA	1	2.00	R
		1	1.63	R			1	2.75	S
	WPA	1	2.25	R	RT-19	WPA	1	2.50	R
		1	2.75	S			1	2.75	S
TR-1	DLA	1	2.00	R	RT-20	WPA	1	2.00	R
		1	2.38	R			1	1.75	R
	WPA	1	1.25	R	RT-21	WPA	1	2.00	R
		1	1.38	R			1	2.38	R
TR-2	DLA	1	2.00	R	RT-23	WPA	1	2.75	S
		1	1.38	R			1	3.13	S
	WPA	1	1.50	R	TR-1-2	WPA	2	2.75	S
		1	1.75	R			2	3.00	S
PiT-1	DLA	1	2.00	R	TR-2-6	WPA	2	2.75	S
		1	2.50	R			2	3.13	S
	WPA	1	1.75	R	RT-5-7	WPA	2	2.75	S
		1	2.50	R			2	2.63	S
PiT-3	DLA	1	1.75	R	RT-5-5	WPA	2	2.75	S
		1	1.88	R			2	3.13	S
	WPA	1	1.75	R	RT-22-10	WPA	2	2.25	R
		1	1.88	R			2	1.75	R
PiT-4	DLA	1	2.00	R	RT-19-10	WPA	2	3.00	S
		1	1.88	R			2	3.13	S
	WPA	1	2.25	R	RT-16-2	WPA	2	3.25	S
		1	2.13	R			2	3.50	S
PiT-5	DLA	1	2.00	R	RT-16-1	WPA	2	3.25	S
		1	1.50	R			2	3.50	S
	WPA	1	1.25	R	RT-11-4	WPA	2	2.25	R
		1	1.50	R			2	2.50	R
PiT-6	DLA	1	1.25	R	RT-10-7	WPA	2	3.00	S
		1	1.38	R			2	3.38	S
	WPA	1	1.50	R	RT-10-15	WPA	2	3.25	S
		1	0.50	R			2	3.50	S
PiT-7	DLA	1	1.75	R	PiT-7-5	WPA	2	2.75	S
		1	2.25	R			2	2.75	S
	WPA	1	1.50	R	PiT-3-8	WPA	2	3.00	S
		1	1.50	R			2	3.00	S

RT-2	DLA	1	1.25	R	PiT-2-4	DLA	2	3.50	S
	WPA	1	2.00	R		WPA	2	2.50	R
RT-3	DLA	1	2.75	S	PiT-12-10	DLA	2	2.50	R
	WPA	1	2.00	R		WPA	2	3.25	S
	DLA	1	2.00	R		DLA	2	3.50	S

LSMEANS

WPA (Experiment 1) = 0.541, WPA (Experiment 2) = 0.473; DLA (Experiment 1) = 0.493, DLA (Experiment 2) = 0.497

- a Two different experiments were performed to check the reliability of the assays but only five genotypes were common due to limited availability of F₁ seeds.
- b Disease score of whole plant assay (WPA) represents the average disease score of plant samples (n=4) kept at 25/14°C in greenhouse while disease score of detached leaf assay (DLA) represents the average disease score of wounded and non-wounded leaf samples (n=8) kept at 25/14°C in controlled environment conditions.
- c Genotypes were categorised based on the average disease score of leaf and plant samples where genotypes with an average disease score higher than 2.5 were considered susceptible in both the cases.

Table 3: Incubation period (IP) (first symptom appearance) of *Cercospora traversiana* in whole plant assay (WPA) (kept at 25/14 °C) and detached leaf assay (DLA) (wounded and non-wounded leaves combined for mean analysis) of various fenugreek genotypes.

Experiment 1					
Genotypes	WPA	DLA	Genotypes	WPA	DLA
TR-1	9.8 cdefgh	18.0 jklmno	RT-4	11.5abcd	19.1 efghijklm
TR-2	10.3 bcdefgh	19.0efghijklmn	RT-5	10.8abcdef	18.9 fghijklmn
TR-3	9.0 fgh	17.3 mno	RT-6	9.3 efgh	20.8 bcdefg
TR-4	9.0 fgh	17.0 no	RT-7	9.8 cdefgh	19.0 efghijklmn
TPi-1	8.5gh	18.7 hijklmn	RT-8	12.3ab	17.3 mno
TPi-2	11.3abcde	20.6 bcdefgh	RT-9	10.8 abcdef	19.0 efghijklmn
TPi-3	8.3h	20.8 bcdefg	RT-10	9.3 efgh	19.9 cdefghij
TPi-4	9.0 fgh	19.3 efghijklm	RT-11	10.8 abcdef	19.3 efghijklmn
PiT-1	12.5a	17.5 lmno	RT-12	11.3abcde	18.6 hijklmn
PiT-2	9.5 defgh	21.0 bcde	RT-13	10.0cdefgh	21.4 bcd
PiT-3	9.8 cdefgh	20.8 bcdefg	RT-14	10.5abcdefg	20.1 cdefghi
PiT-4	9.5 defgh	16.1 o	RT-15	10.3 abcdef	18.6 hijklmn
PiT-5	9.3 efgh	19.1 efghijklm	RT-16	11.5abcd	19.5 defghijkl
PiT-6	9.5 defgh	21.9 bc	RT-17	10.3 bcdefgh	17.0 no
PiT-7	8.5gh	17.4 mno	RT-18	9.8 cdefgh	17.6 klmno
PiT-8	11.0abcdef	20.1 cdefghi	RT-19	10.8 abcdef	18.5 ijklmn
PiT-9	9.8 cdefgh	24.0 a	RT-20	11.0abcdef	19.0 efghijklmn
PiT-10	9.3 efgh	16.0 o	RT-21	9.8 cdefgh	19.6 defghijk
PiT-11	11.3abcde	19.3 efghijklm	RT-22	9.8 cdefgh	20.9 bcdef
PiT-12	10.8 abcdef	18.8 ghijklmn	RT-23	9.5 defgh	18.4 ijklmn
RT-1	9.3 efgh	22.6 ab	L3717	11.8abc	20.4 cdefghi
RT-2	9.0 fgh	18.5 ijklmn	PI138687	10.8 abcdef	20.6 bcdefgh
RT-3	10.0abcdef	18.6 hijklmn	Tristar	8.3h	19.0 efghijklmn
Mean	10.1 ± 0.77	19.2 ± 0.76			
LSmean	2.16	2.09			
F-value	1.81**	4.65**			

Experiment 2					
TR-1-2	10.8abc	16.5	PiT-12-10	11.3 a	14.9
TR-2-6	10.8 abc	15.3	RT-10-15	10.0 abc	15.5
RT-5-7	10.5 abc	14.8	PiT-7-5	10.8 ab	15.5
RT-5-5	10.5 abc	15.0	PiT-3-8	9.9 abcd	16.5
RT-22-10	10.5 abc	17.6	PiT-2-4	10.3 abc	16.0
RT-19-10	10.4 abc	16.1	RT-15	9.3 cd	16.6
RT-16-2	10.4 abc	15.8	RT-22	11.3 a	16.9
RT-16-1	9.4 bcd	15.9	L3717	10.0 abc	16.9
RT-11-4	10.6 abc	16.8	PI138687	10.3 abc	15.6
RT-10-7	10.5 abc	16.3	Tristar	8.5 d	15.8
Mean	10.3 ± 0.52	16.0 ± 0.56			
LSmean	1.38	1.57			
F-value	1.73*	1.50 NS			

Note: Means with different superscripts within an experiment are significantly different from each other. Data were analysed separately for WPA and DLA, and each of the two experiments within them. NS-Non- Significant, ** and *- Significant at 1% and 5% level of probability respectively.

Table 4: Mean disease score (DS) and incubation period (IP) of wounded and non-wounded detached leaves irrespective of genotypes in both experiments.

State of leaves	DS $\pm$ SE		IP (days after inoculation) $\pm$ SE	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Wounded	2.04 $\pm$ 0.04a	3.13 $\pm$ 0.06a	18.8 $\pm$ 0.19b	15.4 $\pm$ 0.18b
Non-Wounded	1.71 $\pm$ 0.04b	2.61 $\pm$ 0.06b	19.7 $\pm$ 0.19a	16.8 $\pm$ 0.18a

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

SE = Standard error

For Review Only

Table 5: Means for different components of disease resistance after treating individual whole plants with conidial suspension (2×10^5 conidia/ml) of *Cercospora traversiana* in experiment 1.

Genotype	AUDPC (Disease score)	AUDPC (Lesion Numbers)	AUDPC (Lesion Diameter)
TR-1	35.0bcde	30.5defgh	8.54
TR-2	30.0cdefgh	30.4 defgh	8.53
TR-3	26.3 efgh	31.1 defgh	8.12
TR-4	30.0cdefgh	30.7 defgh	8.50
TPi-1	33.8 bcdef	32.6 cdefgh	8.67
TPi-2	28.8 defgh	30.4 defgh	8.68
TPi-3	32.5 bcdefg	29.7 fgh	8.74
TPi-4	27.5 defgh	30.4 defgh	9.09
PiT-1	33.8 bcdef	30.4 defgh	8.11
PiT-2	37.5bcd	30.7 defgh	7.78
PiT-3	36.3bcde	29.2 gh	7.87
PiT-4	36.3 bcde	31.7 cdefgh	8.60
PiT-5	33.8 bcdef	31.9 cdefgh	7.65
PiT-6	32.5 bcdefg	28.7 h	8.31
PiT-7	41.3 b	33.9 bcdef	6.87
PiT-8	32.5 bcdefg	33.5 bcdefg	7.47
PiT-9	33.8 bcdef	39.1a	7.41
PiT-10	35.0 bcde	35.5abc	7.75
PiT-11	22.5 gh	33.6 bcdef	7.62
PiT-12	26.3 efgh	32.8 cdefgh	8.05
RT-1	32.5 bcde	37.7ab	7.07
RT-2	40.0 bc	33.0 cdefg	7.99
RT-3	30.0 cdefgh	33.6 cdefg	8.22
RT-4	27.5 defgh	31.0 defgh	7.97
RT-5	28.8 defgh	31.7 cdefgh	8.17
RT-6	32.5 bcdefg	29.9 efgh	6.97
RT-7	28.8 defgh	33.4 bcdefg	7.35
RT-8	20.0 h	29.9 efgh	7.45
RT-9	23.8 fgh	33.6 bcdef	8.84
RT-10	30.0 cdefgh	33.8 bcdef	8.05
RT-11	28.8 bcde	31.9 cdefgh	7.07
RT-12	20.0 h	33.6 bcdef	7.99
RT-13	31.3 bcdefg	34.1 bcde	8.22
RT-14	30.0 cdefgh	31.5 cdefgh	7.97
RT-15	26.3 efgh	32.5 cdefgh	8.17
RT-16	23.8 fgh	30.0 efgh	6.97
RT-17	32.5 bcdefg	31.0 defgh	7.35
RT-18	32.5 bcdefg	30.8 defgh	7.45
RT-19	27.5 defgh	33.9 bcdef	8.84
RT-20	32.5 bcdefg	32.9 cdefgh	9.51
RT-21	27.5 defgh	31.3 cdefgh	9.00
RT-22	32.5 bcdefg	34.5 bcd	7.77
RT-23	33.8 bcdef	32.9 cdefgh	8.57
L3717	26.3 efgh	32.5 cdefgh	8.98
PI138687	28.8 defgh	31.2 cdefgh	8.72
Tristar	61.3 a	37.3 ab	8.07
LSmean	10.63	4.31	1.61
F-value	2.99**	2.05**	1.05 NS
CV	18.54	9.53	14.12

Note: NS-Not Significant, **- Significant at 1% level of probability
Means with different superscripts within a column are significantly different from each other.

For Review Only

Table 6: Correlation coefficient calculated using different parameters of resistance in relation to Cercospora leaf spot of fenugreek when whole plants were inoculated in experiment 1.

Parameters	AUDPC (Disease Score)	AUDPC (Lesion Diameter)	AUDPC (Number of lesions)	Incubation period
AUDPC (Disease Score)	1			
AUDPC (Lesion Diameter)	0.027NS	1		
AUDPC (Number of lesions)	0.261**	0.0139 NS	1	
Incubation period	-0.557**	0.0291 NS	-0.196*	1
Note: NS = Not Significant; * = Significant at $p \leq 0.5$; ** - Significant at $p \leq 0.01$

Fig. 1.

Fig. 2.

Fig. 3.