STRATEGIES AND CONSIDERATIONS FOR MULTI-LOCATION FHB SCREENING NURSERIES

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ABSTRACT

Screening of cereals for reaction to Fusarium head blight (FHB) occurs world-wide and employs diverse methods, or, at best, variations on a basic method. Is there one protocol that is suitable/appropriate for all environments? Or is it best to create an epidemic in any way that can be certain of success, but attempt a uniform analysis to compare reactions of genotypes within and across specific environments? The FHB index commonly used and developed by Charles Snijders of The Netherlands in the 1990s has served us well, but it incorporates only visual symptoms of the disease, i.e. incidence and severity. In societies becoming increasingly conscious of food safety and security, should we consider including additional factors such as Fusarium-damaged kernels (FDK) and deoxynivalenol (DON) as part of the determination of a genotype's reaction to FHB? For a screening nursery to work well there must be a knowledge base of both the pathogen and the host within a specific environment, in order to manipulate factors to create optimal conditions for disease to occur. Some factors to discuss include inoculum, inoculation method (what types of resistance are we screening for?), timing and number of inoculations, application of misting/irrigation, rating (field/lab – single/multiple), and incorporation of FDK and DON into the analysis. One method of analysis (ISK, Kolb, Illinois) proposes that a proportion of the incidence (I), severity (S), Fusarium-damaged kernels (FDK or K) be added to give a ranking of genotypes. To this we should also consider including DON evaluations. Alternatively, we have been experimenting in Canada with 'GGEbiplot' (Genotype – Genotype X Environment), a software package developed at the AAFC Eastern Cereals and Oilseeds Research Centre in Ottawa, Canada. Biplots are used to visualize relationships among genotypes, environments and traits. It appears that either of these methods might be an improvement on the screening method originally proposed by Snijders, and they could also incorporate measures of damage caused by toxin accumulation and FDK.

CORN KERNEL INOCULUM PRODUCTION METHODS FOR A LARGE UNIFORM FHB DISEASE NURSERY

Background and Production Requirements

The following method for corn inoculum is used by pathologists at the Cereal Research centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba, Canada. This method allows us to prepare and freeze the corn kernel inoculum in the off season. We can then apply it over a 1-2 day period in the early summer at end of tillering (3 weeks prior to flowering). With respect to labour, the corn method is complementary to the spray inoculation method. We begin to prepare the conidial inoculum 2-3 weeks before anthesis which usually occurs after the corn inoculum has been applied.

The original application rate prescribed for corn kernel inoculum was 40 g/m^2 or 161 Kg/acre. From 2000 to 2005 we have used a rate of about 20 g/m^2 with good success.

Preparation of corn for FHB Inoculation

Use 26.4 liter Rubbermaid tubs. Into each tub pour ~8-9 Kg of dry corn then add tap water to a level of 4-6 inches above the corn (Figure 1). We usually do this step mid day as 16 hours of imbibition seems to provide for a better substrate for fungal colonization than 24 or even 48 hours of imbibition.

The following morning we drain the tubs, and cover them with 2 layers of industrial strength aluminum foil beneath the plastic lid. We sterilize the corn by autoclaving at 10 to 15 atmospheres for 1 ½ to 2 hours (Figure 2).

We allow the autoclave to cool overnight and remove the sterile corn the following day. We inoculate the corn mixture in a laminar flow hood (or clean room).

Inoculation of sterile corn kernels

Fungal cultures from potato dextrose agar (PDA) plates or millet, pre-inoculated and colonized with *Fusarium graminearum* can be used.

PDA Plate Method
Isolates are sub-cultured on to PDA from master synthetic nutrient agar (SNA) plates (Figure 3). The isolates will take about 1 week to colonize the entire Petri dish (Figure 4). Good culture growth occurs under fluorescent lighting at room temperature.

Using aseptic techniques blend 3-5 plates per tub of sterile corn in 150 ml sterile water with 0.2 grams of streptomycin sulfate and thoroughly mix into the corn.

Millet Method
Fusarium graminearum-infected millet is prepared in much the same the way as the corn kernel inoculum via the PDA plate method. To a large Mason jar add approximately 350-400 g millet and soak in water overnight. Drain the millet through a single layer of cheese cloth or muslin. Replace the lid of the jar loosely, but do not tighten! Choose the wet cycle and autoclave the millet for about 1 hour. Allow to cool. Add a single isolate of *F. graminearum*, about 30-50 ml of sterile water and 0.1 g of streptomycin sulphate to a blender and mix for about 30 seconds. Pour this mixture into the millet and shake to mix. Do not seal the jar when incubating the millet. The incubation period for millet is about 1 week. To dry the millet we empty the jar into a 6 or 8 litre sterilized Rubbermaid container and leave the sample in a laminar flow hood overnight.

The timeline for preparation of millet inoculum is as follows:

Day	Time	Procedure
Day 1	Afternoon	Soak millet in Glass Mason Jar
Day 2	Morning	Autoclave millet and allow to cool
Day 2	Afternoon	When cool inoculate millet with *F.graminearum*. Cover loosely with lid during incubation
Day 6 to Day 8	Afternoon	Layout millet in laminar flow hood and allow to dry.

Before using the colonized millet for corn inoculum, we test its viability by placing 50 kernels directly onto PDA and observe the colony development around the millet seed after 3-5 days (Figure 5).

Millet Inoculation
Corn kernel inoculation with millet: To each tub of corn add 30-60 ml (~25-50 g) of millet (Figure 6). Because the millet is dry it adheres to the individual corn kernels and colonizes uniformly throughout the corn.

Incubation, Drying, and Storage

After inoculation in the laminar flow hood the corn medium is then re-covered and allowed to incubate for no less than 2, and no more than 3 weeks. The FHB requires an aerobic environment, so tightly sealing or stacking the tubs will inhibit development. Room temperature (20-25 ˚C) is excellent for fungal development and the FHB will tolerate low (10 ˚C) overnight temperatures as long as the daytime temperatures get above 20 ˚C.

The end product after 2-3 weeks of standing is a white/pink/yellow mycelial mass that is surprisingly dense (Figure 7). At this point the sample should also be purely *F. graminearum.* However, *Rhizopus, Cladosporium,* and *Penicillium* are secondary invaders which may occur. If a secondary infection should occur, try to remove the growth from the culture as this is tolerable at this stage.

Following the incubation period, the corn kernel inoculum is thinly spread in the greenhouse to dry for 3-8 days depending on the amount of corn, kernel water content, green house temperature and humidity (Figure 8). The drying period is when most secondary fungal infections occur. A strong secondary infection can over-run and spoil an entire batch. The best way to minimize secondary infections is to dry the corn as rapidly as possible. Possible solutions include spreading the corn very thinly (only 1-2 kernels thick), using a fan, increasing temperature and lowering humidity. For our specific situation we mix the corn and break up newly formed mycelium corn aggregates daily for 2-3 days. This helps dry the corn more rapidly and evenly. After the corn has dried it is packed in mesh bags and stored in a cold room at 0 ˚C (or below) until it is required (Figure 9).

Figure 1. Corn soaking in 26.4 litre Rubbermaid tubs.

Figure 2. The soil sterilizer (autoclave) holds four 26.4 litre Rubbermaid tubs

Figure 3. *Fusarium graminearum* isolates grown on Synthetic Nutrient Agar are used as "master" cultures to produce many second generation subcultures on PDA.

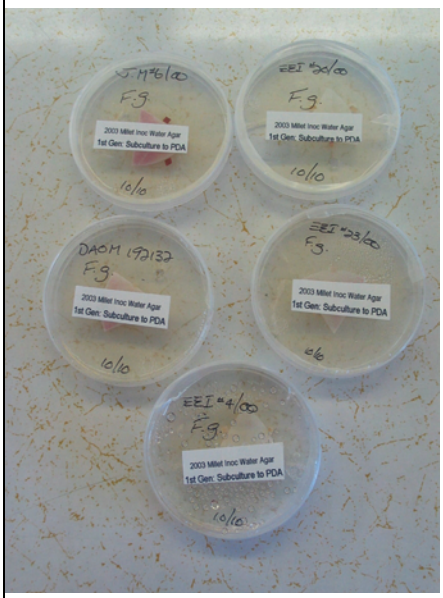

Figure 4. *Fusarium graminearum* subcultures (from SNA master cultures) growing on PDA. 12-20 plates are used per batch of corn (4 Rubbermaid tubs).

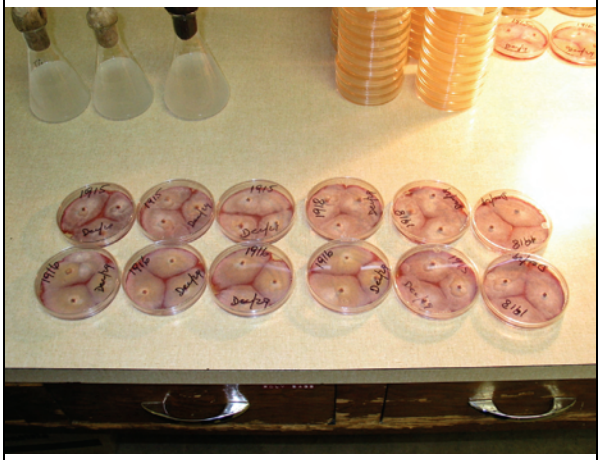

Figure 5. Plate to check level of successful millet inoculation with *Fusarium graminearum*

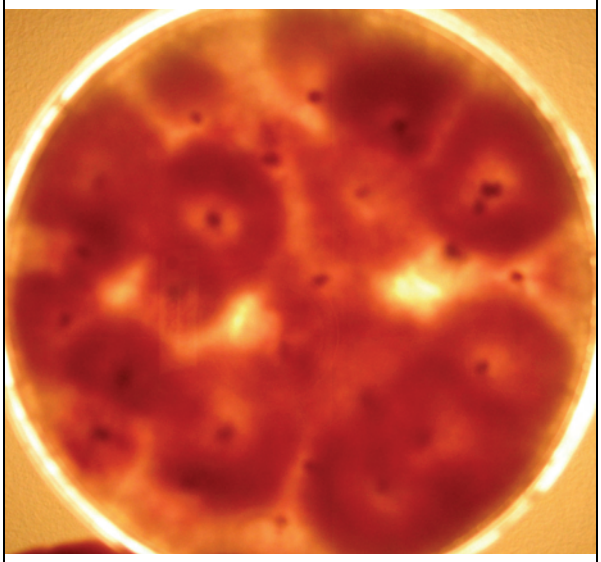

Figure 6. Corn is inoculated with dry *Fusarium graminearum*-infected millet in a laminar flow hood.

Figure 7. Corn kernel inoculum after 2 weeks incubation.

Figure 8. 32 kg (dry weight) of corn inoculum spread over a 2.5 X 0.75 m2 area. A medium weight polythene sheet is surface-sterilized with 95% alcohol. Mycelium continues to develop on kernels. Aggregates of corn are broken up daily

Figure 9. Dried *Fusarium*-infected corn kernels ready to be stored in a cold room or freezer.

Figure 10. Macroconidial suspension of *Fusarium graminearum* attached to vacuum line.

MACROCONIDIAL INOCULUM PROTOCOL FOR FUSARIUM GRAMINEARUM

New isolates of *Fusarium graminearum* are collected annually during late summer in southwestern Manitoba. Isolates are collected from fields that are randomly inspected for fusarium head blight (FHB). Diseased heads are threshed and the Fusarium-damaged kernels (FDK) are surface sterilized using a 0.3% NaCl solution and allowed to dry. These kernels are then plated on streptomycin-amended potato dextrose agar (PDA/S) and placed under cool white light at room temperature for 5-7 days. Isolates are then identified to the species level. Colonies are started from a single germinated conidium, which ensures a pure culture (single spore culture), and grown. The isolate is then tested for pathogenicity by inoculating healthy plants at anthesis with a liquid macroconidial suspension at a standard dilution of 50,000 spores/ml.

The CMC medium for *Fusarium graminearum* inoculum increase consists of:

$NH_4 NO_3$ (ammonium nitrate)	1.0 g
$KH_2 PO_4$ (potassium phosphate)	1.0 g
$MgSO_4$-7H2O (magnesium sulphate)	0.5 g
Yeast extract	1.0 g
CMC (carboxymethyl cellulose)	15 g
H_2O (distilled)	1 litre
Streptomycin sulfate (once solution cools to 50 °C)	0.2 g

Pour about 1/3 of the distilled water into a blender. While the blender is running at LOW speed, SLOWLY add the CMC. Pour this mixture into a 2 litre Erlenmeyer flask. The rest of the dry ingredients may now be added to this mixture. Pour half of the remaining water into the blender and run at low speed for a few seconds to remove any of the mixture that has adhered to the sides of the blender, then add to the first mixture in the flask. Pour the remaining amount of water into the blender & repeat as before. Cover with foil or cotton plug and autoclave for 30 minutes.

After autoclaving, cool medium to 50 ˚C or cooler and add 0.2 g streptomycin sulfate which has been added to 5 ml sterile water.

Add 1 Petri-plate fresh *Fusarium graminearum* culture (shredded). Sterilize a scalpel and cut or shred the culture into 1cm pieces or smaller and add to the CMC. Place a sterile rubber stopper with 2 sterile glass tubes inserted. One tube should reach almost to the bottom of the flask, the other should be well above the solution itself. The other ends of the tubes should be protruding several inches beyond the top of the rubber stopper (Figure 10). On the long tube, place a wad of sterile cotton batting, cover with a double thickness of cheese cloth and secure with an elastic band. On the short tube, attach a piece of flexible hose and connect to a vacuum line. Allow this to bubble gently and continuously for about 4-7 days. Perform a spore count, using a hemacytometer. We use a standard solution of 50,000 spores / ml. For inoculation, add 0.2 ml Tween 20 per 100 ml inoculum. **This inoculum should be used within 2 weeks and stored in a fridge or cold room.**

Conidial suspension can be inoculated 2 ways:

- Single or Double Floret Injections (SFI, DFI)
- Spray inoculations

DFI is used to measure Type-2 resistance (resistance to spread of infection) of the plant. The top third of the spike is referred to as the "Inoculation Zone". The remainder of the spike is rated, on a percent basis, for infection. Using an Eppendorf pipette, 10 µl of inoculum is injected between the lemma and palea of the florets located at the bottom of the top third: one on either side of the main spike (Figure 11).

Spray inoculation is used to measure Type-1 resistance (resistance to initial infection) of the plant. Between 3-5 ml of inoculum are sprayed onto the spike via a pump-spray bottle or by an atomizer.

Inoculated plants are placed into a humidity chamber (100% RH) for 24 hours to enable the pathogen to colonize the host. Infected heads are rated 21 days post inoculation. **Check the plants around 7-10 days post inoculation to observe whether infection is taking place.**

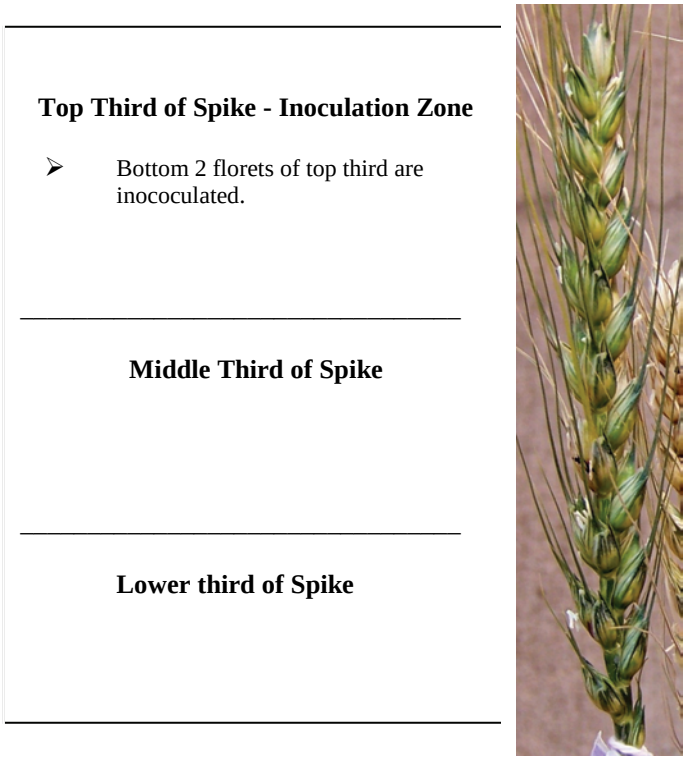

Figure 11. Location of florets for single or double floret inoculation

ANALYSIS OF FUSARIUM HEAD BLIGHT NURSERY DATA

The fusarium head blight (FHB) index, in one form or another, has been used since the 1990s to express the reaction of wheat lines to FHB (Snijders 1990). A typical method has used the product of scores for percent incidence and severity divided by 100 to express the index on a scale of 1-100. However, there are advantages to including measures of damage to the grain and levels of deoxynivalenol (DON) accumulation that would provide an overall indication of the potential problems for grain end-use.

Kolb and Boze (2003) suggest using Fusarium-damaged kernels (FDK) in addition to incidence and severity (or ISK - Incidence, Severity, and Kernel damage). ISK uses a weighted mean with weights of 0.3, 0.3, and 0.4 for incidence, severity, and kernel damage respectively.

ISK= (0.3 Inc(%)) + (0.3 Sev(%)) + (0.4 FDK(%))

If we add a DON measure to make it DISK we might assign the following proportions:

(0.2 Inc(%)) + (0.2 Sev(%)) + (0.3 FDK%) + (0.3 DON(ppm))

We used the data from the 2003 Uniform Regional Scab Nursery (URSN) grown at Glenlea, Manitoba and obtained the following correlations based on genotype means (Table 1):

Table 1. Pearson Correlations among traits for FHB reaction USRN 2003 in MB (n=40)

	FDK(A)	INC(labA)	SEV(labA)	INC(fldA)	SEV(fldA)
DON (log)	0.88	0.77	0.80	0.77	0.69
FDK (A)		0.71	0.83	0.72	0.69
INC(labA)			0.80	0.79	0.50
SEV(labA)				0.75	0.83
INC(fldA)					0.62

log – logarithmic, A – arcsin square root transformed to stabilize variances, lab – lab, fld – field.

The experiment, consisting of 40 genotypes, was grown in in single row plots in a randomized complete block design with 4 replicates. Rows were rated for incidence and severity in the field and the harvested grain samples were returned to the lab for further testing.

In the preceding table the components of the FHB Index, incidence and severity, based on counts made in the lab (lab) were compared to field (fld) ratings, which were based on a visual estimate of incidence and severity. The correlation between DON and FDK is relatively high. Incidence and severity between field and lab are less strongly correlated.

In the data set, DON was measured only on bulk samples from reps 1 and 2 and reps 3 and 4. Incidence, severity, and FDK were measured on 4 replicates, but means of reps 1 and 2 and reps 3 and 4 were used in computing ISK and DISK (Table 2) and in the ANOVA (Table 3).

At Glenlea we use the term visual rating index (VRI) to differentiate between an index based on field ratings versus lab ratings (FHB index). The VRI is an estimate of incidence and severity of FHB in a row or plot. The FHB index is a counted value for incidence and severity based on a random sample taken from the field and counted in the lab. Comparing just the field ratings of incidence and severity with the combined measure of VRI, and ISK, the correlation between VRI and ISK is high (Table 2)

The addition of DON values to the equation results in very high correlations among the VRI, ISK and DISK (Table 2). Further consideration might be given to identifying the most appropriate weights to be assigned parameters that comprise ISK and DISK.

In the ANOVA, DISK was the variable with the highest F value, indicating the greatest discrimination among genotypes (Table 3).

Table 2. Pearson Correlations among traits for FHB reaction USRN 2003 using DISK (n= 40)

	AFDK	AINC	ASEV	AVRI	AISK	ADISK
LDON	0.88	0.77	0.69	0.80	0.85	0.90
AFDK		0.72	0.69	0.80	0.84	0.88
AINC			0.62	0.86	0.89	0.88
ASEV				0.92	0.90	0.88
AVRI					0.99	0.98
AISK						0.99

L – logarithmic, A – arcsin square root transformed to stabilize variances

Table 3. ANOVA of 2003 URSN based on 2 combined replicates.

Name	F value
LDON	4.8
AFDK	9.8
AINC	5.9
ASEV	9.1
AVRI	10.1
AISK	11.1
ADISK	12.4

L – logarithmic, A – arcsin square root transformed to stabilize variances

2D-biplots

2D-biplots can be used to provide a visual representation of data (Yan and Tinker 2006). Practically, it is a two-dimensional display of a two-way table by both row and column. Singular value decomposition (SVD) is used as in principal component analysis. In the biplots presented here the singular values are partitioned with the columns making it easier to see relationships among the columns or traits. The biplot visualizes patterns among row factors and patterns among column factors and patterns of interactions underlying the row and column patterns.

Any 2-way table can be represented using a 2D-biplot if it can be sufficiently approximated by a rank 2 matrix.

In figure 12 below, the data for the 2003 URSN grown at Glenlea, Manitoba, in 2003 are presented. The genotypes are in mixed case (or blue) and the parameters in upper case (or red). Principal component 1 (PC1) versus PC2 form the primary biplot and in this case explain a large proportion of the variation, 86.3%.

The biplot is based on the data in Table 1. Genotypes such as Oslo that are closest to the traits (incidence and severity from the field and from the lab ratings, FDK, and DON content) are more susceptible to FHB than those that are a greater distance from the traits, such as ND2710. Genotypes such as SD3739 have lower incidence, but higher severity, while 98S003-12 has higher incidence and lower severity.

Figure 13 provides information on fewer parameters, including incidence and severity from the field, FDK and DON. Just the checks and one Canadian cultivar are named to make the picture easier to see. The cosine of the angle between parameters gives information on their relatedness. Acute angles show a positive correlation, obtuse angles show a negative correlation, and right angles no correlation. The length of the vector describes the discriminating ability of the parameter. A short vector may indicate that the trait is not related to other parameters, that there is a lack of variation or that it is not well represented in the biplot. For these data, all traits are positively correlated.

The biplot in figure 14 represents 23 variety means for 4 traits (incidence, severity, FDK and DON) for each of two seeding dates in 2003 in the Ottawa (OT) FHB nursery. The measurements are coded as 31, 32 for the first and second seeding dates in 2003. The first principal component is closely related to the average of the standardized traits (mean and standard deviation standardized) ranging from low to high incidence, severity, DON, FDK on the left to the right, respectively. The second principal component relates to consistency across the traits where the most consistent entries are closer to zero. The bold uni-directional line indicates the means of the standardized traits while the line at right angles relates to consistency.

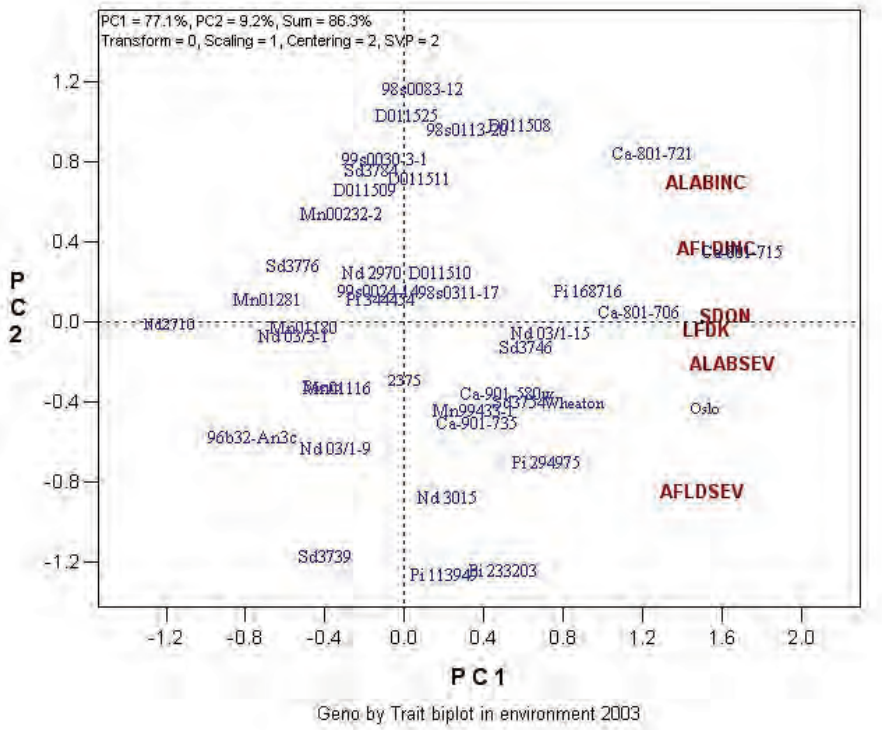

Figure 12. Genotype by trait biplot of 2003 URSN grown at Glenlea, Manitoba.

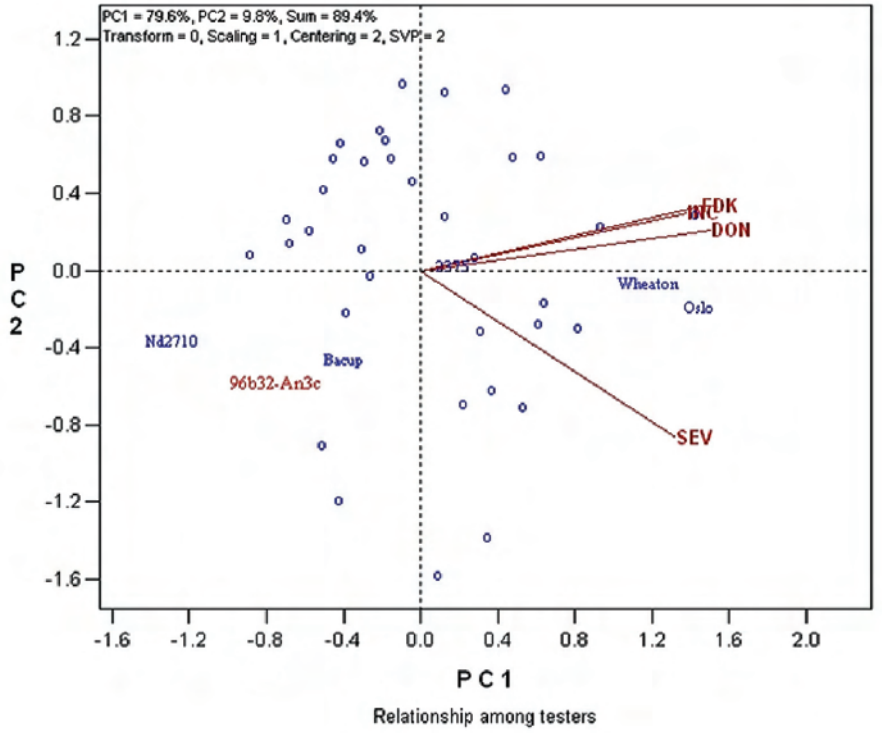

Figure 13. Biplot showing relationship among traits of field incidence and severity, FDK and DON for 2003 URSN grown at Glenlea, Manitoba.

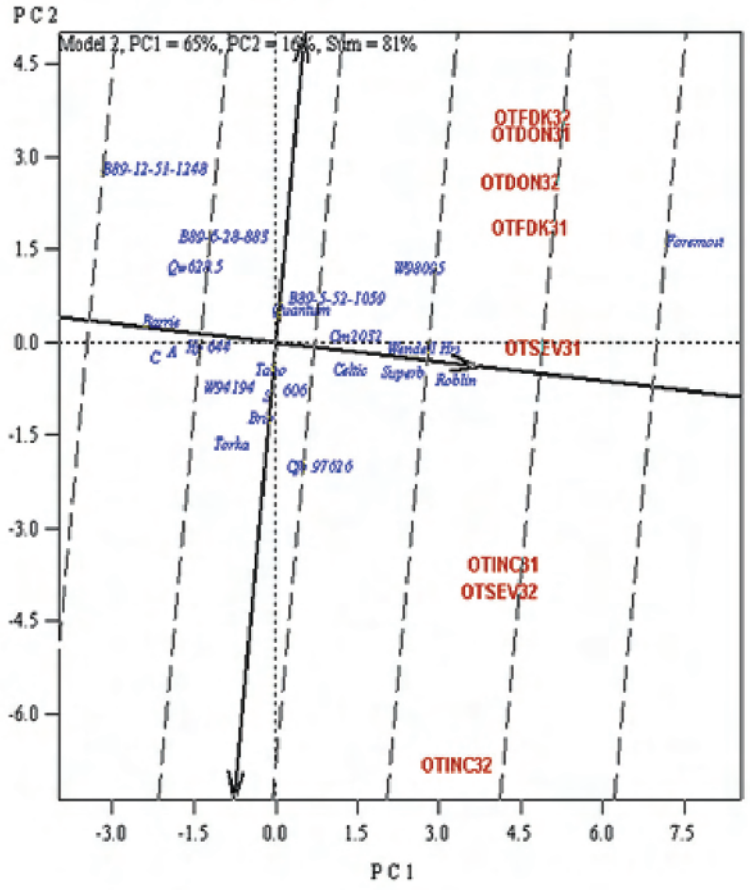

Figure 14. Biplot showing effect of two seeding dates on incidence, severity, FDK and DON in 23 genotypes in the 2003 FHB nursery in Ottawa, Ontario.

SUMMARY

Using ISK and DISK, other important effects of FHB damage, other than incidence and severity, can be considered when making decisions concerning advancement of lines with resistance to FHB. The biplots can show how well a genotype performs against different traits, in different environments or for different seeding dates. All these measures provide more information for genotypes than the reliance on incidence and severity alone.

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