Sclerotinia stem test protocol for canola

Developed by Lone Buchwaldt and modified by Pathology Sub-committee members to suit WCC-RRC's need for sclerotinia testing of canola lines in the co-op system.
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Experimental design

Sclerotinia stem-tests are carried out under field conditions. Establishment of a healthy and uniform plant stand is required. Irrigation and misting is generally not needed for symptom development unless weather conditions are hot and dry during flowering. The negative effect of wind out can be mitigated by planting the test by planting guard rows around the test site or by planting in a sheltered or low-lying and area. Fertilizer application, weed and insect control should be carried out as for a commercial canola crop. However, all fungicide treatments should be avoided. The seed can either be planted directly in the field or in a greenhouse followed by transplanting of seedlings at the 3-4 true leaf stage.

Test locations: Tests can be located in canola growing areas of Alberta, Saskatchewan and Manitoba.

Plot lay-out*:* All lines are planted in 6 replications in a complete randomized block design. Data is needed from 5 plants per line per replication. Planting of extra seed is recommended to ensure that enough plants are at the full flower stage at the time of inoculation.

Susceptible and resistant checks: The susceptible is 45H29 and partially resistant check is 45S52 both from Pioneer. In cases when a late flowering co-op line is included, a suitable late flowering susceptible check will be identified for comparison.

Inoculum

Fungal isolate: Sclerotinia sclerotiorum isolate #321 is recommended for inoculation to allow comparison between test locations. The isolate was collected in a commercial canola field in Olds, Alberta in 1992 (Kohli and Kohn, 1995 Mol Ecol. 4: 69-77). Sclerotia of this isolates can be obtained from Lone Buchwaldt (email: Lone.Buchwaldt@agr.gc.ca).

Inoculum production: Sclerotia are divided into batches matching the number of expected inoculation dates. They are surface sterilize in 10% Javex (10 ml Javex + 90 ml water) and rinsed 2-3 times in sterile water. Sclerotia are cut in half and each half placed on PDA in the centre of a 9 cm Petri plate. The plates are sealed with Parafilm and incubated up-side-down (to avoid condensation) at 20-22^{o}C in a 16/8 hour light/dark cycle (an incubator or lab bench is equally good).

Age of mycelium cultures: The cultures are ready for inoculation when the mycelium is 1-2 cm from the edge of the Petri plate which normally takes 4 to 6 days. Cultures that have reached the edge of the plate are too old for inoculation. If needed, the growth of cultures can be slowed down by placing them at 5-8^{o}C (in a fridge); however, this should only be for a maximum of 24 hours. A cooler can be used for transport of the cultures to the field, but direct contact with ice should be avoided.

Mycelium plugs: Plugs should be cut with a 7 mm diameter cork borer approximately 5 mm behind the growing margin under clean but not necessarily sterile conditions. Ensure that each plug contains a similar amount of mycelium by holding the plates up against a light source while

cutting thereby avoiding areas with sparse hyphal growth. For consistency, only one or two circles of plugs should be cut from each culture. Between 15 and 30 plugs can be obtained per Petri plate depending on the size of the culture.

Inoculation

Time of inoculation: Each plant is inoculated when it is at full flower. Full flower is defined as the stage when petals senesce at the same rate as new flowers open. This corresponds to 65 on the BBCH canola growth scale; see http://en.wikipedia.org/wiki/BBCH-scale_(canola). There is usually a 7 day window for inoculation at individual test locations. However, at some locations it may be necessary to inoculate on 2 or 3 different days due to variable flower time between lines or even between plants of the same line.

Preparation of Parafilm: First cut several 2 meter long 3 cm wide ribbons of Parafilm, remove the paper, combine 6-8 layers of ribbon and cut them into 3 x 4 cm pieces. Stretch each piece to 4 x 7 cm and place them on a tray. Place one plug with the mycelium facing up in the middle of each piece of Parafilm. The inoculum should be used within one hour of preparation.

Site of inoculation: The middle internode of the main stem is inoculated. This indernode is identified as half way between the soil surface and the lowest side branch, about 30-50 cm above the soil surface. The internode is inoculated by attaching a 7 mm plug to the stem with the mycelium facing the stem surface. The two ends of the Parafilm are twisted around one another on the opposite side of the stem while ensuring that the plug keeps its shape.

Weather considerations: Inoculation is best carried out at temperatures between 18^{o} and 23^{o}C. If the weather is hot and dry then inoculation should be postpone to the evening or to a cooler day.

Verification of inoculation: Evidence of successful inoculation can be verified after 5-7 days by looking for small lesions under the mycelium plugs. If uniform infection is not achieved, it is possible to re-inoculate the stems using the internode above the previously inoculated internode given that the plants are still flowering.

Disease rating

Lesion length: The length of each stem lesion is measured in millimeters (no decimals) 21 days after inoculation (dai). Measurements made one day before or after is acceptable i.e. 20 to 22 dai. The longest part of the lesion is measured including the dark margin that separates the necrotic and healthy stem tissue (Figure 1).

Wilted plants: It may be difficult to determine the upper edge of the infected area if the plant is wilting. In these cases it is often possible to measure from the site of inoculation to the lower edge of infection. This length is then multiplied by 2 and recorded as the total lesion length.

Lesion firmness: The appearance of each lesion is recorded as black (bl), firm (f), soft (s) or collapsed (c) (**Fig 2**). The firmness is assessed by applying a slight pressure to the lesion with two fingers (**Fig 3**). A cross section of a firm lesion shows infection is confined to the stem surface while soft lesion affects the vascular tissue and the stem pith (**Fig 4**). Generally, collapsed lesions girdle the stem, while soft lesions do not.

Many small, black flecks: The stem under the plug can sometimes show many small, black flecks indicating many attempts of infection. In such cases, the longest fleck is measured (**Fig 2**).

Escapes: If no symptoms can be seen at the site of inoculation it is most likely an escape due to problems with the inoculum or weather conditions, and should therefore not be attributed to resistance of the stem. These escapes are rare, but the missing values should be recorded with 'e' not zero (0).

Thin stems: Small plants with thin stems (< 6 mm diameter) should be avoided and not inoculated since they are more susceptible than normal-sized stems of the same line.

Other observations: In order to determine if a data set should be excluded it is essential to describe damages to the plant stand caused by blackleg or other diseases, insect pests, weeds, hail, excessive moisture, drought etc. It is also important to mention variability in the test caused by human error.

Guidelines for the WCC/RRC Pathology Sub-committee

The committee maintains a list of collaborators who have the capacity to conduct the stem test.

Number of co-op lines: Each test can accommodated up to eight co-op lines plus the susceptible and resistant check lines for a total of 300 plants (10 lines x 5 plants x 6 replications). If more co-op lines need evaluation, they will be divided into two or more groups with similar flowering dates and planted in separate tests.

Coded names: Seed of co-op lines should be sent to WCC/RRC co-op coordinator, Raymond Gadoua, CCC. Seed envelopes will be labelled with code names and distributed to collaborators. A key for converting to actual names will be available after statistical analysis and first discussion by the Pathology Sub-committee.

The disease rating data, planting and inoculation dates should be emailed to the stem test coordinator, Lone Buchwaldt, AAFC (Lone.Buchwaldt@agr.gc.ca). Any problems regarding plant growth, weather, human errors and other comments should be included in this email.

Statistical analysis: A GLM (general linear model) analysis of stem lesion length and % soft + collapsed lesions will be conducted for individual test location and for lines over locations combined. Lines will be separated using Fisher's LSD analysis.

Elimination of test locations: Data from a test location will be eliminated if the average stem lesion length is less than 20 mm on the susceptible check line 45H29. Other reasons for exclusion of data are poor or uneven plant growth, extreme weather conditions and human errors. Data from all other test locations will be included in the final evaluation.

Minimum number of test locations: Five is the minimum number of test locations for evaluation of each co-op line. Data can be obtained from field tests in one or more years. Data from indoor testing are not permitted.

Criteria for PR-labeling of a co-op line: A co-op line is considered partially resistant to sclerotinia when the average stem lesion length at 21 days is significantly less than the susceptible check 45H29 in the analysis of test locations combined.

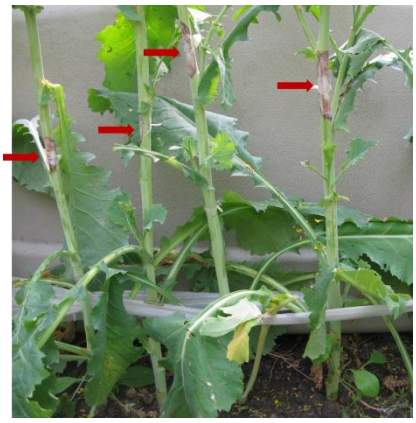

Figure 1. The middle internode of the main stem (red arrow) inoculated with a mycelium plug of *S. sclerotiorum* held in place with a piece of Parafilm.

Figure 2. The four different notations used to describe lesion appearance: black, firm, soft and collapsed.

Figure 3. A slight pressure with two fingers on the stem lesion is used to assess whether the lesion is firm, soft or collapsed.

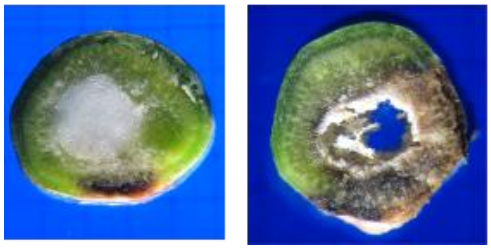

Figure 4. Cross sections of canola stems inoculated with a *S. sclerotiorum* showing a firm and a soft lesion, respectively.