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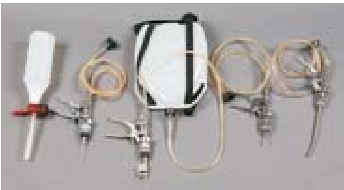
Screening Corn for Resistance to Common Diseases in Canada



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Screening Corn for Resistance to Common Diseases in Canada

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Preface

Since 1995, researchers of the corn improvement program of Agriculture and Agri-Food Canada (AAFC) have been developing a multiple-pest breeding program for corn. Previous pest resistance breeding at AAFC focused on developing resistance to ear rots caused by *Fusarium* species and details of techniques developed for this disease were published in the AAFC Technical Bulletin 1996-5E, "Screening Maize for Resistance to Gibberella Ear Rot". However, other diseases of corn annually reduce yields and grain quality in Canada. A summary of the most common diseases is available in the AAFC Publication No. 2088 /E 2001, " Common Diseases of Corn in Canada". AAFC has now developed field screening techniques for several of these diseases and has standardized the techniques for routine use in a breeding program. The techniques allow for good differentiation between genotypes, ranging from very susceptible to highly resistant and are now being used in AAFC's corn breeding program to develop populations and inbred lines with improved resistance.

This publication describes field screening techniques for common diseases of the ear (gibberella ear rot, fusarium ear rot, common smut), stalk (gibberella, fusarium and anthracnose stalk rot) and leaves (eyespot, common rust and Northern corn leaf blight) of corn grown in Canada. Details of inoculum production, inoculation technique and evaluation of resistance are given for each disease. As well, a general summary on nursery designs, isolation of pathogens, pathogenicity tests, safe handling of inoculum and infected plant material, and statistical analyses and data interpretation is given.

This booklet is designed primarily to assist researchers in corn breeding; however, the techniques described can be and have been used to study resistance mechanisms, inheritance of resistance, epidemiology and basic pathology of the most common diseases of corn in Canada.

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1.0 Ear Diseases

1.1 Gibberella and Fusarium Ear Rot

Gibberella Ear Rot (Pink Rot, Red Rot) causal agent: *Fusarium graminearum* Schwabe [sexual state: *Gibberella zeae* (Schwein.) Petch]. Fusarium Ear Rot (Kernel Rot) causal agent: 3 species are involved, *F. verticillioides* (Saccardo) Nirenberg [= *F. moniliforme* J. Sheld. (sexual stage: *G. moniliformis* Wineland)]; *F. proliferatum* (Matsushima) Nirenberg (sexual stage: *G. fujikuroi* var. *intermedia* Kulmann); and, *F. subglutinans* (Wollenweb. & Reinking) Nelson, Toussoun and Marasas (sexual stage: *G. subglutinans* Nelson, Toussoun and Marasas).

The typical symptom of gibberella ear rot is a pink-to-reddish coloured mold, often starting at the ear tip or from an insect wound and growing down the ear. Cobs can become quite spongy while husks become bleached and adhere tightly to the kernels. A powdery, cottony-pink mold may form later. Black coloured perithecia (fungal fruiting bodies that produce sexual spores) may be visible on husks.

The typical symptom of fusarium ear rot is a whitish to pink coloured mold, often starting at the ear tip or butt of the ear, commonly from an insect wound. *Fusarium verticillioides* symptomatic kernels tend to be randomly scattered on the ear unlike other ear rots that spread from an initial point of entry. Infected kernels may also exhibit a 'starburst' symptom in which white streaks radiate from the point of silk attachment to the kernel. Similar to gibberella ear rot, husks may become bleached and adhere tightly to the kernels and black coloured perithecia may be visible on husks at the later stage.

1.11 Inoculum Production

A liquid suspension of macro- and/or microconidia is commonly used as inoculum. Best fungal growth is achieved in a low-sugar liquid medium that is subject to some agitation (shaking, rotating, bubbling) to prevent mycelial growth and clumping. A typical medium is a modified Bilay's (Reid et al., 1996) which consists of 2.0 g potassium dihydrogen phosphate (KH_2PO_4); 2.0 g potassium nitrate (KNO_3); 1.0 g potassium chloride (KCl); 1.0 g magnesium sulphate ($MgSO_4$); 0.0002 g/L each of: ferrous sulphate ($FeSO_4$), ferric chloride ($FeCl_3$), manganese sulphate ($MnSO_4$), and zinc sulphate ($ZnSO_4$); 1.0 L distilled water; and, 1.0 g dextrose. The medium is dispensed in 150 ml aliquots into 500 ml Erlenmeyer flasks, autoclaved for 20 minutes, checked for a pH=5, then four to six pieces of 1 cm^2 potato dextrose agar (PDA) containing mycelium and conidia of a single isolate is added. Cultures are agitated at 25°C for 1 hr at 4 hr intervals under natural light supplemented with fluorescent light. Conidial concentrations can reach several million per ml in one week. Prepared inoculum can be stored at 2-4°C (in a refrigerator) for a maximum of four weeks before decrease in conidial viability occurs. Prior to inoculation, the mixture is filtered through two layers of cheesecloth to remove mycelial clumps and diluted with sterile water to the desired conidial concentration. A concentration of 2.5×10^5 conidia/ml is commonly used.

1.12 Inocultion Techniques

The inoculation techniques for Gibberella and Fusarium ear rot are the same. A separate inoculation technique is required for each mode of fungal entry, i.e. silk vs. kernel.

1.121 Silk Channel Inoculation

Two ml of conidial suspension are injected into the silk channel of each primary ear 4-7 days after silk emergence when silks are elongated, pollinated, and may have some tip browning but are not dry. Timing of inoculation is critical since inoculations made too late result in little or no disease symptoms. Care must be taken to ensure that the inoculum is injected at right angles to the silk channel, Otherwise conidia will be injected down the silk channel onto the kernels and evaluation for silk resistance will not be accomplished. A useful apparatus for doing these inoculations is a graduated, 10 ml, self-refilling, automatic vaccinator attached to a 2.5 L backpack container (Nasco Co., Fort Akinson, WI). The drenching nozzle that comes with the vaccinator is removed and replaced with an 18-gauge, 5.6 cm long, Luer-lock stainless steel needle (Reid et al. 1996) (Fig. 1). Secondary ears are not inoculated since they are not present in all genotypes and often mature later than primary ears. After inoculation, ears can be spray painted with a small dot of red spray paint on the lower husk/shank area to mark inoculated plants.

Figure 1. *Silk channel inoculator used for gibberella and fusarium ear rot and common smut inoculations. A graduated, 10 ml, self-refilling, automatic vaccinator is attached to a 2.5 L backpack containing the fungal spore suspension. Two ml of inoculum is injected into the silk channel 4-7 days after silk emergence.*

1.122 Kernel Inoculation

Two ml of a conidial suspension is injected into 3-4 kernels at the center of each primary ear at the blister stage of kernel development, approximately 10-15 days after silk emergence. Timing of inoculation is critical since inoculations made too late result in little or no disease symptoms. Care must be taken to ensure that the inoculum is injected into the kernels and not into the cob (rachis) or symptoms will be difficult to see on the kernels. A useful apparatus for doing these inoculations is a graduated, 10 ml, self-refilling, automatic vaccinator attached to a 2.5 L backpack container (Nasco Co., Fort Akinson, WI). The drenching nozzle that comes with the vaccinator is removed and replaced with a modified injector end consisting of four needles that have sealed ends and an opening on the underside near the tip to allow puncture and injection without plugging the needle with husk and kernel tissue (Reid et al. 1996) (Fig. 2). Secondary ears are not inoculated since they are not present in all genotypes and often mature later than primary ears. After inoculation, ears can be spray painted with a small dot of red spray paint on the lower husk/shank area to mark inoculated plants.

Figure 2. *Kernel inoculator used for gibberella and fusarium ear rot inoculations. A graduated, 10 ml, self-refilling, automatic vaccinator is attached to a 2.5 L backpack containing the fungal spore suspension. Two ml of inoculum is injected via four needles into the kernels 10-15 days after silk emergence. The four needles have sealed ends and openings on the underside near the tip to allow puncture and injection without plugging the needle with husk and kernel tissue.*

1.13 *Evaluation of Resistance*

For both diseases, plants are evaluated for resistance at normal grain harvesting moisture (approx. 24%), about 6-8 weeks after inoculation. Ears are hand husked while still attached to the stalk and the severity of ear rot symptoms is evaluated using rating scales based on the percentage of kernels with visible symptoms of infection such as rot and mycelial growth (Fig. 3). This is more difficult to do with *F. verticillioides* since symptomatic kernels tend to be more scattered on the ear; thus, a visual estimation of the percentage of kernel surface infected is required.

In a breeding program, the acceptable level of visible infection for selection of resistant plants is largely dependent on the inoculation technique used. In the case of silk channel inoculations, ears with no visible infection (rating=1) reflect resistance to the spread of infection down the silk channel. Ears with infected kernels imply that silk resistance was not present or was not sufficient to stop the fungus from reaching the kernels before kernel resistance developed and/or kernels hardened and were no longer receptive. The environment (e.g. humidity, temperature) can also delay progression of the fungus down the silk channel.

With kernel wound inoculation techniques, there is always some infection. In this case a resistant plant would be one in which the infection does not spread from the wounded kernels to non-wounded kernels (rating=2). This can be manifested as a shrinking/abortion of the wounded kernels with or without visible signs of infection such as mycelial growth.

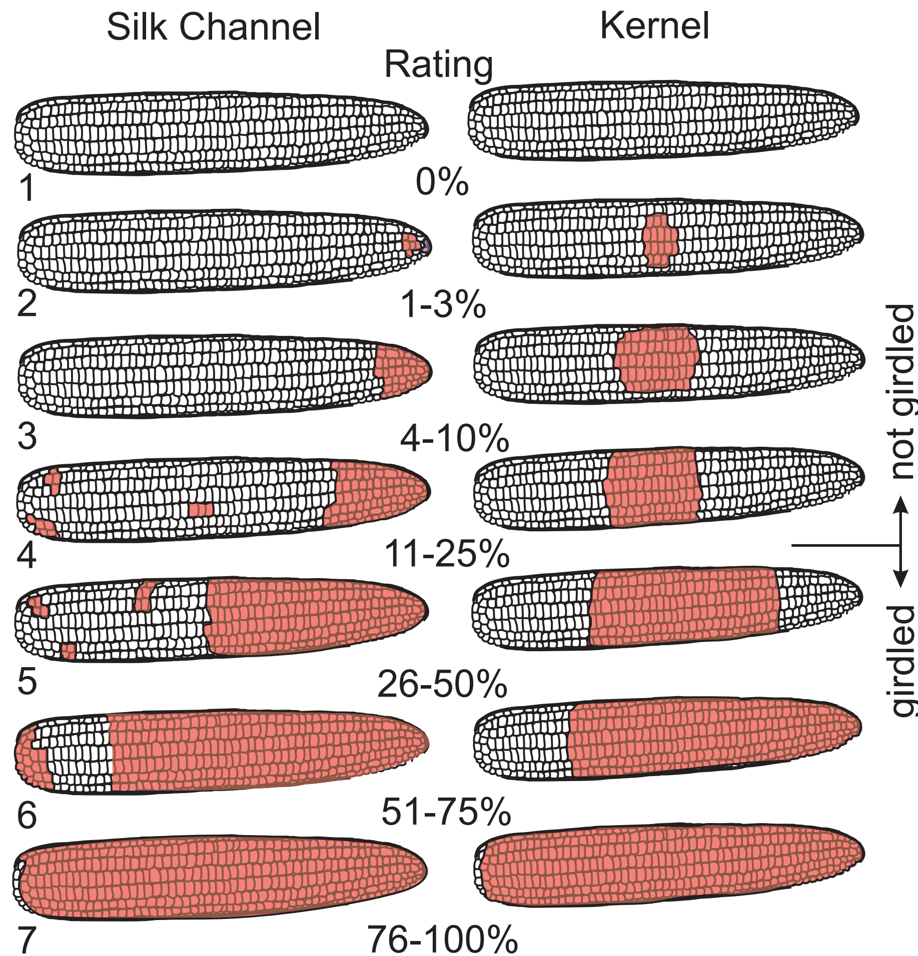

Figure 3. *Gibberella and fusarium ear rot silk and kernel inoculated disease severity rating scales based on the percentage of kernels with visible disease symptoms. The silk channel scale can also be used for assessing disease severity with common smut inoculations where the ratings are based on the number of kernels with galls.*

1.2 Common Smut (Boil Smut, Blister Smut)

Causal agent: *Ustilago zeae* (Beckm.) Unger [= *U. maydis* (DC.) Corda]

Symptoms of common smut can occur on all plant parts above the ground, especially young and actively growing plant tissues. Large 2-10 cm galls (swollen, distended growths) are formed on stalks, tassels and ears. The galls are first covered by a silvery-white membrane, which changes to a grey mass containing black powdery spores. When mature, these galls will erupt and release the spores. Galls may also form on leaves but are generally small, brown and hard. Early infection may kill young plants. Plants with large galls on the lower stalks may be stunted, barren or produce small ears. Infection of ears is usually from spores germinating on the silks and mycelia growing down the silks to infect the kernels.

1.21 Inoculum Production

Mature galls are collected from infected corn ears each field season, dried in a laboratory oven at 28-32ºC for 10-15 days and stored at 4-6ºC until further use. Two days before inoculation of field plants, 15 ml of potato-dextrose agar (PDA) media are poured into 9 cm diameter petri dishes. About 36 hours before inoculation, one gall from each diseased ear is held above a petri dish and slightly tapped to dislodge the spores (teliospores) onto the agar. The petri dish is then shaken to evenly distribute the spores and then turned briefly upside down to dislodge any large particles of gall tissue that may have fallen onto the agar. The plates are then cultured at 24-28ºC with 12 h light/darkness for 30 h to allow sporidia formation. After culturing, each plate is washed twice with 15 ml of sterilized water and the resultant wash is filtered through four layers of cheesecloth and diluted with sterile water to a concentration of 5×10^5 sporidia/ml. One ml of 0.5% Tween 20 is added to every 500 ml of suspension to improve the ability of the suspension to adhere to the ears after inoculation. Suspensions can be stored for a maximum of 4 hours at 4-6ºC before use.

1.22 Inoculation Technique

For evaluating resistance to smut infection of the ear, use the ear rot silk channel injection technique as outlined in Section 1.121 (Fig. 1).

1.23 Evaluation of Resistance

Plants are evaluated for resistance at normal grain harvesting moisture (approx. 24%), about 6-8 weeks after inoculation. Ears are hand husked while still attached to the stalk and the severity of common smut symptoms is evaluated using rating scales based on the percentage of kernels which have galls, where: 1= no galls on the ear; 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 25-50%, 6= 51-75%, and, 7= 76-100% of kernels have galls (Fig. 3). A rating of 1 indicates that silk channel resistance exists.

2.0 Stalk Diseases

2.1 Gibberella and Fusarium Stalk Rot

Gibberella stalk rot causal agent: *Fusarium graminearum* Schwabe [sexual state: *Gibberella zeae* (Schwein.) Petch]. Fusarium stalk rot causal agent: 3 species are involved, *F. verticillioides* (Saccardo) Nirenberg [= *F. moniliforme* J. Sheld. (sexual stage: *G. moniliformis* Wineland)]; *F. proliferatum* (Matsushima) Nirenberg (sexual stage: *G. fujikuroi* var. *intermedia* Kulmann); and, *F. subglutinans* (Wollenweb. & Reinking) Nelson, Toussoun and Marasas (sexual stage: *G. subglutinans* Nelson, Toussoun and Marasas).

Gibberella stalk rot infected plants often wilt and the leaves may change from a light to a dull green colour while the lower stalk becomes dry and the pith tissue disintegrates to a shredded appearance. Distinctive symptoms of gibberella stalk rot are a tan to dark brown discolouration of the lower internodes and a pink to reddish discolouration of the pith tissue. Bluish-black coloured perithecia (fungal fruiting structures that release sexual spores) or reddish-white asexual spores form on the stalk surface. Plants may lodge if the infection is severe.

Symptoms of fusarium stalk rot are not much different from gibberella stalk rot. Plants may wilt, the leaves change from a light to a dull green colour, lower stalks dry and the pith tissue disintegrates to a shredded appearance. Brown streaks appear on the lower internodes and the rotted pith tissue may be whitish-pink to salmon in colour as opposed to the distinct red-pink colour of gibberella stalk rot. Infected plants may lodge. Symptoms usually appear late in the season.

2.11 Inoculum Production

The inoculum production techniques for gibberella and fusarium stalk rot are the same as those used for gibberella and fusarium ear rot (Section 1.11).

2.12 Inoculation Technique

The inoculation techniques for both gibberella and fusarium stalk rot are the same. Plants are inoculated with a conidial suspension (2.5×10^5 spores/ml) by injecting 2 ml of inoculum at a 45° angle downwards into the middle of the first internode above the uppermost aerial root node 4-7 days after silk emergence. Since regular syringe needles will get plugged with pith tissue when inserted into the stalk, inoculations are easier if a 5 cm long, 3 mm diameter needle with a sealed end and an opening on the underside near the tip (allows puncture and injection) is used. This needle can be connected to a graduated, self-refilling automatic vaccinator attached to a 2.5-L backpack container (Nasco Co., Fort, Atkinson, WI) (Fig. 4).

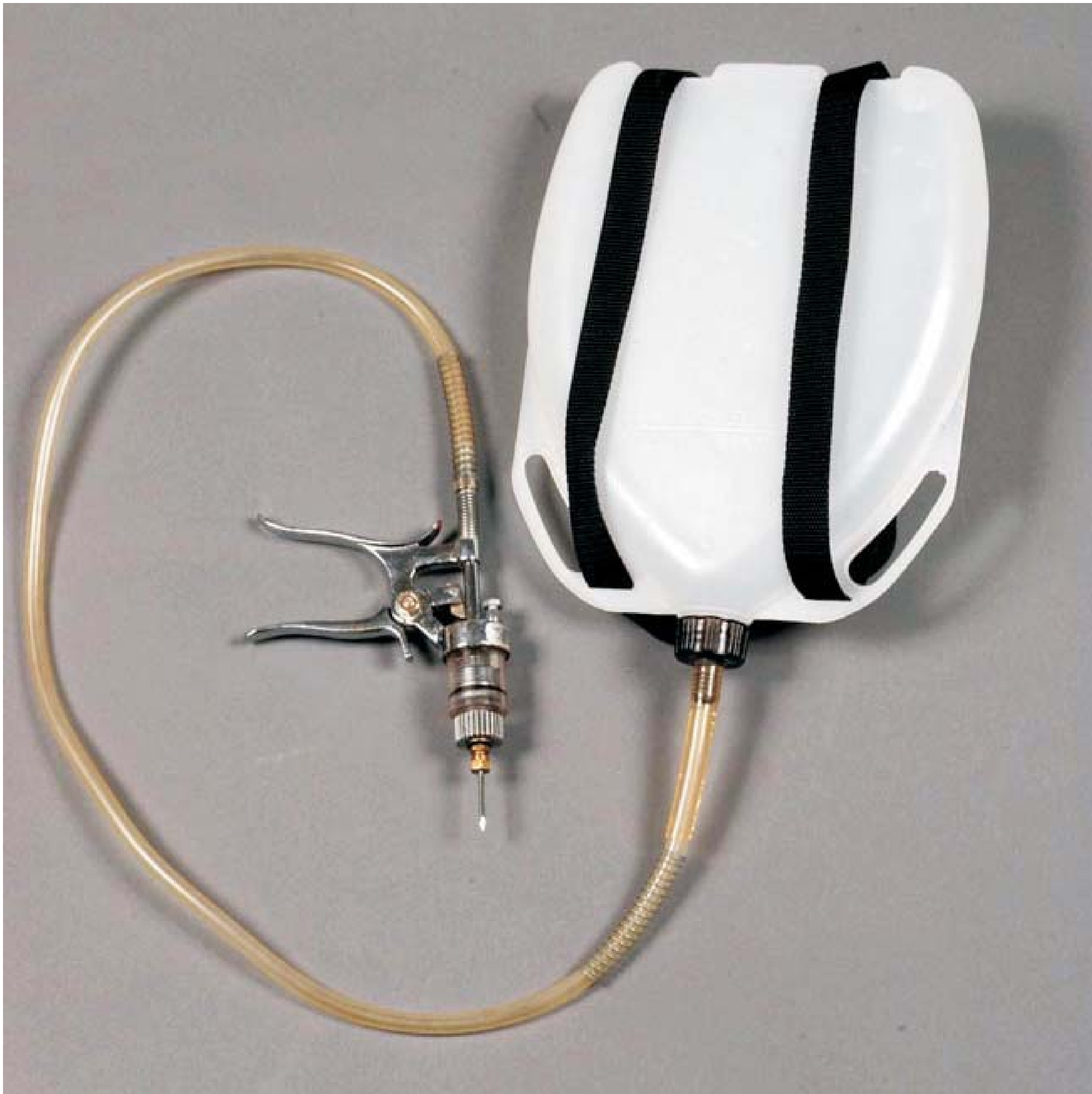

Figure 4. *Inoculator used for gibberella, fusarium and anthracnose stalk rot inoculations. A graduated, 10 ml, self-refilling, automatic vaccinator is attached to a 2.5 L backpack containing the fungal spore suspension. The stalk is punctured and two ml of inoculum is injected. The 3 mm diameter steel needle has a sealed end and an opening on the underside near the tip to allow puncture and injection without plugging the needle with pith tissue.*

2.13 *Evaluation of Resistance*

At normal grain harvesting moisture (approx. 24%) or about 6-8 weeks after inoculation, stalks are split longitudinally from just below the ear to the ground. The severity of stalk rot is estimated using a rating scale based on the degree of discoloration in the internodes, where 1= no visible spread of the pathogen from inoculation point; 2= 1- 25%, 3= 26- 50%, 4= 51- 75%, 5 = >75% of inoculated internode symptomatic; 6 = symptoms have spread to one adjacent internode; and, 7= symptoms have spread to two or more adjacent internodes (Fig. 5).

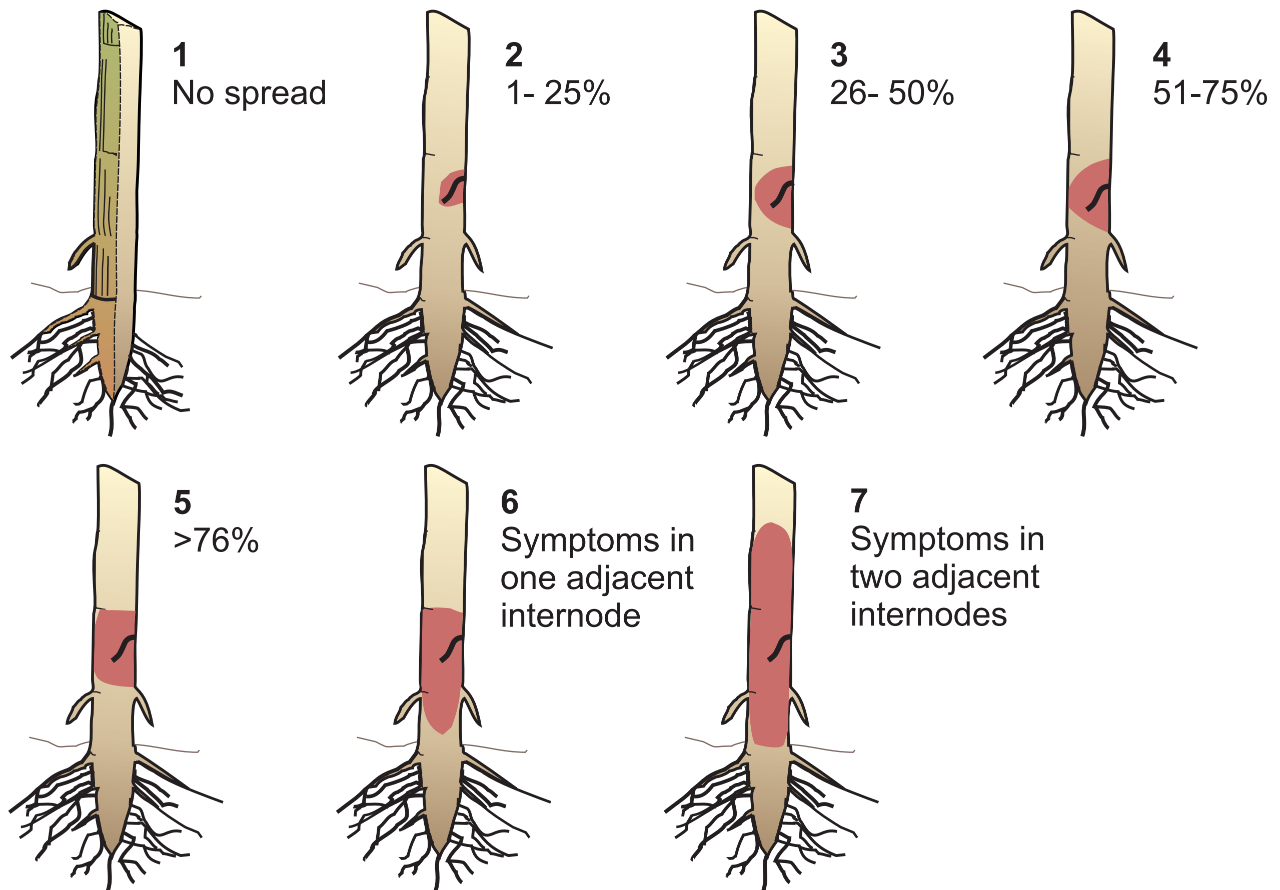

Figure 5. *Gibberella, fusarium and anthracnose stalk rot disease severity rating scale based on the degree of discolouration in the internodes and the spread of symptoms from the inoculated internode.*

2.2 Anthracnose Stalk Rot

Causal agent: *Colletotrichum graminicola* (Ces.) G.W. Wils. (sexual state: *Glomerella graminicola* Politis)

Anthracnose stalk rot is characterized by a distinctive blackening of the lower stalks as a result of black streaks that appear late in the season. The pith turns dark brown and has a shredded appearance. Numerous, black, spiny asexual fruiting bodies (acervuli) form on the surface of the dead tissue. Like most stalk rots, the most obvious symptom is a sudden death of plants before grain maturity. Since the leaves wilt and die after infection, the plant has the appearance of being 'frosted'. Another common symptom is plant lodging.

2.21 Inoculum Production

Colletotrichum graminicola is cultured in 9 cm diameter petri dishes with 15 ml oatmeal agar medium (boil 75g of rolled oats in 1000ml of water, add 18-20g agar) at room temperature (22-25°C) with 12 hr light/dark for 10-14 days. After culturing, each plate is washed twice with 15 ml of sterilized water and the combined wash is filtered through four layers of cheesecloth and diluted with sterile water to a concentration of 2.5×10^5 spores/ml.

2.22 Inoculation Technique

The inoculation technique for anthracnose stalk rot is the same as that used for gibberella and fusarium stalk rot (Section 2.12) (Fig. 4).

2.23 Evaluation of Resistance

Resistance evaluation for anthracnose stalk rot is the same as that used for gibberella and fusarium stalk rot (Section 2.13) (Fig. 5).

3.0 Leaf Diseases

3.1 Eyespot (Brown Spot)

Causal agent: *Aureobasidium zeae* (Narita & Hiratsuka) J.M. Dingley (= *Kabatiella zeae* Narita & Hiratsuka)

Symptoms of eyespot consist of round-to-oval lesions (2-5 mm in diameter) with a tan-to-cream coloured centre and a brown or purple margin surrounded by a yellowish halo, giving the characteristic 'eyespot' appearance. These lesions are easy to recognize if the leaf is held up to a light. The lesions may fuse to form large necrotic areas. The upper leaves may wither and die prematurely. Symptoms can sometimes be confused with non-infectious physiological leaf spots or insect damage.

3.11 *Inoculum Production*

Aureobasidium zeae inoculum is produced in a liquid culture with a modified carboxyl methyl cellulose (CMC)-maltose-yeast medium. CMC and maltose are difficult to dissolve in water, so the medium is made by adding 2.0 g CMC, 2.0 g maltose, 0.6 g yeast extract, 0.6 g peptone, 0.4 g KH_2PO_4, and 400 ml of distilled water into a 1 L flask which is then sterilized by autoclaving at 15 psi for 15-20 minutes. After the medium cools down, six to eight 1 cm^2 pieces of potato dextrose agar (PDA) containing mycelium and conidia of a single isolate of *A. zeae* are added to each flask. Cultures are agitated on a shaker table at 25°C for 1 hr at 4 hr intervals under natural light supplemented with fluorescent light. In two to three weeks the layer of mycelium and conidial spores on the walls of the flask turns black in colour. The flask is then shaken to dislodge this layer into the liquid which is then filtered through four layers of cheesecloth and diluted with sterilized water to a concentration of 2.5×10^5 conidia/ml.

3.12 Inoculation Technique

To achieve good infection levels, plants are inoculated twice, once at the 6-8 leaf stage and again at the 10-12 leaf stage. Two ml of conidial suspension (2.5×10^5 conidia/ml) is dispensed into the whorl (plant tissues are not damaged) of each plant by using a graduated, 10 ml, self-refilling, automatic vaccinator attached to a 2.5 L backpack container (Nasco Co., Fort Akinson, WI) (Fig. 6). The original 18 cm long, 0.5 cm diameter stainless steel drenching nozzle that comes with the vaccinator is used for dispensing the inoculum; this nozzle extends the reach of the operator so that inoculum can be dispensed with minimal bending over.

Figure 6. *Inoculator used for eyespot and common rust inoculations. A graduated, 10 ml, self-refilling, automatic vaccinator is attached to a 2.5 L backpack containing the fungal spore suspension. Two ml of inoculum are dispensed into the whorl, once at the 6-8 leaf stage and again at the 10-12 leaf stage.*

3.13 *Evaluation of Resistance*

Disease severity is evaluated at the soft dough stage (approx. 3 weeks after silk emergence) using a rating scale based on the percentage of leaf area with visible symptoms where **1**= no symptoms, **2**= just a few lesions (< 1% of the leaf area with symptoms), **3**= several lesions, but not linked together (1- 5% infected leaf area), **4**= many lesions some linked together to form a necrotic (dead) area (6- 20% infected leaf area), **5**= necrotic areas linked together and a few leaf tips are dead (21-50% infected leaf area), **6**= 50% of the leaf tips are dead (> 50% of leaf area with symptoms), and **7**= most of the leaves are dead and plant is usually dead (Fig. 7).

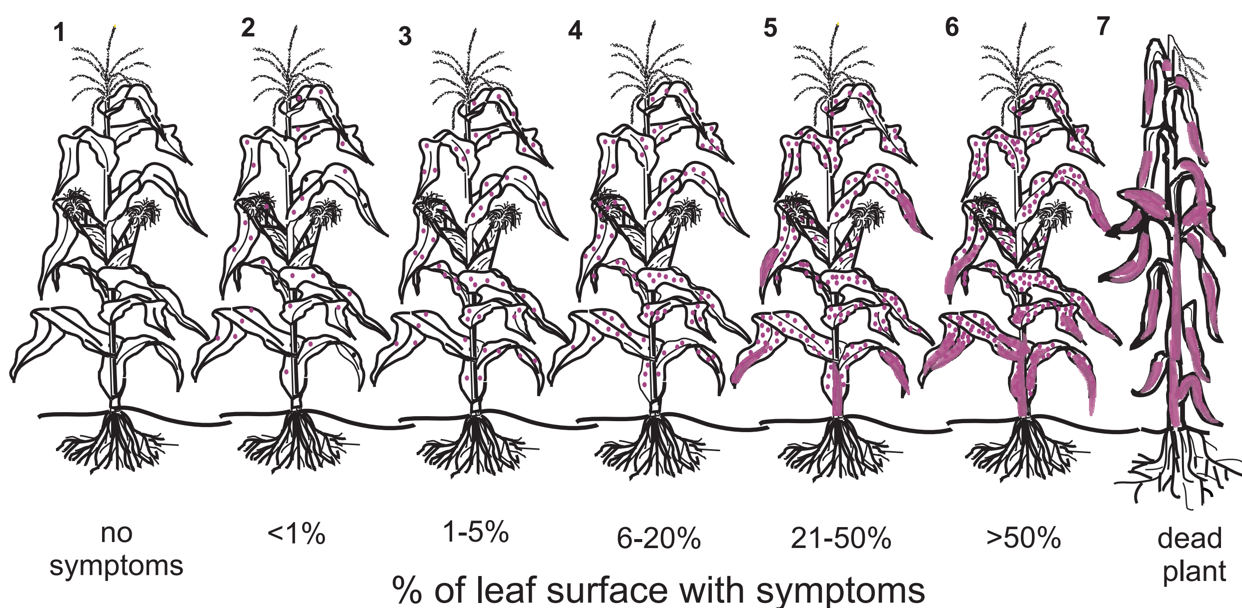

Figure 7. *Disease severity rating scales for eyespot and common rust. Ratings are based on the percentage of leaf surface with symptoms.*

3.2 Common Rust

Causal agent: *Puccinia sorghi* Schwein.

Symptoms of common rust on leaves begin as small, discoloured flecks that soon turn into small, round to elongated, reddish brown pustules full of red coloured urediniospores. Pustules can be observed on both leaf surfaces as well as on husks, leaf sheaths and stalks. The pustules turn black as the plant matures and are frequently clustered in bands around the leaves as a result of infection that took place when the leaf tissue was in the whorl. Younger leaves are more susceptible than mature leaves. When the disease is severe, leaf tissue around the pustules turns yellow, withers and dies. Some hybrids have resistance genes; these plants may develop a hypersensitive response consisting of several small (pin-prick) pale coloured lesions.

3.21 Inoculum Production

Puccinia sorghi is an obligatory parasite, so the pathogen must be kept alive and cannot be stored in a dormant state as with the other pathogens outlined in this guide. Sweet corn, which is generally susceptible to common rust, is a useful over-wintering host when planted in the greenhouse. Urediniospores can survive up to six months in the refrigerator (4-6°C); so from January to mid-June, urediniospores can be collected from greenhouse grown sweet corn plants previously inoculated with rust spores. Prior to field inoculation, all the collected spores are mixed together and divided into two batches for inoculation at two stages of plant development. Before inoculation, the spores are suspended in sterilized water with 1 ml of 0.5% Tween 20 added to every 500 ml of water followed by agitation (stirring) for 15 minutes to improve the ability of the suspension to adhere to the plants after inoculation. The suspension is then filtered through two layers of cheesecloth to discard any large particles or plant tissues and diluted with sterilized water to 2.5×10^5 spores/ml. The amount of sterilized water to use for suspension preparation will depend on how successful the spore collection was; we usually suspend our spores in 6 liters of water.

3.22 Inoculation Technique

The inoculation technique used for common rust is the same as that used for eyespot (Section 3.12) (Fig. 6).

3.23 Evaluation of Resistance

At the soft dough stage about 3 weeks after silk emergence, plants are rated for both specific (based on pustule type) and general resistance (based on disease severity). Six pustule types (Fig. 8) are classified as: immune (I)= no rust symptoms; highly resistant (HR) = hypersensitive reaction, having yellowish pin-point fleck symptoms; resistant (R)= very small pustules with light-green or yellowish or brown necrotic borders and still covered by the cuticle of the leaf; moderately resistant (MR) = small pustules mostly covered by the host cuticle but a few may have emerged and a few urediniospores are present; moderately susceptible (MS) = larger pustules, some are linked together and some have emerged from the leaf cuticle, a few urediniospores are present; and susceptible (S) = large pustules, mostly linked together and broken out with lots of urediniospores. The rating scale for general resistance is the same as that used for eyespot (Section 3.13) (Fig. 7) except that lesions are replaced by pustules.

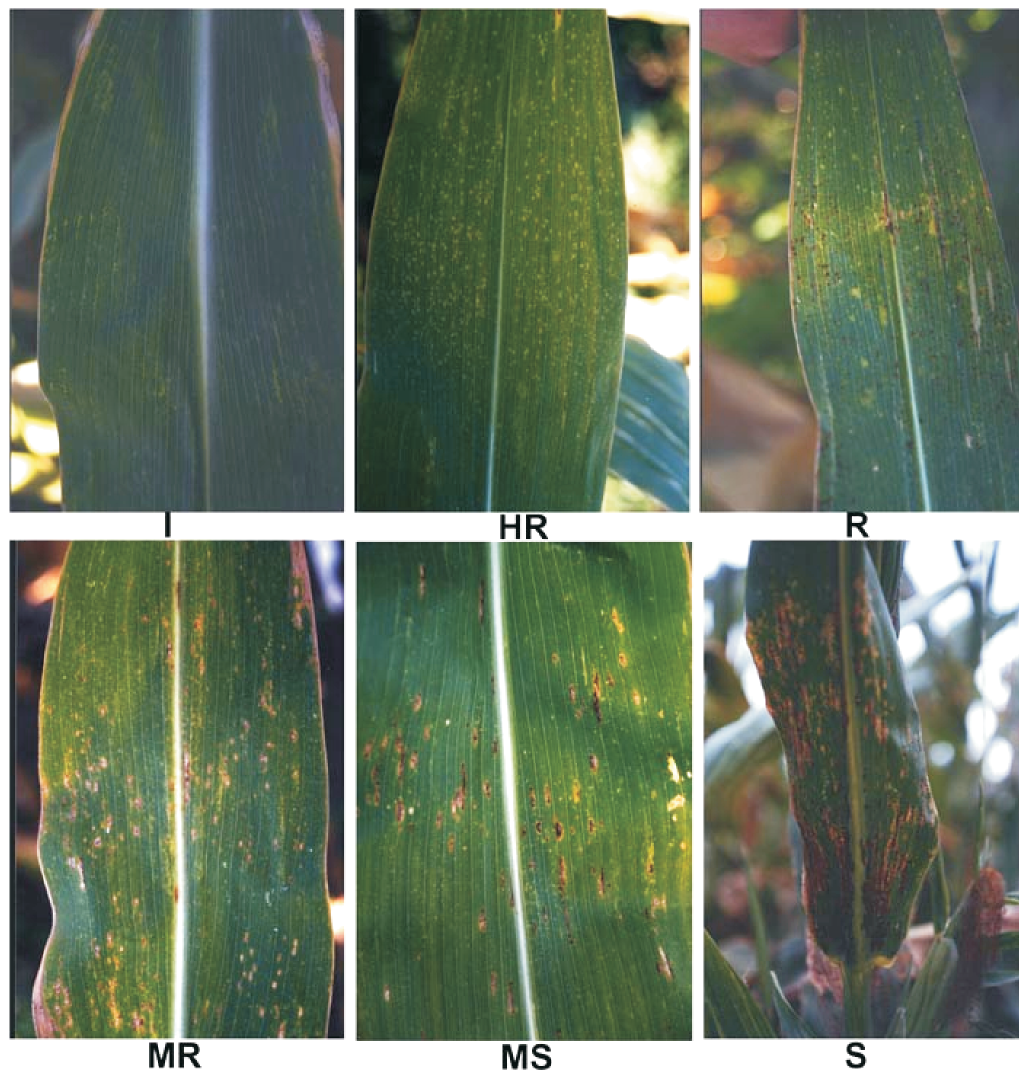

Figure 8. *Pustule types associated with specific resistance to common rust. I = immune, HR = highly resistant, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible.*

3.3 Northern Corn Leaf Blight (White Blast, Crown Stalk Rot or Stripe)

Causal agent: *Setosphaeria turcica* (Luttrell) K.J. Leonard & E.G. Suggs [asexual state: *Exserohilum turcicum* (Pass.) K.J. Leonard & E.G. Suggs = *Helminthosporium turcicum* Pass.]

Typical symptoms of Northern corn leaf blight consist of long, elliptical, grayish-green or tan coloured lesions appearing first on the lower leaves. Lesions may be as large as 4 X 15 cm. When infection is severe, entire leaves die and individual lesions are difficult to see; this is sometimes referred to as a 'burning' of leaves. Lesions may also occur on husks but the kernels are not infected. A severely infected plant may turn grayish-green in colour and die pre-maturely. Plants with specific resistance gene(s) to Northern corn leaf blight exhibit different symptoms, usually a long stripe or narrow elliptical green-yellowish lesion with or without a grayish center.

3.31 Inoculum Production

The easiest way to produce inoculum for Northern corn leaf blight is to collect infected leaves, grind them into a powder and store the powder at room temperature for use in the following growing season. To initiate an abundance of infected leaves in the first year, the plants are inoculated with infected corn kernels. Kernels are boiled in water for 1-2 hours until the pericarp starts to break, then used to fill 1L flasks half way. The flasks are sterilized in an autoclave at 15 psi for 45 minutes and when cool, six to eight 1 cm^2 pieces of potato dextrose agar (PDA) containing mycelium and conidia of a single isolate of Northern corn leaf blight are added to each flask. The flasks are cultured for 2-3 weeks at room temperature with 12 hr light/12 hr dark conditions until all the kernels appear colonized by fungi. Three days before inoculation, the kernels are removed from the flasks, spread on a tray to separate kernel masses and allowed to dry to increase production of spores. Inoculation with a leaf powder is preferred over inoculation with infected kernels since the powder preparation is much less labour intensive and birds often eat the kernels after inoculation.

3.32 Inoculation Technique

To achieve good infection levels, plants are inoculated twice, once at the 6-8 leaf stage and again at the 11-12 leaf stage by placing approximately 0.2 g (1.5 ml) of infected leaf powder or three cultured kernels into the whorl of each plant. A useful inoculation tool for the powder application is the Bazooka (Sistrunk Inoculators, Starkville, MS 39759, USA) (Fig. 9); two doses of powder from the Bazooka are equivalent to 0.2 g.

Figure 9. *Bazooka inoculator used for Northern corn leaf blight inoculations. Two doses (0.1 g per dose) of ground infected leaf tissue are dispensed into the whorl of each plant.*

3.33 Evaluation of Resistance

At the soft dough stage, about 3 weeks after silk emergence, plants are rated for both specific resistance (based on lesion type) and general resistance (based on disease severity) according to Wu et al. (1991). There are four lesion types (Fig. 10): resistant lesion (R), stripe or narrow elliptical green-yellowish lesion; moderately resistant lesion (MR), narrow, long, elliptical gray lesion with green-yellowish border; moderately susceptible lesion (MS), long, elliptical, gray lesion with green-yellowish border; and susceptible lesion (S), long, elliptical, gray or tan coloured lesion. The rating scale for general resistance is similar to that used for eyespot and rust but the lesions are larger and the higher ratings are based on the spread of symptoms from the lower to upper leaves on the plants. The 7 ratings are: **1**= no symptoms; **2**= < 1%; **3**= 1-10%; **4**= 11-25% of leaves symptomatic; **5**= > 50% of the lower leaves are symptomatic, < 25% of center (the four leaves closest to the primary ear) and upper leaves are symptomatic; **6**= lower leaves are dead, > 50% of the center leaves, < 25% of upper leaves are symptomatic; and, **7**= plant is dead (Fig. 11).

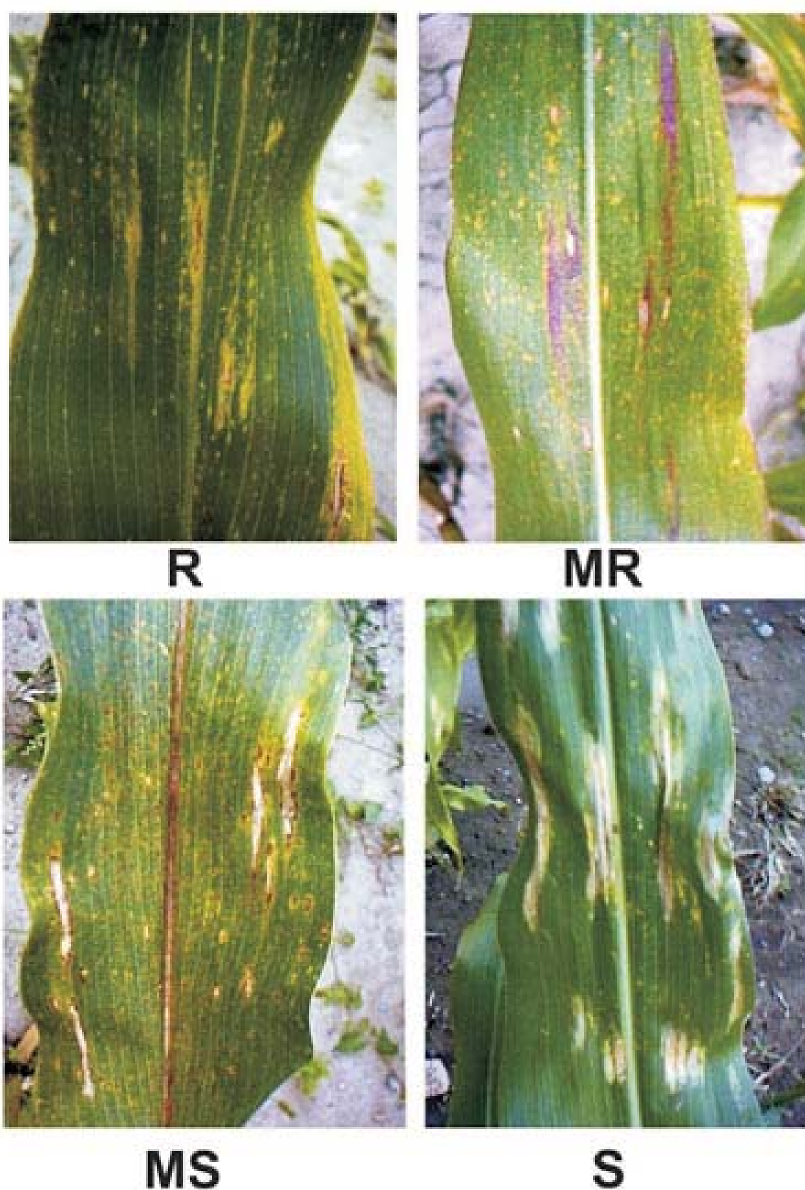

Figure 10. *Lesion types associated with specific resistance for northern corn leaf blight.*
R = resistant,
MR = moderately resistant,
MS = moderately susceptible,
S = susceptible.

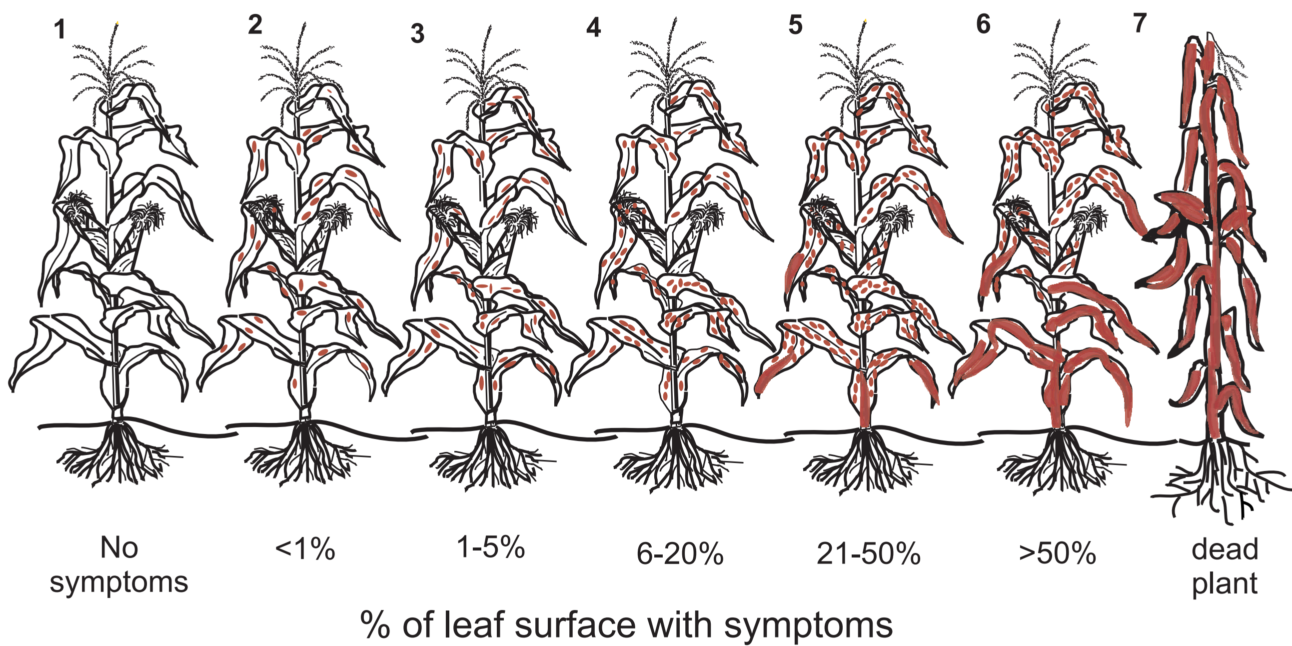

Figure 11. *Disease severity rating scale for northern corn leaf blight. Ratings are based on the percentage of leaf surface with symptoms and the spread of symptoms from the lower to upper leaves.*

4.0 Nursery Design and Plot Maintenance

In disease screening nurseries, one of the most important considerations is the creation of an environment that favours disease development and promotes an outbreak. Soil type can influence disease development. A flat, uniform, fertile field that results in good plant emergence and optimal plant health is best. With-in plot variability of disease severity is significantly reduced if care is taken to make plants as uniform as possible and if timing of inoculation is consistent. Appropriate management practices must be practised to ensure that only healthy plants are inoculated. This includes proper fertilization based on a soil test, plant spacing, weed control, and even pesticide use to control insects and other diseases. Stressed plants may have increased disease severity, making resistance difficult to determine.

For all of the diseases outlined above, best results are obtained if adequate soil and air moistures are maintained by the use of irrigation. Irrigation is essential to provide adequate moisture for fungal growth, and in some cases, to lower the temperature of the crop canopy to a level more suitable for growth of some fungal species. The amount and duration of irrigation will depend on the amount of natural rainfall and daily temperatures. In general, irrigation should be provided for at least four weeks after inoculation; disease progress should be monitored during this period to determine if more or less irrigation is required.

In breeding programs that screen for more than one pathogen, care should be taken to minimize the spread of disease from one area to another. This is of little concern when dealing with ear and stalk rot pathogens since most of these will not spread to neighbouring plants during a given growing season. However, this is not the case with leaf pathogens. Eyespot does not spread much within a field but common rust and especially Northern corn leaf blight can rapidly spread from the point source of inoculation. It is advisable to plant Northern corn leaf blight fields in isolation from other corn fields. At harvest, inoculated plants for all diseases should be removed from the field or ploughed under to minimize winter survival of the pathogen and reduce future inoculum potential.

The sample size or number of plants required to accurately assess resistance is determined by the disease in question, the corn genotype, the disease rating protocol, and the purpose of the resistance evaluation. In basic screening (i.e. resistant or not) for most diseases, a single-row plot of 15 plants will suffice for genetically uniform inbreds and hybrids; however, a minimum of 30 plants are needed for segregating or less uniform populations. If the disease is rated by incidence (percentage of plants with symptoms), then a minimum four-row plot (50-60 plants) should be used. In a breeding nursery, single rows of developing lines will suffice for selection but better results will be obtained if at least two rows are planted per line. In a typical nursery row of 15 plants, two-row plots will give you at least 20 plants from the center of the rows from which to determine disease severity and allow for selection. The first 1-3 border plants at the ends of each row should not be used for selection or rating as these plants are more subject to environmental influence. At least four rows should be used for disease evaluations of segregating populations or lines in the early stages of development. Competition between different corn genotypes, especially between inbreds, hybrids and populations, must be reduced by grouping similar genotypes together and adding border rows between groups. Susceptible and resistant check genotypes should be planted every 30-50 rows to allow comparisons among genotypes, replicates, locations, and years.

5.0 Isolation of Pathogens

To conduct inoculations as outlined previously, pure cultures of each pathogen must be maintained to produce inoculum. Single-spore isolations from collected samples are used to produce individual isolates which are then used alone or in combination with other isolates depending on the gene-to-gene relationship between pathogen pathogenicity and host resistance. Since the main purpose of most breeding programs is to discover breeding material with multiple resistance genes, a mix of two or more isolates is often used. Isolates must be tested for their virulence prior to use in screening. Virulence tests are conducted by inoculating a set of inbreds or hybrids having known levels of susceptibility/resistance. This test can be done either in the field or in the greenhouse.

The isolation of *Fusarium* species is well described in many publications and a commonly used selective medium is pentachloro nitrobenzene (PCNB) (Booth, 1971). Use of recently infected plant tissue is often critical for the isolation of some diseases such as the causal agents of eyespot and anthracnose stalk rot. The spores of *A. zeae* and *C. graminicola* are small and similar to those of many other fungi that may be contaminating older lesions and are not eliminated with leaf surface sterilization (Arny et al., 1971; Dale, 1963). The isolation of Northern corn leaf blight is relatively easy because the spores are larger than those of many other fungi (White, 2000). Potato Dextrose Agar (PDA) and Cornmeal Agar (CMA) are two commonly used media for the isolation and culture of eyespot and Northern corn leaf blight fungi. Common rust is an obligatory parasite, thus a culture method is not available yet. Rust must be over-wintered in the greenhouse; a separation of the races of *P. sorghi* is not necessary for routine screening in Canada.

The causal agent of common smut, *U. maydis*, has a heterothallic character. Locus *a* controls the mating type of sporidia and *b* controls the pathogenicity (White, 2000). This heterothallic character has made its pathogenicity more complicated; paired multisporidial cultures are generally more virulent than the monosporidial cultures comprising them. Since it is not clear how many single spore isolates are needed for common smut evaluations, it is better to use mixed spores to produce inoculum.

6.0 Safe-Handling of Inoculum and Infected Plant Material

The spores produced by most plant pathogens can cause allergies and the inflammation of lung tissue. Inoculum made with *Fusarium* species and *Fusarium*-infected plant tissue will also contain mycotoxins. These toxins are toxic to humans and inhalation of spores or dust from contaminated plants can be extremely hazardous. Precautions should be taken to minimize contact with all pathogen cultures and infected plant material by any route (oral, inhalation, or skin). Handling of fungal cultures, especially those of *Fusarium* species, should only be done in a biological containment hood which draws air away from the user and through a filter. Disposable gloves and other protective clothing (labcoat, coveralls) should be worn when working with liquid cultures, especially during filtration.

Inoculation equipment should be cleaned with 70% ethanol after use each day. Do not mix inoculating equipment between fungal species. All laboratory surfaces, where culture or suspension transfers have been made, should be cleaned with 70% ethanol or other disinfectants immediately after use.

During harvest, especially of *Fusarium*-infected and smut infected ears, inhalation is more hazardous than ingestion of infected plant material and dust. Gloves, coveralls and dust masks should be worn when harvesting because dust and fungal particles are released from the ear as the husk is pulled off. Whenever possible, stand upwind when handling mold-contaminated ears. When combining contaminated field plots, the combine operator should also wear protective clothing and a mask, especially if the combine does not have a positive-pressure closed-in cab, in which air filters are changed frequently.

Grinding of infected plant material such as grain and diseased leaves, should take place in a room equipped with a ventilation/exhaust system capable of handling dust removal. Protective clothing, especially a dust mask, should be worn and where appropriate, cleaned after use. Hands, face and other exposed areas of the body should be washed with soap and water immediately after grinding. Never eat or drink in areas where pathogen cultures or infected plant material are present.

7.0 Statistical Analysis and Data Interpretation

In corn breeding programs, disease screening is usually conducted to determine if a genotype is resistant or susceptible; thus the conclusion is more qualitative than quantitative. Therefore, sometimes the highest average rating from all replicates is used to represent the disease severity of a given genotype. For many diseases, such as eyespot, common rust, Northern corn leaf blight, and even stalk rot, the disease severity of a given genotype varies little within a replicate, thus ratings are often conducted on a row basis and two replicates are often sufficient for a basic disease screening. For more quantitative studies or for more variable diseases, especially those subject to large environmental influence such as ear rot and common smut, plants should be rated individually and an average rating calculated for each row within each of 3-4 replicates. Methods will vary depending on research objectives, e.g. individual plant ratings may be more desirable with segregating populations.

A randomized complete block design is usually used and data is analysed and presented as a range of resistance or a ranking of genotypes. Since a rating scale is used for assessing disease severity, there are various methods one can choose for analysis of the data. Nonparametric analyses can be performed; however, they can be limiting in the degree of analyses available. The data can be transformed and then analysed, or standard parametric statistics can be performed on the data provided that residual error terms are distributed normally.

Due to environmental influences, the ability to differentiate among genotypes may vary somewhat from year to year, making it desirable to evaluate material over two or more years. Mean ratings for a given genotype vary from year to year, but rankings among genotypes are less likely to vary, even across environments/locations in a given year. Thus it is important to use check genotypes that show differences under different environments, and it is also important to test error mean squares for homogeneity before pooling data over years. A minimum of two years of field data should be obtained for each genotype except in breeding nurseries where selections are being used to advance inbred development or improve populations.

Resistance and susceptibility are two relative terms. They vary with the disease in question, with the degree of yield loss and with the level of resistance available in the current pool of germplasm. For gibberella and fusarium ear rot, the acceptable level of mycotoxins in the grain is very low; thus, a resistant plant is one that has virtually no visual infection. For common smut, if disease severity is greater than 3.0, then the yield damage is greater than 10%, which is not acceptable. For stalk rot diseases, genotypes with disease severity ≤ 3.0 are rare, so severity ratings as high as 5 can be acceptable. For leaf diseases, there are some genotypes with excellent resistance (disease severity ≤ 3.0); however, yield damage is low even with disease severity ratings as high as 5, these genotypes can be classified as having intermediate resistance.

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Notes