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Characterization of Flax Germplasm for Resistance to Fusarium Wilt

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Abstract

Fusarium wilt of flax (*Linum usitatissimum* L.) caused by *Fusarium oxysporum* f. sp. *lini* (*Fol*) is an economically important disease that can result in severe yield losses. Due to the pathogen's ability to survive in soil for long periods, it is essential to identify fusarium wilt resistant flax varieties. The objectives of the study were to phenotype and compare a recombinant inbred line (RIL) population of flax in a controlled environment and in field wilt nurseries. Disease reaction of a subset (160) of RIL lines developed from cultivars 'Aurore' (moderately resistant) and 'Oliver' (susceptible) was assessed under controlled environment conditions to two *Fol* isolates. Disease severity was determined and the area under the disease progress curve (AUDPC) was calculated. The population varied in response from resistant to highly susceptible, indicating that resistant to wilt was probably polygenic. Twenty-eight days after inoculation, 14% and 5% of the RILs were severely wilted (scores of 8 and 9, with isolates 131 and 81, respectively). Plant height was negatively correlated with AUDPC (r^2= -0.13155 for 131 and r^2=-0.29841 for 81). Similarly, in the field in wilt nurseries, at Saskatoon and Morden, evaluation of the full set of 200 RILs, the disease reaction varied from resistant to susceptible, with 21% and 42% of RILs severely wilted (rated 8 and 9) at each site at the green boll stage. The results from the two locations were significantly different, although moderately correlated (r^2=0.6127). The 160 RILs in controlled environment inoculated with isolates 131 and 81 showed a higher correlation for disease severity at 28 days after inoculation, with the wilt nursery in Saskatoon (r= 0.40028 and r^2=0.38046) as compared to Morden (r^2=0.33016 and r^2=0.21140) at green boll stage. Differences in environmental and experimental conditions (such as seeding date) at the two locations, as well as different *Fol* strains in the soil combined with the subjectivity of the grading system may explain the differences between locations.

Introduction

Cultivated flax (*Linum usitatissimum* L. subsp. *usitatissimum*), commonly known as linseed, is one of the oldest cultivated crops, grown either for fibre or oil. Fibre is obtained from flax straw and used to make paper and linen, while the oil is attained from the seeds and has been used in production of quality linoleum flooring and paints (Newkirk, 2008; Growing Flax, 2002). At present the finest flax is also used as a food or as an animal feed because of its high omega – 3 fatty acid content.

Canada has been the leading flax producer in the world for over two decades, producing 614, 800 MTs of linseed in 2013/2014 (Flax Statistics). While Canada was ranked first in linseed

production in the world, France produced the most flax fibre in 2012 (FAOSTAT, 2012). The majority of the flax produced in Canada, is grown in Saskatchewan with, 492 800 MT production (Flax Statistics).

Pasmo and wilt have been identified as the main diseases affecting the plant growth and yield of flax in Canada. *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lini* (*Fol*), can infect flax at any growth stage and may result in 100% disease incidence in certain cultivars (Kommedahl *et al.*, 1970). The pathogen can be seed-borne or soil-borne, invades through roots and develops in the xylem. Mycelial growth in the xylem interferes with water conduction, thus resulting in wilting of the plant (Growing Flax, 2002). Depending on the plant growth stage, death of seedlings or premature death of plants can occur randomly or as distinct patches throughout the field. Plants grown from cracked or split seeds are more susceptible to wilting than intact seeds, whereas temperature is considered to be the main factor affecting disease development (Kommedahl *et al.*, 1970; Rashid and Kenaschuk, 1993).

Due to the ability of the pathogen to survive as a saprophyte for many years, in plant debris in or on the soil, it is difficult to eradicate the disease from a field once infected. The most commonly practiced disease control method is the use of resistance varieties, while crop rotation for at least three years helps lower the inoculum level, thus reducing disease incidence and severity (Diseases of Oil Seed Crops, 2012; Growing Flax, 2002). However, the resistance among varieties can differ due to the variability of pathogen races in different geographical regions with varying temperatures and environmental conditions (Rashid and Kenaschuk, 1993, Kommedahl 1970).

Flor in 1940 studied flax rust and wilt diseases and presented the gene-for-gene theory based on the observations of flax and *Melampsora lini* interactions. However, the genetics and the resistance mechanisms of fusarium wilt have never been fully understood, although resistance to the disease was developed by selection and recombination (Kroes, 1997). Diederichsen and Fu (2008), have shown that germplasm from North America has above average resistance to Fusarium wilt. While the germplasm from East Asia also has above average resistance, accessions from the Indian subcontinent and Europe have lower than average resistance to the disease. Research was carried out to develop resistant cultivars since none of the existing cultivars show resistance to all the isolates of *Fol* in western Canada and because resistance has broken down on occasion (Mpofu and Rashid 2001).

Recombinant inbred lines (RILs) or doubled haploid populations (DH) are developed from crosses between resistant and susceptible parents. Earlier attempts at identifying major genes conferring resistance using segregating populations of resistant and susceptible parents have been unsuccessful and it has been determined that the resistance was due to polygenic effects (Kommedahl, 1970). However, Spielmeyer *et al.* (1997) suggests that major gene effects may be hindered by the presence of different pathotypes in the field, segregation of many genes from genetically diverse parents affecting resistance or unreliable infection because of heterogeneity of field conditions. Resistance in a cross between a Linola line and cv. Glenleg was attributed to two major genes, with a significant number of resistant doubled haploid (DH) lines showing a more extreme phenotype than the resistant plant (Spielmeyer *et al.* 1997).

Materials and Methods

A field and growth chamber experiments were conducted to assess the disease reaction of recombinant inbred lines to *Fol* and to determine the correlation between the two environments

Preliminary Disease Assessment in the Growth Chamber

A protocol provided by Dr. Khalid Rashid, of AAFC, Morden, MB, was used to carry out the preliminary disease assessment to identify two pathogen isolates and to screen a recombinant inbred line (RIL) population with the selected isolates. Canadian flax varieties Bison and Novelty, were used as resistant and susceptible checks in the study.

Fifteen liters of vermiculite were supplemented with 8 L of 0.4 g/L Murashige and Skoog basal medium and were autoclaved at 121°C (gravity cycle, 1 hour) to prepare the growth medium. Four inch square pots were filled with the autoclaved mixture and 8 pots were fitted in a tray on plastic saucers to prevent mixing of the water leaching from the pots. All trays were covered with transparent lids to maintain humidity and to prevent the drying of the vermiculite.

Nine seeds were sown in a pot and plants thinned to seven seedlings, one week after seeding. The trays were placed in the growth chamber at 16 h day at 23°C temperature and 8 h night at 18°C.Plants were watered daily and to provide sufficient nutrients for the plants to grow, 50 ml of 0.4 g/L of MS medium was added to each pot 3 weeks after sowing.

Two replicates of a variety x isolate combination were placed in each of two growth chambers, and was replicated in another chamber at the same time. The two replicates in either chamber were sown and inoculated on subsequent days. All the variety x isolate treatment (or control) combinations were randomized within a bench, using excel.

Seventeen *Fol* isolates provided by Dr. Khalid Rashid (AAFC, Morden, Manitoba), collected from different fields in Manitoba and Saskatchewan, Canada, were sub-cultured on Potato Dextrose Agar (PDA) medium. A sterile 10 mm cork borer was used to cut agar plugs from the growing edge of a colony and 5 plugs were transferred to 125 ml of autoclaved Czapek Dox medium in s 250 ml Erlenmeyer flask. Three such flasks were prepared per isolate at a time and were incubated at 25°C on the shaker at 100 rpm at 16:8 hour day/ night. After 7 days, the spore suspension was filtered through sterile cheese cloth into a sterile flask and the filtrate of the three flasks combined. A haemocytometer was used to count the number of spores per ml of the spore suspension by counting the total number of spores in five squares, four times and the average was used to determine the concentration of the spore suspension. Finally the spore concentration was adjusted to 10^6 spores/ml, using sterile distilled water. A fresh batch of spore suspension was prepared for each experiment to maintain high germination and virulence of the spores.

Seven day old seedlings of Aurore, Oliver, Bison and Novelty were inoculated with 15 ml of each isolate spore suspension, using a 5 ml pipette. Spore suspension was added to the surface of the vermiculite so that it was distributed throughout the pot. In each replicate of each experiment all 4 varieties had a control pot that was inoculated with 15 ml of sterile distilled that was used to prepared the spore suspension. Lids were replaced after inoculation.

Disease assessment was conducted 7 days after inoculation using a disease grading scale provided by Dr. Khalid Rahsid, and continued every 7 days for 28 days after inoculation for each pot, and the heights of the 7 plants in a pot were also measured. Disease scores were used to calculate the **A**rea **U**nder **D**isease **P**rogress **C**urve (AUDPC).

RIL Phenotyping in the Growth Chamber

A subset of 160 of the 200 RILs (Aurore x Oliver) was used for the controlled environment phenotyping due to limitation in space and seeds. Seeds of the RILs increased under controlled conditions in the phytotron were used for Fusarium wilt screening in the growth chamber. Parents of the RILs, French varieties Aurore and Oliver were included along with the checks Bison and Novelty.

The experiment had two replications and was repeated once. Within each replication controls of the checks and parents were included for comparison. Seeding and inoculation of the replicates in the two chambers were carried out over two consecutive days to accommodate the work load.

Isolate 131 and 81 were selected to conduct the RIL phenotyping based on the differential interactions they showed with the resistant and susceptible parent and check and their growth and spore production in Czapek Dox medium. Seedlings and spore suspensions were prepared in the same way as in preliminary disease assessment.

Seven days old seedlings of the 160 RILs and parents and the checks were inoculated with 15 ml of the *Fol* spore suspension and 15 ml of sterile distilled water was added to control pots of the parents of the RILs and the checks. After inoculation, the trays were covered again with the lids.

Disease severity of each pot was recorded using the same grading scale and the heights of the individual plants in the pot were also recorded 4 times, starting 7 days after inoculation to 28 days, at 7 day intervals. From the disease scores the AUDPC was calculated for each RIL.

RIL Phenotyping in Wilt Nurseries

The original 200 French RILs and the parents of the RILs and varieties Novelty and Bison were used for wilt nursery phenotyping in Saskatoon and Morden and the plants were grown in a Modified Augmented Design (MAD) due to shortage of seed and the limited space in the wilt nursery, in 2013. Varieties Bison and Novelty were used as plot and sub-plot controls, while Aurore and Oliver were included as test plots.

Three disease assessments were conducted at each location, the first at the seedling stage, the second at the early flowering stage and third at the late flowering/green boll stage using the grading scale. Also the vigour of the RILs was recorded at the same time from 1-5, 1 the most vigorous plants and 5 the least vigorous along with plant stand recorded at the seedling stage with a full stand (high emergence and seedling survival) rated 1 and very poor stand rated 5. Using the disease data, AUDPC was calculated. Plants were not allowed to mature and were removed from the wilt nursery soon after the third assessment. This experiment will be repeated at the same two locations, in the same design summer, 2014.

Data Analysis

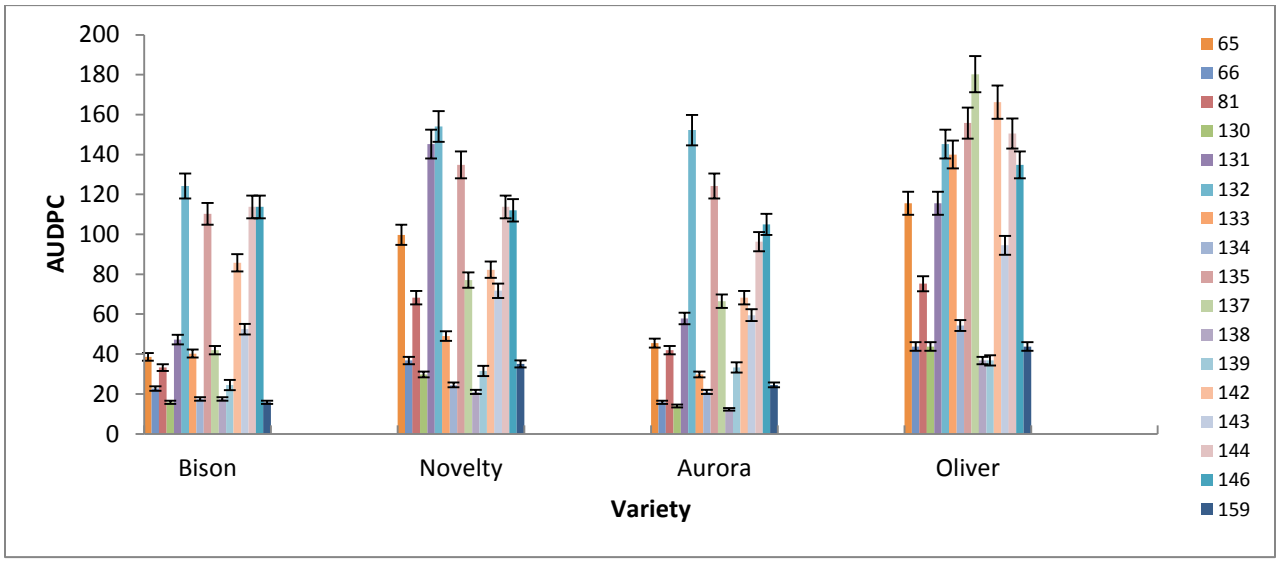
Data collected from the growth chamber and wilt nursery experiment, were analysed using SAS Version 9.3. Disease progression and severity at 28 days after inoculation of the seventeen isolates were analyzed to determine significant differences. With the presence of significant interactions between varieties and the isolates, the Tukey–Kramer test was conducted to identify significant differences among interactions. Isolate 131 was tested for significant differences among RILs using proc mixed, while isolate 81 was analyzed using proc glimmix. Finally, the Pearson's correlation coefficients among the disease grading at 28 days, AUDPC and height was assessed at the 5% significant level for both isolates.

Phenotypic data from the wilt nurseries were first adjusted according to the plot and subplot controls of the MAD design and tested for variance between the adjusted and raw values using Agrobase software. There was a significant difference between locations, and a separate analysis was done for each location.

Finally, the 160 subset that was used in growth chamber phenotyping was extracted from the wilt nursery screening, and the Pearson's correlation coefficient was determined for the two isolates and the wilt nursery data.

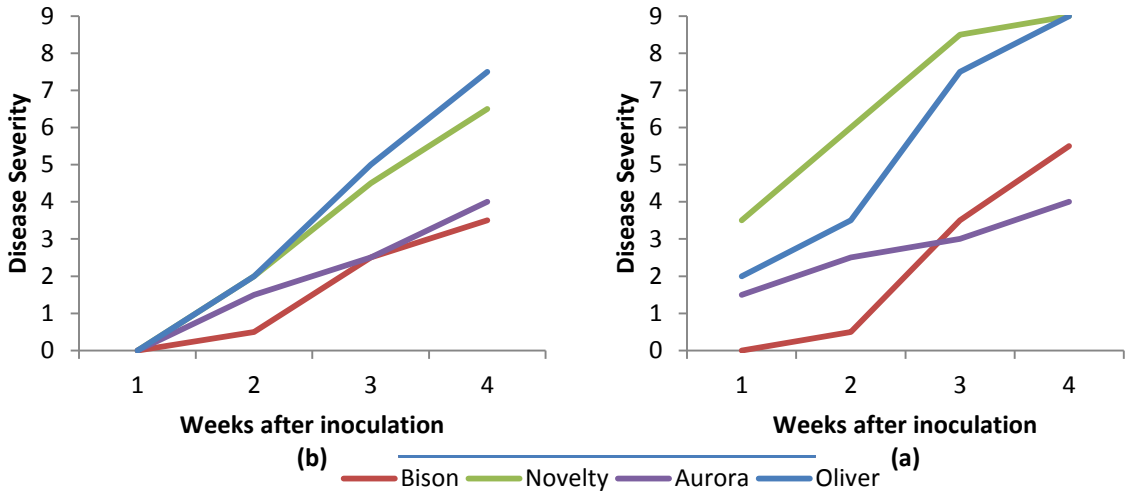
Results and Discussion

Differential disease development from an isolate on the two parents and the checks were tested to identify two isolates to phenotype and all the isolates showed significant interaction with the variety and all isolates showed significant F values at 5% significance. Comparisons between the isolates by varietal interactions gave several isolate by variety combinations that were not significantly different from each other. Therefore, the second condition, the disease progression or the disease reaction at 28 days after inoculation was considered, which was also significant for the isolates by variety interactions.



Graph 5.1.1 AUDPC of the 17 isolates with each variety

Finally, the spore production of the isolates in Czapek-Dox medium was taken into consideration to decide which two isolates to use to test RILs, since that had been a limiting factor for some isolates.



Graph 5.1.2: a) Disease progression of the four varieties with isolate 131; b) Disease progression of the four varieties with isolate 81

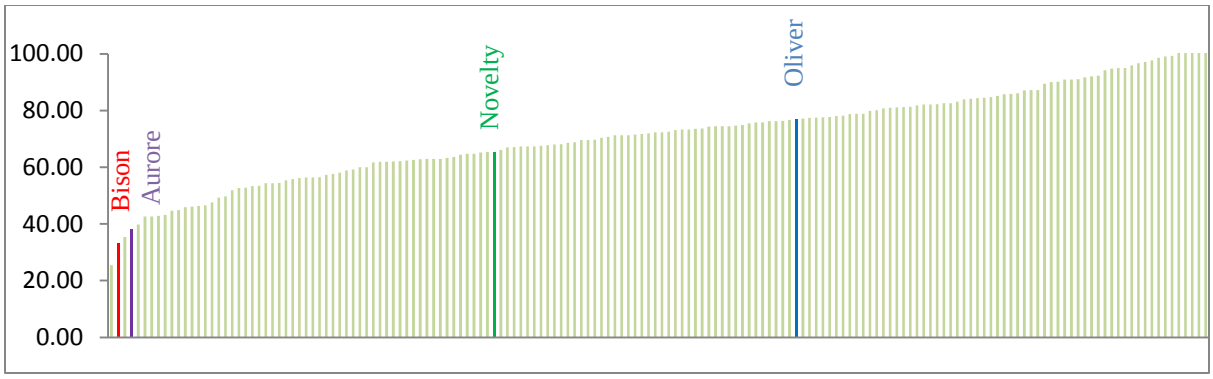
Based on the three aspects, isolate 131 was initially selected for the phenotyping the RILs in the growth chamber. RILs developed severe disease reactions with this isolate and therefore when selecting the second isolate more consideration was given to select a less aggressive isolate that was showing differential reactions between parents and checks. Thus, isolate 81 was selected, which caused less disease severity on the varieties tested, 28 days after inoculation.

RIL Phenotyping in the Growth Chamber - Isolate 131

AUDPC for RILs with isolate 131 was not normally distributed. However, the when tested with residuals, data was normally distributed (Shapiro – Wilk test) and showed homogenous variance at the 5% significant level for Levene's test of homogeneity.

RILs showed a significant effect on variables, AUDPC and disease grading at 28 days, with F values of 1.84 and 1.95, respectively at the 5% probability level, when analyzed in a mixed model.

Disease progression and the disease severity at 28 days both showed a gradual increase rather than separation of resistant and susceptible lines.

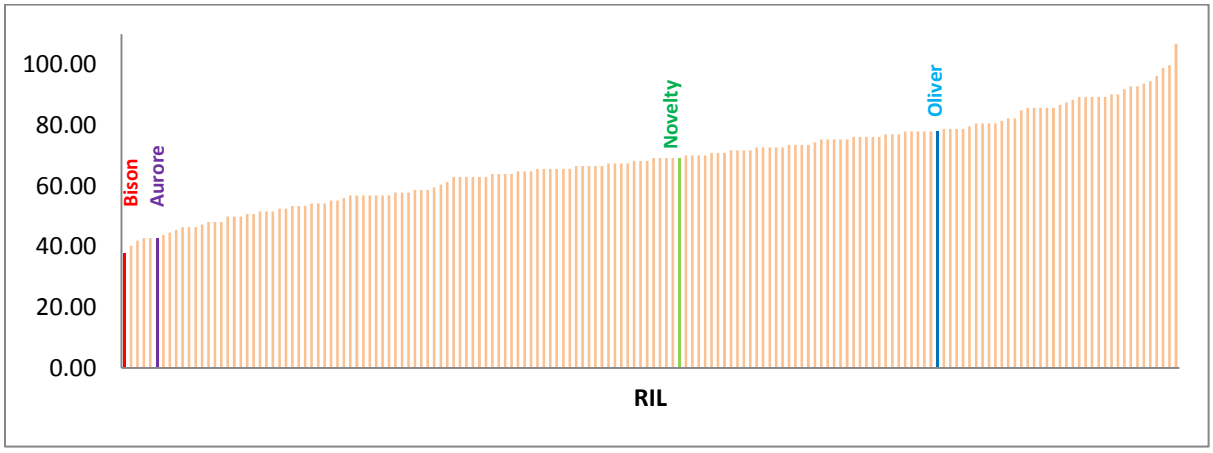


Graph 5.2.1.1 AUDPC of the RILs with isolate 131

Of the 160 RILs used in the growth chamber experiment, 13.8% had an average disease grading of 8 or higher, with another 33.1% between 7 and 8. Since this was high disease reaction, a less aggressive isolate was selected for the second round of RIL phenotyping.

RIL Phenotyping in the Growth Chamber - Isolate 81

Similar to isolate 131, RILs showed gradual variation from resistant to susceptible for both AUDPC and disease severity.



Graph 5.2.2.1 AUDPC of the RILs with isolate 81

Five percent of the RILs used for phenotyping with isolate 81 showed disease severity of 8 or above, with another 35% graded between 7 and 8.

RIL Phenotyping in Wilt Nursery

Since the wilt nursery experiment was designed as a MAD design, initial adjustments were carried out using Agrobase software, to adjust the test plots according to variations in plot and subplot controls. There was no significant difference between the raw and adjusted data.

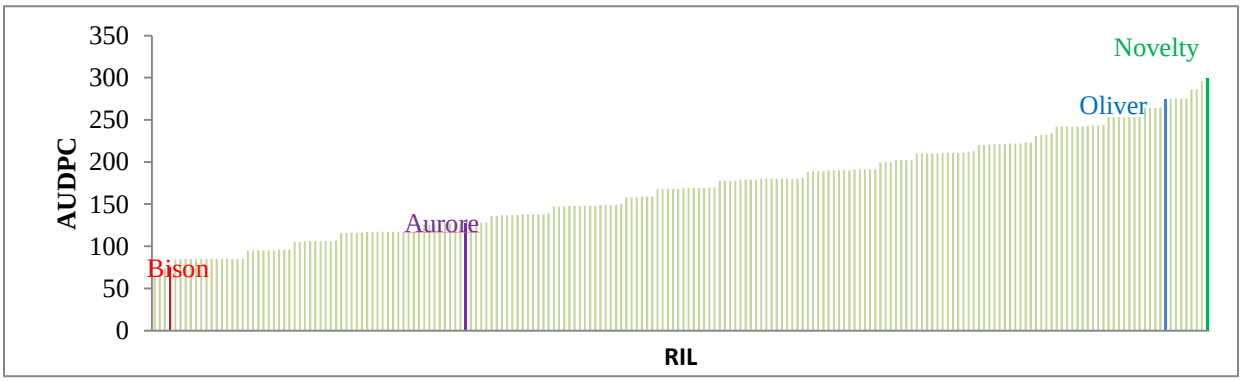
Adjusted values were used for the other analyses. Both locations deviated from a normal distribution but with homogenous variance and was analyzed using the generalized linear mixed

model. However, there was significant difference between the two locations at F = 370.01 and the data could not be combined. Therefore, the data from the two locations were considered separately. However, when tested for correlation, both sites showed moderate but significant correlation.

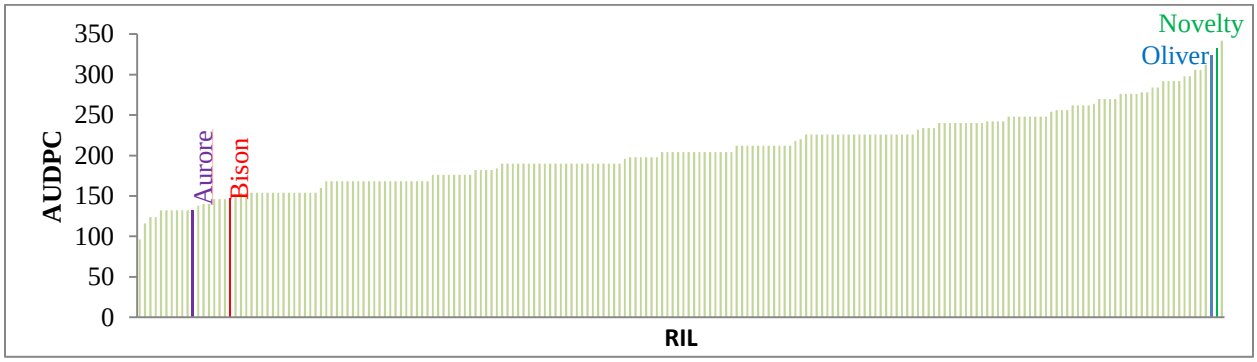
Table 5.3.1. Pearson's correlation coefficients for AUDPC at wilt nurseries in Saskatoon (skaudpc) and Morden (moaudpc)

	skaudpc	**moaudpc**
skaudpc	1.00000	0.61269
moaudpc		1.00000

There was a gradual change at both locations for AUDPC and disease severity, similar to that observed for phenotyping in the growth chamber. Of the 200 RILs used in the field trial, 21.5% at Saskatoon wilt nursery and 33% at Morden wilt nursery showed disease severity of 8 and 9.



Graph 5.3.1 AUDPC of the RILs in the wilt nursery at Morden



Graph 5.3.2 AUDPC of the RILs in the wilt nursery at Saskatoon

Finally, the Pearson's correlation coefficient was determined for AUDPC and disease severity at the final rating (green boll stage for wilt nursery and 28 days after inoculation for phenotyping in

the growth chamber). For both variables, all the experiments showed moderate but significant correlations, but higher correlations were observed between the two nurseries and the two isolates.

Table 5.3.2. Pearson's correlation coefficients for disease reaction in the Saskatoon wilt nursery (audpcsk), Morden wilt nursery (audpcmo), isolate 131 in growth chamber (audpc131) and isolate 81 in growth chamber (audpc81) for 160 subset of RILs

	audpcsk	**audpcmo**	**audpc131**	**audpc81**
audpcsk	1.00000	0.65136	0.40028	0.38046
audpcmo		1.00000	0.33016	0.21140
audpc131			1.00000	0.40194
audpc81				1.00000

Table 5.3.2. Pearson's correlation coefficients for disease severity at the final rating with Saskatoon wilt nursery (wilt3sk), Morden wilt nursery (wilt3mo), isolate 131 in growth chamber (d28i131) and isolate 81 in growth chamber (d28i81)

	wilt3sk	**wilt3mo**	**d28i131**	**d28i81**
wilt3sk	1.00000	0.62397	0.51253	0.40879
wilt3mo		1.00000	0.40816	0.32407
d28i131			1.00000	0.40675
d28i81				1.00000

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