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Preface

Since 1986, scientists of the maize (corn) improvement program of the Eastern Cereal and Oilseed Research Centre (ECORC) (formally the Plant Research Centre), Agriculture and Agri-Food Canada, have been developing field-plot techniques to screen maize inbreds and hybrids for resistance to ear mold caused by *Fusarium graminearum*. Two techniques were developed, one to screen for resistance to infection via the silk and one to screen for resistance to infection via kernel wounds. Both techniques have undergone testing to standardize them for routine use in breeding programs and pathology research. These techniques allow for good differentiation between genotypes, ranging from very susceptible to highly resistant and are now being used in maize breeding programs to develop inbred lines with improved resistance to *F. graminearum*. In addition, both techniques have been used successfully to infect maize ears with *F. moniliforme*, *F. subglutinans*, and *F. culmorum*.

Although this booklet is designed primarily to assist researchers in maize breeding, the techniques described have been used to study resistance mechanisms, inheritance of resistance, epidemiology and basic pathology of this important maize disease.

Summary

The development of suitable techniques for the screening and evaluation of maize for resistance to gibberella ear rot could greatly facilitate breeding for resistant hybrids. Development of such techniques requires many studies and rigorous testing. This review describes two field screening techniques which have been standardized for routine use in breeding programs. The techniques are differentiated on the basis of the mode of fungal entry simulated: silk vs. kernel infection. The silk channel inoculation technique consists of injecting a *Fusarium graminearum* macroconidial suspension into the silk channel of primary ears. The kernel inoculation technique consists of stabbing the centre of the ear with four stainless steel pins previously dipped in a macroconidial suspension. Details of inoculum production, inoculation site, inoculum concentration and volume, timing of inoculation, plot maintenance, statistical analyses, evaluation and interpretation of results, mycotoxin analysis, and safety procedures are discussed.

Résumé

La sélection génétique d'hybrides de maïs résistants peut être améliorée par le développement de techniques adaptées pour l'évaluation et l'identification de lignées de maïs résistantes á la fusariose (gibberella) de l'épi. La conception de telles techniques nécessite de nombreuses études et une évaluation rigoureuse des résultats. Ce bulletin décrit deux techniques de démarquage au champs qui doivent faire partie intégrante d'un programme de sélection pour l'amélioration du maïs résistant á la fusariose de l'épi. Ces techniques se distinguent sur la base de deux modes de simulation d'attaques fongiques, soit une infection via les soies de l'épi ou bien une infection directe sur les grains. La technique d'inoculation par le col des soies consiste en une injection d'une suspension de macroconidies de *Fusarium graminearum* appliquée sur l'épi principal. Le type d'inoculation par les grains consiste en un marquage, au moyen d'un pionçon muni de quatre pointes en acier inoxidable, au centre de l'épe et au travers de grains, qui a été trempé dans une solution macroconidienne. Les différentes étapes de ces techniques sont discutées, á savoir, la production d'inoculant, le choix du site d'essai, le volume et la concentration de l'inoculant, la cédule d'inoculation, l'entretien des parcelles, les analyses statistiques et l'interprétation des résultats, les analyses de mycotoxines et enfin les normes de sécurité á respecter.

Introduction

Fusarium graminearum Schwabe, the asexual state of *Gibberella zeae* (Schw.) Petch, is an important ear-rotting pathogen of maize (corn) in many areas of the world, including Canada (Gordon 1959, Sutton et al. 1980a), the United States (Hesseltine and Bothast 1977, Koehler 1959), southern and eastern Europe (Milic et al. 1969), central and southern Africa (Marasas et al. 1979), the former USSR (Manannikova 1979), and China (Tanaka et al. 1988). The disease itself is called gibberella ear rot or pink mold. *Fusarium graminearum* is also a causal agent of stalk rot in maize and head blight (scab) in wheat.

The major symptom of *F. graminearum* infection on maize ears is a characteristic pink- to reddish-coloured mold on kernels and between husks and kernels (Fig. 1). Silks and husks may adhere tightly to the kernels in severely infected ears, and mold growth may be visible on husks at the tip of the ear. Kernel infection is usually found near the tip of the ear or around tunnels made by insect feeding. If the season is long and wet, small, round, black perithecia (fruiting structure in which ascospores are formed) of *G. zeae* may develop on the surface of infected husks.

Growth of *F. graminearum* requires periods of warm temperatures (optimum 24°C–26°C) with persistent wetness. Rainfall and warm temperatures during July and August (silking and early kernel development) are key factors in epidemics of gibberella ear rot (Koehler 1959, Miller 1994, Sutton 1982, Tuite et al. 1974). The major sources of *F. graminearum* inoculum seem to be infested host debris such as old stalks and ears of maize or debris from a preceding wheat crop (Sutton 1982). Such refuse may give rise directly to infectious mycelium, or may serve as a food base for sporulation and dissemination. Nearby infected wheat fields can also be sources of inoculum. The use of infected grain as seed results in poor stands and diseased seedlings (blight). During epidemics, it is believed that the major means of inoculum dispersal is aerial; ascospores and macroconidia are therefore the most important inoculum types. Birds and insects can also be vectors of *F. graminearum* and wounds created by feeding may predispose the ear to further fungal invasion (Attwater and Busch 1983, Enerson and Hunter 1980, Sutton et al. 1980b). Damage of ears by hail has also been found to increase the incidence of infection (Abbas et al. 1988). Fungal entry into maize ears can occur through two major modes: (1) by growth of mycelium down silks to the kernels and cob (rachis) from spores

Fig. 1. Maize ear infected with *Fusarium graminearum*, exhibiting the discolouration of husk (A), mycelial growth between husk leaves (B), and characteristic pink to reddish-coloured mold on kernels (C) associated with gibberella ear rot.

germinating on the silks; and (2) by entry through wounds (Hesseltine and Bothast 1977, Koehler 1942, Sutton 1982).

Although the incidence and severity of gibberella ear rot can be somewhat sporadic and localized from year to year, ear rot does reduce the total yielding potential of hybrids and losses in grain quality may be appreciable due to mycotoxins produced by this pathogen. This is of considerable concern to livestock producers. Swine are the most sensitive to *F. graminearum* mycotoxins. Two major mycotoxins are produced by this pathogen: zearalenone and deoxynivalenol. Zearalenone causes swine estrogenic syndrome, as well as male infertility, reduced litter size, feed refusal, and haemorrhagia (Mirocha and Christensen 1974, Prelusky et al. 1994). The trichothecene toxin deoxynivalenol (DON, vomitoxin) causes vomiting, feed refusal, and decreased weight gain in swine (Prelusky et al. 1994, Vesonder et al. 1981). Trichothecenes are also immunosuppressants and inhibitors of protein synthesis and thus can predispose animals to other diseases and mask underlying toxicoses (Pestka and Bondy 1990). Besides causing direct and indirect economic losses, this fungus can also affect the health of grain handlers and processors. It is therefore imperative to develop and devise adequate control and protection measures against this disease.

Various management strategies which give some degree of ear rot control include plowing under of residues, weed control, crop rotation with non-graminaceous crops, and balanced soil fertility. Once infection has taken place, various strategies might be tried to reduce further fungal growth and contamination; these include proper grain drying, storage of grain at low moisture levels, and sanitation of feed preparation and delivery systems (Enerson and Hunter 1980, Martin and Johnston 1982, Shurtleff 1984). Dilution of contaminated grain with clean grain has been used but it is not a fully satisfactory control method and is often not practical for growers who produce their own feeds (Charmley and Prelusky 1994). Decontamination of grain by chemical treatment is not economically feasible. Since the pathogen utilizes the maize matrix, infected kernels are significantly lighter than uninfected ones, and thus density segregation can be used to remove damaged kernels at harvest. Lighter infected kernels can be removed when combine-harvesting, thus potentially reducing the toxin content in the harvested grain (Charmley and Prelusky 1994, Trenholm et al. 1988). A considerable proportion of deoxynivalenol is in the cob

or rachis and thus will be removed at harvest when cobs are discarded (Reid et al. 1996a).

The best way to control gibberella ear rot is to prevent infection in the field. This can be achieved with the development of resistant hybrids through genetic improvement and breeding. It is the most economical and efficient means of controlling maize diseases and is the control measure that is most readily accepted by growers.

Availability of reliable screening methods for detecting resistance is the cornerstone of any disease resistance breeding program. One might consider using mycotoxin levels in the grain to screen for resistance. However, chemical analysis of mycotoxin levels is very time-consuming and expensive. Mycotoxin assay kits based on monoclonal antibodies have been developed for qualitative assays of individual toxins and are faster and cheaper than chemical analyses of mycotoxins. However, these kits are not yet inexpensive enough to use in a breeding program.

Currently, the only way to screen for resistance to gibberella ear rot is in the field. There are two reasons for this: (1) satisfactory levels of infection and reliable genotypic differentiation have not been achieved under greenhouse conditions; and (2) there is no laboratory technique or seedling test that can be used to screen for the type of resistance that is exhibited in a fully grown field plant.

Due to the sporadic nature of gibberella ear rot epidemics, artificial inoculation techniques are needed to enhance incubation and infection and to overcome variability of infection during years when natural contamination is too low to identify genotypic differences. This variability has been a major limiting factor in breeding for resistance since methods of inoculation and screening have not been consistent. A diversity of results, many of which are conflicting, have been obtained with different inoculation and post-inoculation treatments. Growth stage of the host, inoculum type, position of inoculum, wounding of ear, and environmental conditions are among the factors affecting development and expression of disease.

The development and evaluation of any inoculation and selection technique for gibberella ear rot resistance involves a number of essential steps:

1. Develop an appropriate method of maintaining the pathogen in pure culture to provide inoculum.
2. Determine the most efficient and reproducible method of applying the inoculum to the plant.
3. Determine the inoculum dose or density required to produce the desired amount of infection.
4. Determine the part of the ear to be inoculated.
5. Determine the optimum stage of plant growth or time for inoculation since disease incidence and severity can be affected by the maturity of the host plant.
6. Determine if different isolates of the pathogen differ in aggressivity, and whether there are important genotype by isolate interactions.
7. Control the field environment whenever possible to produce uniform and optimum infection and disease development, e.g. by providing overhead irrigation during the incubation period.
8. Maintain appropriate management practices to ensure that only healthy plants are used for testing.
9. Determine the optimum number of plants and replicates required to give statistically significant results.
10. Develop an accurate scale or index for measuring the response of plants to infection.

The most important consideration is the effectiveness of the technique in demonstrating differences in disease reactions among genotypes for the type of resistance (silk vs. kernel) being assessed.

Silk vs. Kernel Inoculations

Evaluations of maize genotypes for resistance to gibberella ear rot should take into account the two modes of fungal entry, i.e. growth down the silks and entry through kernel wounds. Resistance to one mode of infection does not imply resistance to the other mode. Silk resistance alone is not sufficient since infections through the kernel can occur at most stages of ear development. Kernel resistance alone may not be sufficient since infections through the silk when kernels are not yet fully developed can result in extensive infection of kernels and cobs, (Reid and Hamilton 1996a).

Screening for silk resistance has usually involved one of three techniques: (1) insertion of a colonized substrate (e.g. toothpicks or cereal kernels overgrown with mycelium) or a pipecleaner impregnated with macroconidia into the silk channel (region within the husk between the tip of the cob and tip of the husk where the silks emerge); (2) spraying a conidial suspension on the exposed silks; or (3) injection of a conidial suspension into the silk channel.

The use of colonized substrates does not simulate natural infection since the inoculum source is mycelium, not spores. Colonized substrates or pipecleaners placed in the silk channel are often displaced away from the ear tip as silks elongate, resulting in a low level of infection (Sutton and Baliko 1981). Spraying of a conidial suspension on silks more closely simulates natural infection. This technique augments natural infection by providing for a greater volume, a higher concentration of spores, a more specific time of application, and a more uniform distribution of inoculum. Unfortunately, low levels of infection are achieved with this technique. To counteract this, spraying of a conidial suspension on silks is often followed by bagging of the ear to prevent desiccation. However, bagging often results in excess water on ear surfaces, encouraging bacterial growth and reducing the level of infection. Injection of a conidial suspension into the silk channel has been found to give the most consistent results of the three techniques.

Screening for kernel or wound resistance often involves puncturing the husk, kernels, and cob followed by insertion of a colonized substrate (toothpick) or spores (saturated pipecleaner) into the wound. High levels of infection are obtained when a colonized toothpick is inserted into the centre of the ear and it thus is difficult to distinguish resistant from susceptible genotypes. The insertion of toothpicks or spore-impregnated pipecleaners through the husk, kernels and cob, circumvents any physical barriers or resistance factors that could otherwise exclude the pathogen. Techniques which produce a point source wound to kernels and not to the cob more closely approximate infection from insect damage. Recently, methods have been developed to avoid wounding the cob by puncturing just the husk and kernels followed by application of a conidial suspension (Chungu et al. 1996, Reid and Hamilton 1996a, Schaafsma et al. 1993).

The Eastern Cereal and Oilseed Research Centre has developed two screening techniques, one to evaluate resistance to infection through the silk and one for infection through wounded kernels. Both techniques allow for good differentiation between genotypes and have been successfully used to identify genotypes with extremely high levels of resistance to either silk or kernel infection (Reid et al. 1995b). The following sections describe in detail the application of these techniques and the interpretation of the results.

Inoculum Production

Macroconidia are the easiest inoculum propagules of *F. graminearum* to produce in mass quantities. The technique and medium used for producing macroconidia depends on the facilities available and the amount of inoculum required. Ultimately, a liquid suspension of conidia is required. Best fungal growth is achieved in a low-sugar liquid medium that is subjected to some agitation (shaking, rotating, bubbling) to prevent mycelial growth and clumping.

To produce a macroconidial suspension of *F. graminearum*, liquid medium consisting of the following can be used:

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: ferric sulphate ($FeSO_4$), ferric chloride ($FeCl_3$), manganese sulphate ($MnSO_4$), and zinc sulphate ($ZnSO_4$)

1.0 L distilled water

1.0 g dextrose

Other carbohydrate sources, such as 2.0 g of soluble starch or sucrose, can also be used. The medium is dispensed at 150 mL into 500 mL erlenmeyer flasks, autoclaved for 20 minutes (pH=5), then a 1 cm^2 piece of potato dextrose (PDA) agar containing mycelium and macroconidia of a single isolate of *F. graminearum* is added. Cultures are shaken at 25°C for 1 hr at 4 hr intervals under natural light supplemented with fluorescent light [Sylvania Cool White (F40 CW); GTE. Corp.]. Conidial concentrations can reach 2 x 10^6 spores/mL in one week

depending on strain. Prepared inoculum can be stored at 2–4°C (refrigerator) for a maximum of four weeks before decreases in spore viability occur. 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 typical flask of 150 mL of 2×10^6 conidia/mL suspension when diluted to a concentration of 5×10^5 conidia/mL (final volume of 450 mL) will provide enough inoculum to inoculate 225 plants at 2 mL per plant.

Sterile water is used for a control since by the time of inoculation the inoculum largely consists of spore suspension in diluted spent medium. Sedimentation or centrifugation of the conidia and resuspension in sterile water, to eliminate the addition of any medium to the inoculated ear, is not recommended since most of the conidia will lyse in such conditions and little infection is achieved.

Number of Isolates

Some *F. graminearum* isolates may be more aggressive than others, inducing greater disease severity. There may also be some interaction between maize genotypes and pathogen isolates, but most isolates will consistently differentiate or rank the most resistant and the most susceptible genotypes. Inconsistencies in ranking tend to be restricted to those genotypes with moderate susceptibility (Atlin et al. 1983, Mesterhazy and Kovacs 1986, Reid et al. 1993). If a single sufficiently aggressive isolate is used, a plant breeder will be able to identify genotypes with useful resistance and those with severe susceptibility. If a mixture of isolates is used, conidial suspensions of each isolate are prepared and mixed just before inoculation.

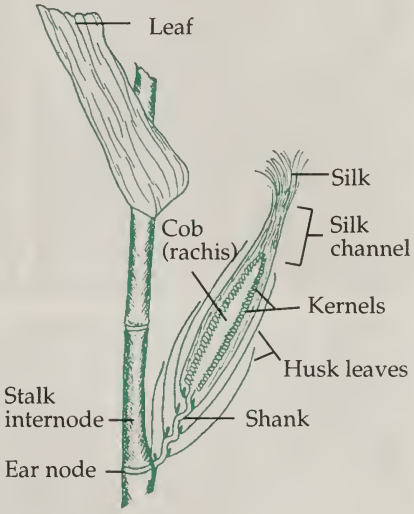
Any isolate(s) used for screening should have been isolated originally from naturally infected maize ears and should be able to cause typical ear rot symptoms in susceptible maize and produce mycotoxins in infected kernels. Virulent *F. graminearum* isolates can be obtained from the Canadian Collection of Fungus Cultures, Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, K1A OC6. Isolates should be freeze-dried or frozen in liquid nitrogen for storage.

Silk Channel Inoculation

The most satisfactory silk inoculation technique involves the injection of a conidial suspension of *F. graminearum* into the silk channel (Fig. 2) inside the husk cavity and above the cob. In a breeding nursery, pollinations are conducted as usual, then ear shoot bags can be lifted to perform inoculations and replaced.

Two mL of inoculum are injected into the silk channel of each primary ear using a graduated, 10 mL, self-refilling, automatic vaccinator attached to a 2.5 L backpack container (Nasco Co., Fort Atkinson, WI) (Fig. 3). An 18-gauge Luer-lock stainless steel hypodermic needle is attached to the vaccinator. One individual can inoculate 300-400 ears per hour. Secondary ears are not inoculated since they are not present in all genotypes and they often mature later than primary ears.

Fig. 2 . Longitudinal section of a maize ear showing location of silk channel.



Inoculation Site

For kernels to become infected with the silk channel technique, macroconidia placed within the silk channel must germinate and hyphae must grow down the silks to infect developing kernels. The rate of progression of the fungus down the silk channel is a function of the degree of inherent silk resistance, silk age, and environment. For example, if silk resistance is not complete but is sufficient to delay the progression of the fungus down the silk until the kernels have hardened then infection will be minimal. Once the fungus

reaches the kernels, the severity of infection is a function of the inherent resistance of the kernels, kernel maturity, and environment. This inoculation technique cannot be used for genotypes with little or no silk channel.

To successfully assess silk resistance, care should be taken to ensure that the inoculator needle is held at right angles to the silk channel (Fig. 3C), otherwise macroconidia will be injected

Fig. 3. Silk channel inoculation of maize ears with a macroconidial suspension of *Fusarium graminearum* using a graduated, self-refilling, automatic vaccinator (A) attached to a 2.5 L backpack (B). Two mL of suspension is injected into the silk channel while holding the vaccinator at right angles to the long axis of the ear (C).

down the silk channel onto the kernels. If this happens disease severity ratings will be high and a measurement of resistance to infection via the silk will not be obtained. Injections are made into the centre of the silk channel, which can be estimated by feeling for the tip of the cob and injecting halfway between that point and the end of the silk channel, where the silks emerge from the husk. Care should be taken to avoid wounding too low, as this would flood the kernels and possibly wound the cob tip. Occasionally the needle may become plugged with silk and husk tissue. This can be avoided by periodically forcibly squeezing the vaccinator trigger and injecting inoculum into a waste container to dislodge any tissue.

Inoculum Volume

The silk channel cavity of most maize genotypes will hold about 2 mL of inoculum. Higher volumes of inoculum significantly increase the amount of infection in more susceptible genotypes (Fig. 4). This is largely due to the probability that with higher volumes, inoculum will be forced down the silk channel reaching the cob tip and kernels, thus overcoming the natural barrier of the silk and any silk resistance. Genotypes with useful silk resistance will therefore, not be selected as they will appear susceptible. Moreover, the ability to differentiate between genotypes will be reduced and selection will be ineffective.

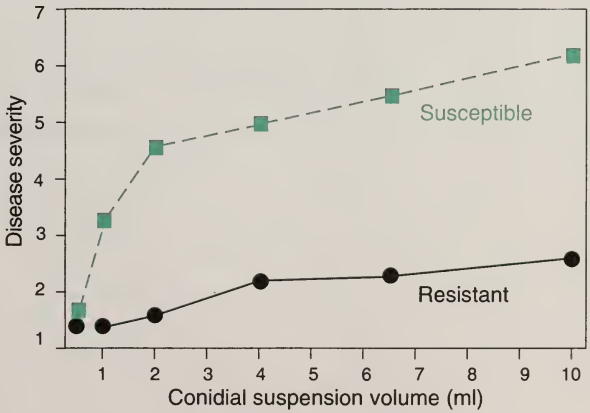


Fig. 4. Effect of inoculum volume on gibberella ear rot disease severity for two maize hybrids inoculated with the silk channel technique. Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

Inoculum Concentration

A concentration of 5 x 10^5 spores/mL has been observed to give maximum differentiation among genotypes, ranging from resistant to very susceptible, although most concentrations will differentiate between the most resistant and the most susceptible (Reid et al. 1995a). Higher concentrations significantly increase the amount of infection in susceptible genotypes (Fig. 5), especially in years with overall higher levels of infection. Lower concentrations will also differentiate between genotypes, but the degree of differentiation may be lower in years which are less conducive to fungal growth.

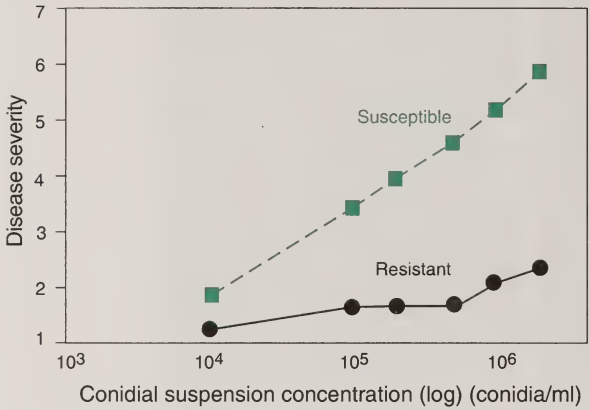


Fig. 5. Effect of inoculum concentration on gibberella ear rot disease severity for two maize hybrids inoculated with the silk channel technique (2 mL volume). Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

Timing of Inoculation

Stage of plant growth at inoculation is the most important technical parameter to consider for gibberella ear rot (Reid et al. 1992). Silk channel inoculations are best if done 4–7 days after silk emergence when there is a peak in expression of susceptibility (Fig. 6). This period corresponds to the stage when silks are elongated, pollinated and may have some tip browning but are still green (not dry). The silks of maize senesce rapidly after pollination and this physiological change seems to alter the suitability of silk for growth of ear-rotting organisms. Infections are insufficient when inoculations are made more than 8 days after silk emergence, especially if the silks have dried out; assessments are then incorrect or no differentiation is observed. Timing is critically important when

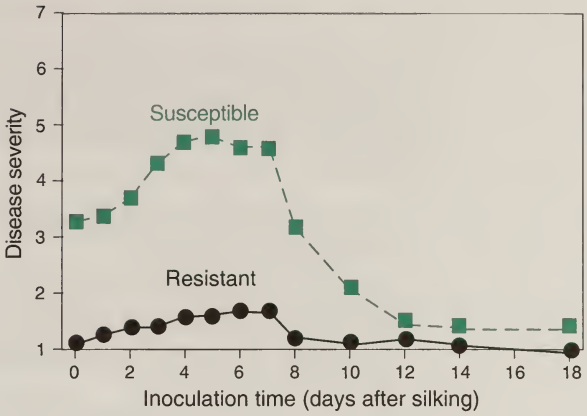


Fig. 6. Effect of time of inoculation after silk emergence on gibberella ear rot disease severity for two maize hybrids inoculated with the silk channel technique. Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

genotypes with different maturity are to be evaluated. Timing can be based on the number of days from 50% silk emergence (50% of the plants of a given genotype with emerged silk) or, in large screening programs where this is not feasible, timing can be based on the physical appearance of the silk.

Timing of inoculation among plants of a given genotype is also important. More consistent results will be obtained if all ears of a given genotype are inoculated at the same time, so that the environmental conditions at time of inoculation are consistent. Rows 3.8 m long with 14 plants are convenient and all plants in the row are inoculated when 10 of the centre 12 plants are ready for inoculation. If the rows are from a segregating population, this is not possible and the row should be visited several times until all desired plants are inoculated. To keep track of which plants have been inoculated and to avoid double-inoculations or escapes, a small dot of spray paint (red is easiest to see at harvest) is sprayed onto the lower husk/shank area to mark the inoculated plants.

Summary of Instructions for Silk Channel Inoculations

The following steps describe the inoculation of maize silk channels with a macroconidial suspension of *F. graminearum* using a self-refilling automated vaccinator attached to a 2 L backpack:

1. **Prepare conidial suspension at least one week in advance of date of inoculation.**
2. **Assemble vaccinator by attaching rubber hose and clamps. Assemble backpack by attaching straps. Thoroughly rinse vaccinator, hose and backpack with 70% ethanol, then with sterile water. Let dry.**
3. **Do a spore count of conidial suspension and dilute to 5×10^5 spores/mL with sterile water. Use rubber gloves for this step and the rest of the procedure (AVOID ALL CONTACT WITH SPORE SUSPENSION).**
4. **Filter through 2 layers of cheesecloth.**
5. **Fill backpack to volume of inoculum required (number of plants to be inoculated x 2 mL).**
6. **Attach vaccinator and rubber hose to backpack. Ensure that all connections are tight to prevent leakage.**
7. **Invert backpack to allow suspension to flow down the hose to the vaccinator Remove all air bubbles from hose and vaccinator by pumping suspension through the vaccinator. Sterilize Luer-lock needle in 70% ethanol and attach to vaccinator.**
8. **Ensure that vaccinator is set at 2 mL by injecting into a graduated cylinder to check volume. Ensure that screw on top of vaccinator is tight to prevent volume changes (this screw may loosen on older vaccinators, it should be periodically checked while in use).**
9. **Select plants to be inoculated (THIS IS THE MOST CRITICAL STEP). These plants will have primary ears with silk approximately one week old, i.e. silk is elongated and has some tip browning (DO NOT INOCULATE PLANTS IN WHICH THE SILK IS BROWN AND DRY). If experiments consists of genotypes of many different maturities, walk through the field every 3 days and record which rows have 50% of the plants with silk emerged, these rows are then inoculated approximately 5–6 days later (note: this time span will be reduced if the growing season is rapid, so do not rely on chronological time alone, combine it with observations on silk appearance). When inoculating hybrids**

or inbreds, inoculate all of the plants that are ready in a row on the same day (do not go back and inoculate slower growing plants since this will create too much variability in the results). When inoculating segregating populations where plants are of different maturities, the row(s) should be visited every 2–3 days and only plants that are ready are inoculated. In a nursery, it is more difficult to determine inoculation time based on silk appearance since ears are covered with paper bags to prevent pollination. In this case, mark the pollination date on the bag with a water-proof marker when making pollinations then return to those plants 5–6 days later, lift bags, inoculate, then replace bags.

10. On the primary ears (usually the highest ear on the stalk) of each plant, locate the centre of the silk channel by feeling for the tip of the cob and insert the vaccinator needle at a right angle to the silk channel in the midpoint between the cob tip and the point where the silks emerge from the husk. If any resistance to penetration is felt, the needle has been inserted into the cob and wounded it; move to the next ear and be careful in locating the silk channel. This inoculation technique cannot be used for genotypes with little or no silk channel.
11. Slowly inject the suspension into the silk channel, keeping the needle at right angles to the channel.
12. Spray a small dot of red paint on the lower husk area or shank of the ear. If inoculation of an entire row is completed this can be indicated by spraying an additional band of paint on the internode below the tassel on the first plant of the row. In a nursery, since the ear is covered with a bag, inoculated plants can be marked by either spraying paint on the stalk above the ear or spraying paint directly on the bag. Do not inoculate a row and then come back and spray paint on inoculated plants, this leads to errors. Nursery aprons are used to carry bottles, inoculators, spray paint, etc.
13. Inoculate the next plant. Every 5–6 rows, check the vaccinator to ensure that no blockage of the needle is occurring by rapidly squeezing the trigger to dislodge any tissue. When the inoculator is not in use, e.g. at breaks or lunch, do not leave it outside in the sun and heat.
14. When finished, thoroughly rinse vaccinator and backpack with 70% ethanol and sterile water. Allow to air dry overnight.

Kernel Inoculation

The kernel inoculation technique (Reid and Hamilton 1996a) involves wounding the husk and kernels by stabbing them with four small (3 mm dia.) stainless steel pins mounted in a rectangular 7 mm x 5 mm pattern on one end of a 2.5 cm x 50 cm cylindrical wooden handle (Fig. 7A). The pin spacing is designed to increase the chance of wounding four kernels when the inoculator is held such that the 7 mm spacing is horizontal. Prior to wounding, the pins are dipped in a conidial suspension. The conidia are carried into the wounds with the pins or by capillary action. Alternatively, a more expensive apparatus can be used to inject the kernels with inoculum (Fig. 7B). Our results have shown no significant difference in disease severity between the two techniques, but the automated apparatus has two advantages, it is faster to use, and it minimizes exposure of the user to inoculum. With both inoculators, only 3–4 kernels are wounded, thus creating a point source of inoculation from which the spread of infection from wounded to non-wounded kernels can be measured. One individual can inoculate an average of 200–300 ears per hour with this technique. As with the silk channel technique, inoculation can be conducted following pollination just by lifting the ear shoot bag.

The depth of puncture (pin length, 5 mm) is suitable for most commercial maize hybrids. Care should be taken to avoid wounding the cob; if this happens, the infection may remain in the cob causing a spongy rot with no visible symptoms apparent on the kernels. Such an ear may be falsely evaluated as resistant. Alternatively, if the cob is wounded the infection may spread to non-wounded kernels through the cob rather than from wounded to non-wounded kernels. If this technique is used on inbreds or genotypes with thin husks, it is recommended that inoculations first be tested on a few border plants to make sure that penetration depth is appropriate. To adjust the depth, a small piece of rubber can be used to shorten the length of the pins. Penetration depth is adjustable on the automated inoculator (Fig. 7B). As with the silk channel technique, only the primary ears are inoculated and spray paint can be used to mark treated plants.

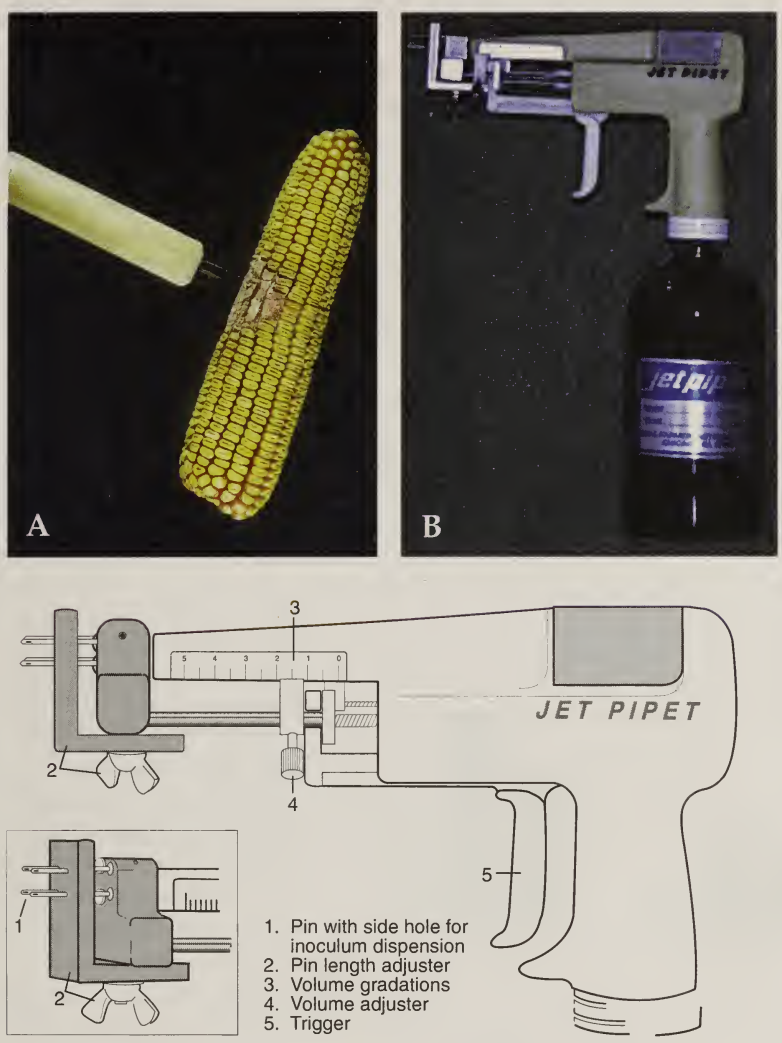


Fig. 7. Two methods of kernel inoculation of maize ears with a macroconidial suspension of *Fusarium graminearum*. (A) Inoculator has four stainless steel pins attached to a cylindrical wooden handle. The pins are dipped in the suspension before stabbing the centre of the ear through the husk and into 3–4 kernels, thus producing a point source of infection from which the fungus spreads. (B) Automatic, self-refilling, graduated kernel inoculator which injects inoculum into 3–4 kernels. Each pin has two holes drilled on the sides to prevent plugging upon wounding. Penetration depth is adjustable.

Inoculation Site

As with the silk channel technique, the position of the inoculator is very important. The pins should enter at a right angle to the long axis of the ear midway between the butt and the tip. Wounds made too high, near the ear tip, may result more often in wounding of the cob than in infection of the kernels, which are less well developed than those at the centre of the ear (Fig. 8). Inoculations made too close to the butt also result in reduced infection levels, probably because kernel hardening progresses from the butt to the tip; and the more mature butt kernels are less receptive to infection. Inoculation in the centre of the ear produces the most uniform results. In susceptible genotypes, infection will rapidly spread to neighbouring non-wounded kernels, often forming a ring around the ear before progressing to tip and butt areas. Again, because the butt kernels harden first, infection tends to spread to the tip area more than the butt (Reid and Hamilton 1996a).

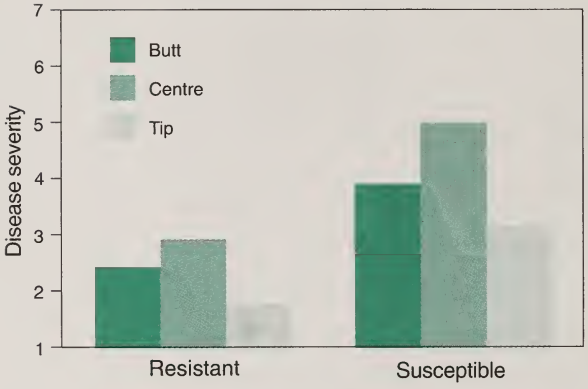


Fig. 8. Effect of inoculation site (butt, centre, or tip of ear) on gibberella ear rot disease severity for two hybrids inoculated with the kernel wound technique. Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

Inoculum Concentration

As with the silk channel technique, increasing the concentration of the conidial suspension increases disease severity (Reid and Hamilton 1996a) (Fig. 9). There is little difference in severity ratings at concentrations from 10^4 to 5×10^5 conidia/mL, mainly because the kernel technique bypasses any morphological resistance barriers. However, as with the silk channel technique, the degree of differentiation may be lower in some years which are less conducive to fungal growth at lower inoculum concentrations. For simplicity, one can use the same concentration recommended for the silk channel technique, i.e. 5×10^5 conidia/mL.

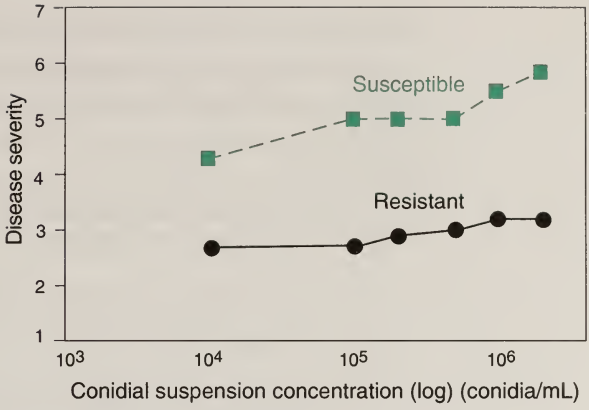


Fig. 9. Effect of inoculum concentration on gibberella ear rot disease severity for two maize hybrids inoculated with the kernel wound technique. Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

Timing of Inoculation

Inoculating at the optimum stage of plant growth is as important with kernel inoculations as with the silk channel inoculations (Fig. 10). Kernel inoculations are made 10–15 days post-silk emergence. This corresponds roughly to the 'blister to early milk stages' of kernel development. Early inoculations result in too much infection since kernels are not yet developed and have a much lower resistance. Inoculations made too late result in little infection since kernels have hardened. Thus, care should be taken to ensure that inoculations are made at the proper time of kernel development and for all plants of a given genotype at the same time.

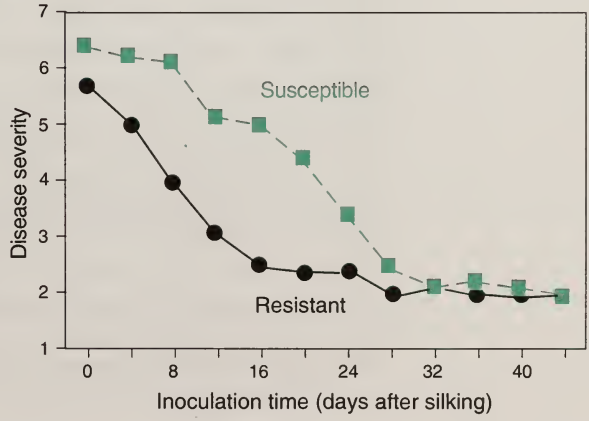


Fig. 10. Effect of time of inoculation after silk emergence on gibberella ear rot disease severity for two maize hybrids inoculated with the kernel wound technique. Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

Summary of Instructions for Kernel Inoculations

The following steps describe the inoculation of maize kernels with a macroconidial suspension of *F. graminearum* by stabbing the ear with four small (3 mm dia.) stainless steel pins:

1. Prepare conidial suspension at least one week in advance of date of inoculation.
2. Clean inoculator and a small (approx. 100 mL) plastic bottle with a screw cap with 70% ethanol, then with sterile water. Let dry.
3. Do a spore count of conidial suspension and dilute to 5 x 10^5 spores/mL with sterile water. Use rubber gloves for this step and the rest of the procedure (AVOID ALL CONTACT WITH SPORE SUSPENSION).
4. Filter through 2 layers of cheesecloth.
5. Fill plastic bottle with suspension and cap tightly.
6. Select plants to be inoculated (THIS IS THE MOST CRITICAL STEP). These plants will have primary ears with silk approximately 10–15 days old, i.e. the silk will appear brown and almost dry, the ear will be thicker and rounder in the centre and butt due to kernel and cob development, and the kernels will be in the blister-early milk stage of development. If experiments consist of genotypes of many different maturities, walk through the field every 3 days and record which rows have 50% of the plants with silk emerged; these rows are then inoculated approximately 10–15 days later (note: this time span will be reduced if conditions are favourable for rapid plant growth so do not rely on chronological time alone, combine it with ear appearance). When inoculating hybrids or inbreds, inoculate all of the plants that are ready in a row on the same day (do not go back and inoculate slower growing plants since this will create too much variability in the results). When inoculating segregating populations where plants are of different maturities, the row(s) should be visited every 2–3 days and only plants that are ready are inoculated. In a nursery, it is more difficult to determine inoculation time based on ear appearance since ears are covered with paper bags to prevent pollination. In this case,

mark the pollination date on the bag with a waterproof marker when making pollinations then return to those plants 5–6 days later, lift bags, inoculate, then replace bags.

7. On the primary ear (usually the highest ear on the stalk) of each plant, locate the midpoint of the ear by feeling for the tip and butt of the cob and inoculating through the husk at that point.
8. Unscrew the bottle with suspension and dip the inoculator into it. Immediately stab the inoculator into the midpoint of the ear at a right angle to the ear. The inoculator is inserted such that the 7 mm spacing between the pins is horizontal. Withdraw the inoculator smoothly. Do not wiggle the inoculator or do anything to increase the degree of wounding. Do not stab to the point where the cob is wounded, only wound the kernels, i.e. once the slightest bit of resistance to entry is felt you have hit the cob; try to avoid this by practising on border plants or rows. Nursery aprons are useful to carry bottles, inoculators, spray paint, etc.
9. Spray a small dot of red paint on the lower husk area or shank of the ear, far enough away from the wound so that no paint enters the wound. If inoculation of an entire row is completed this can be indicated by spraying an additional band of paint on the internode below the tassel on the first plant of the row. In a nursery, since the ear is covered with a bag, inoculated plants can be marked by either spraying paint on the stalk above the ear or directly on the bag. Do not inoculate a row and then come back and spray paint on inoculated plants, this leads to errors.
10. Inoculate the next plant. When the inoculator is not in use, e.g. during breaks or lunch, do not leave the suspension bottle outside in the sun and heat.
11. When finished, thoroughly rinse the inoculator and bottle with 70% ethanol and sterile water. Allow to air dry overnight.

When you are using the automated kernel inoculator follow the steps outlined above but fill the inoculator bottle with suspension and set the volume to 0.5 mL. Stab the plant as usual and slowly inject the suspension.

Disease Severity Assessments

Visual Rating Scales

Various methods have been used to evaluate the severity of gibberella ear rot after inoculation, including whole-row ratings (Koehler 1942), kernel sorting (Koehler 1959), kernel plating (Koehler 1959), and individual ear ratings using visual scales (Ullstrup 1970, Sutton and Baliko 1981, Enerson and Hunter 1980). The latter method is the most widely used since it is the least time-consuming, increments on the scale are easily discernable, data recording is simplified, and it allows for rapid screening of a large number of genotypes.

Ears are harvested at normal grain harvesting moisture (approximately 24%) in October. For most genotypes, it takes 6–8 weeks for disease severity levels to reach a peak and stabilize. Ears are hand husked 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. The scales consist of the same 7 classes for both the silk channel (Fig. 11A) and the kernel technique (Fig. 11B). This allows for direct comparisons between the two techniques. It is easiest and most efficient to husk the ears while they are still attached to the plants. After rating disease severity, ears needed for mycotoxin analysis can be hand-picked and the remaining ears removed for disposal with a combine/forage harvester.

Providing personnel have been adequately trained and inoculations are properly performed, we have not found significant differences in severity ratings among individuals using the techniques described. However, more consistent results are achieved when one individual inoculates a given experiment. If experiments are large and require two or more individuals to inoculate, try to put each person on a separate replicate or block. In addition, it may be useful to conduct a smaller experiment in which the treatment effect is the various individuals. This will give a measure of the variability between people's techniques. However, if individuals are properly trained and the inoculation techniques are properly demonstrated, this kind of variability is minimal to not significant. Since interpretation of the rating scale may vary slightly between users, it may require periodic standardization. It is recommended that one person does all of the ratings within nurseries or experiments.

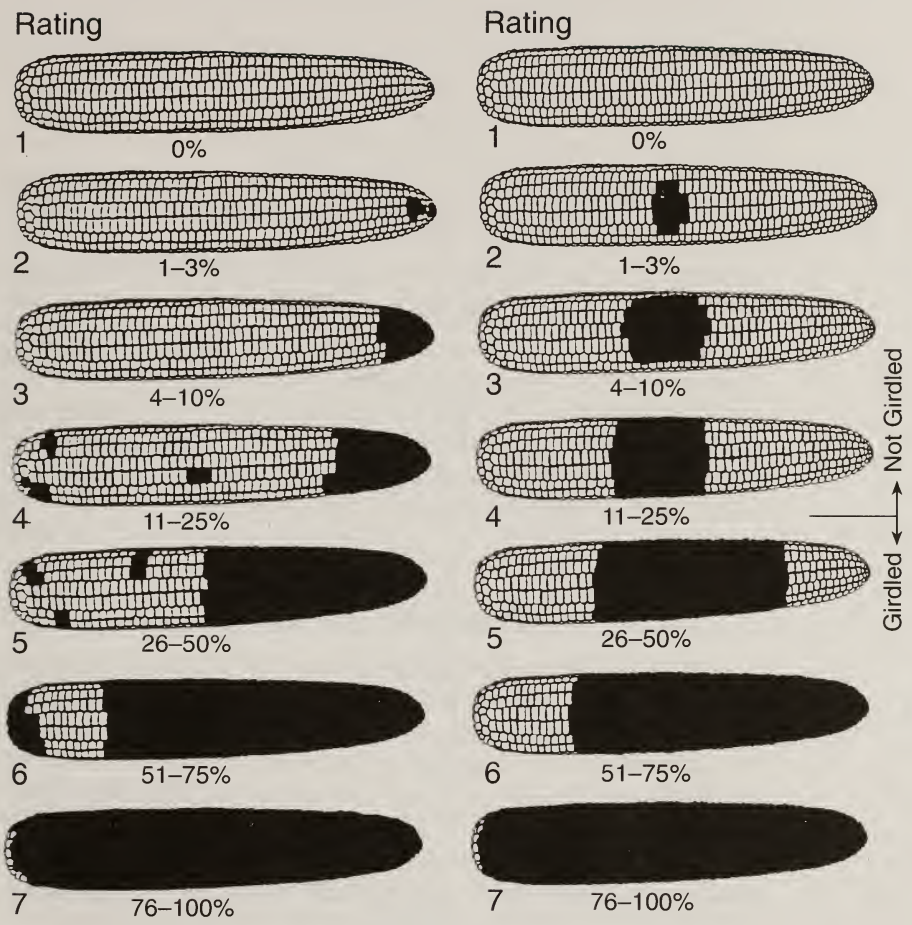


Fig. 11. Disease severity rating scales and percentage of visibly infected kernels for gibberella ear rot after silk channel (A) and kernel (B) inoculations with *Fusarium graminearum*.

Selection of Resistant Plants

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 inoculation, 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 can also delay progression of the fungus down the silk channel, hence the need for further study using kernel inoculations.

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.

Mycotoxin Content

A strong positive relationship ($r>0.80$, $p<0.01$) exists between visible disease symptoms and DON levels for both silk channel and kernel inoculations for most genotypes (Reid et al. 1996a,b). The relationship is exponential in that after a rating of approximately 5 (50% infection) the amount of toxin in the kernels can be very high. This is mostly dependent on the environment. Thus, mycotoxin analyses are not needed during routine screenings. Such a relationship has also been reported by other researchers using wound inoculation techniques (Atlin et al. 1983, Cullen et al. 1983, Hart et al. 1982, Hart et al. 1987). Selection can be based on visual evaluation of disease symptoms. This is easy and rapid and is significantly less expensive than mycotoxin analysis. However, mycotoxin analyses are desirable in the final stages of inbred and/or hybrid development before variety release because the acceptable level of DON in feed is low (in the order of 1 ppm).

For deoxynivalenol analyses, ears are grouped on a per row basis (usually a bulk of 10), bagged in mesh sacks, air-dried for two weeks, and frozen (–20°C) until analysis. Ears (bulk) are hand-shelled and kernels are mixed thoroughly to obtain a random distribution. A 50 g sample is ground to a fine powder

in a Retsch Ultra Centrifugal Mill Type ZM1 (Brinkman Instruments, Rexdale, Ontario) with a 0.75 mm mesh. A sample of this ground tissue is then sent to the appropriate laboratories for mycotoxin analyses.

Plot Maintenance

In disease resistance studies the environment should be controlled as much as possible to produce uniform plant emergence and growth as well as optimum infection and disease development. This is especially important for gibberella ear rot because of the sporadic nature of this disease and its susceptibility to environmental factors.

A humid environment should be maintained using irrigation, at a rate of 2-5 mm daily for the four-week period after inoculation. However, this may not be essential except in areas or years which are very dry. Irrigation may increase disease severity with silk channel inoculations (Fig. 12A); but the degree of increase, if any, is a factor of the precipitation and temperature for the month after inoculation (Reid and Hamilton 1996a). In contrast, irrigation has little significant effect and will sometimes decrease infection with the kernel inoculation technique (Fig. 12B). This may be due to penetration of the inoculum into a substrate since the wounds bypass morphological barriers. In contrast, silk channel inoculation places the fungus in a silk containing cavity from which the mycelium must grow along the silk to infect the ovules/cob, and the ability to do so may be influenced by the moisture content of the silk and the environment. A decrease in disease severity with kernel inoculation under irrigation may be due to water washing the conidia from the ear, out of the wound, before colonization takes place.

It is important to conduct appropriate management practices to ensure that only healthy plants are inoculated. This includes proper fertilization, plant spacing and weed control. Stressed plants may have increased disease severity and resistance may not be apparent. Fields with uniform soil should be used to minimize variation in plant development since inoculations of the same genotype made at different times may result in variable results because of environmental changes. Within-plot variability is significantly reduced if care is taken to make plants as uniform as possible and if timing of inoculation is consistent.

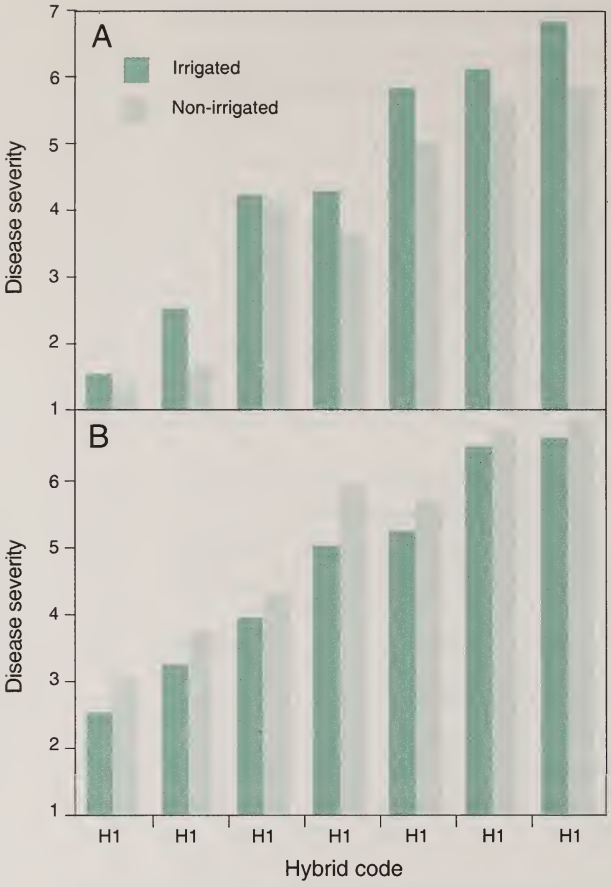


Fig. 12. Effect of irrigation on gibberella ear rot disease severity for seven maize hybrids inoculated with the silk channel technique (A) and the kernel wound technique (B). Disease severity ratings are on a 1–7 scale where 1= no infection and 7= >75% of the kernels visibly moldy (see Fig. 11).

If the inoculated ears are left on the plant following disease assessment, a combine can be used to remove the grain for disposal. Plant debris left in the field should be plowed under to minimize winter survival of the fungus and reduce future inoculum potential.

Experimental Design and Statistical Analyses

Ears are rated individually and a mean rating is calculated for each row within each of four replicates; however, methods will vary depending on research objectives, e.g. individual plant ratings may be more desirable with segregating populations. Entries are planted in single-row plots (3.8 m long) of 12–14 plants. The primary ears of the centre 10 plants are inoculated.

A randomized complete block design is usually used and data are analyzed and presented as a range in resistance or a ranking of genotypes. Relatively good reproduction of infection ratings have been obtained from year to year. The different levels of resistance demonstrated in inoculated hybrids used as checks correlate well with those observed in the field following natural infection.

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 analyzed, or standard parametric statistics can be performed on the data provided 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, seldom vary, even across environments/ locations in a given year. For example, years with average temperatures, following inoculation, of less than 25°C result in low levels of severity thus a susceptible genotype which rated 6 in a warm year may rate 4 in a cool year, and a resistant genotype may rate 2 in a warm year and 1 in a cool year. Thus it is important to use check genotypes that show similar rankings under different environments and it is important to test error mean squares for homogeneity before pooling data over years.

Safety - Handling of Conidial Suspensions and Infected Ears

The spores produced by *F. graminearum*, like those of most molds, can cause allergies and inflammation of lung tissue and these spores also contain the same mycotoxins found in the infected ears. These mycotoxins are toxic to humans and inhalation of spores or dust from contaminated grain can be extremely hazardous. Precautions should be taken to minimize contact with cultures and infected plant material by any route (oral, inhalation, or skin).

Handling *F. graminearum* cultures, especially petri dish cultures, should only be done in a biological containment hood which draws air away from the user and through a filter. Conidial

suspensions may contain mycotoxins, so disposable rubber gloves and other protective clothing (labcoat, coveralls) should be worn when these suspensions are being filtered, diluted and placed into backpacks for silk channel inoculation. Once in the backpack, exposure to the inoculum is minimal. However, some inoculum may drip from the needle during injection , so gloves should be worn in the field. With the kernel inoculation technique, exposure is more probable as the inoculator is dipped into the conidial suspension, and again gloves are the best protection. If the Jet Pipet kernel inoculator is used, exposure is significantly reduced but gloves should still be worn. Inoculating equipment should be cleaned with 70% ethanol after use each day. If inoculations also are being conducted with other *Fusarium* species, e.g. *F. moniliforme* or *F. subglutinans*, designate an inoculator for each species to avoid contamination. All laboratory surfaces, where culture or suspension transfers have been made, should also be cleaned with 70% ethanol or other disinfectants immediately afteruse.

The Canadian Grain Commission, Health Canada, Human Resources Development Canada-Labour Program, and Agriculture and Agri-Food Canada have established guidelines for the handling of grain contaminated by *F. graminearum*. The greatest exposure to mycotoxins is in the handling of infected ears and the grinding of infected grain since inhalation is more hazardous than ingestion of *Fusarium* mycotoxins. Gloves, coveralls and a dust mask 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 contaminated kernels indoors should take place in a room equipped with a ventilation/exhaust system capable of handling dust removal. Protective clothing should be removed and cleaned after use. Hands, face and other exposed areas of the body should be washed with soap and water before eating. Never eat or drink in any areas where contaminated grain or cultures are present. For further information, consult the Agriculture and Agri-Food Canada publication *Reducing Mycotoxins in Animal Feeds* (#1827 E).

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