PROJECT REPORT No. 340

CEREAL SEED HEALTH AND SEED TREATMENT STRATEGIES: EXPLOITING NEW SEED TESTING TECHNOLOGY TO OPTIMISE SEED HEALTH DECISIONS FOR WHEAT

Technical Paper No. 3

Variability in the distribution of seed-borne diseases in wheat seed bulks, and the derivation of sampling procedures for their detection

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INTRODUCTION

New PCR-based diagnostic tests for seed-borne diseases of wheat have recently become available in the UK. The techniques have the advantage of offering rapid throughput of large numbers of tests, creating the opportunity for many seed stocks of winter sown wheat to be tested within the short time between harvested and sowing of new crops. Seed treatment decisions can thus be based on the outcome of a seed test result, rather than the current approach of using prophylactic applications regardless of health status. In any move away from prophylactic treatment, it is essential that the test result is representative of the health status of the bulk of seed that it was taken from. Variability in the distribution of seed-borne diseases in seed bulks may be due to a range of factors, including the health status of the mother seed, conditions during the growth of the crop, and handling of harvested seed.

Two diseases were investigated in the course of this work; seedling blight, caused by *Microdochium nivale*, and bunt, caused by *Tilletia tritici*. *M. nivale* infects ears during anthesis, and colonises developing seeds. Infection is favoured by wet conditions, and inoculum can arise from a number of sources. *T. tritici* can be both seed and soil-borne. Seed-borne inoculum is carried externally, and arises when bunt balls are shattered at harvest, releasing spore clouds, which are distributed through the crop as it is harvested, and onto neighbouring crops, depending on wind direction.

Sampling frequencies and maximum bulk sizes are given for different crop species in the International Rules for Seed Testing, Anon. 2003, but govern characteristics such as germination, purity and "other seed count" rather than diseases. Though some analogies exist between such characteristics and disease, there is no documentation covering sampling frequencies for the detection of disease at different levels, or the degree of confidence with which a test result represents the health status of the bulk from which it was taken.

This work was undertaken to investigate the distribution of *M. nivale* and *T. tritici* in wheat seed bulks, and to devise sampling frequency guidelines to ensure that levels of infection which could lead to unacceptable crop loss can be detected with a satisfactory and defined degree of confidence.

MATERIALS AND METHODS

Advisory tests for *T tritici*

Results from historical advisory *T. tritici* tests were used to investigate the effects of different *T. tritici* treatment thresholds on the number of bulks where seed treatment would need to be recommended. The tests were carried out on seed samples submitted for commercial testing to the Official Seed Testing Station, Cambridge from 1997 to 2002. Results from a total of 1882 samples were used. A working sample of 60g was prepared by mixing and dividing down the seed in a Gamet and Pascal centrifugal sample divider (Pascal Engineering Co. Ltd, Crawley, UK), and counting two repeat sub samples of 300 seeds each, and a further three samples each containing 100 seeds. The latter were examined separately if the 300 seed sample was found to be heavily contaminated with debris otherwise they were pooled. Samples were washed in 60 ml of a 0.1% solution of Tween 20 in water on a rotary shaker for 3 minutes. Liquid was then decanted, and filtered though a 5.0µm cellulose nitrate filter in a Buchner apparatus. The filter was the placed on a microscope slide under a cover slip, and the number of bunt spores in 10 randomly selected fields counted at a magnification of x 200. The number of spores per seed in the working sample was then calculated from the average number of spores per field of view, the area of the field of view, area of the filter and number of seeds examined. The majority of samples tested were from seed intended for farm processing. No details of sampling procedures were known.

Field experiments

Field experiments in each of two years were established so that a high degree of variability in seed contamination with *T. tritici* could be introduced into a harvested bulk. A field of about 1.25 ha on the NIAB, Cambridge farm was drilled with cv Consort on 19th October 1999 at 210 kg/ha. Seed was treated with Sibutol except for a drill width (1.8 m) 90 m in length, which was sown with untreated seed contaminated with bunt spores. Seed contamination was achieved by mixing spores extracted from bunt balls with the seed at a rate of 1.2 g extracted bunt balls / kg seed to give approximately 50,000 spores per seed.. At harvest, about 40% of plants in the bunted strip had bunt infected tillers. The crop was combined on 24th August 2000. A section of the Sibutol treated area was cut first, followed by a 0.25 m x 90 m wide segment from the bunted strip. The harvested grain was then emptied from the trailer on to a barn floor. The rest of the field (but not the remains of the bunted strip) was then cut, and grain placed next to the first load. Healthy grain from another farm crop was added over the first two loads to give a final weight of about 28 t.

A second crop of cv Consort, also treated with Sibutol, was established in the same field on 14th December 2000, and seed contaminated with *T. tritici* was sown to produce a bunt infected strip as before. The crop was combined on 23rd August 2001, beginning with uninfected parts of the field, then adding half of the bunted strip. The trailer was then emptied on to a barn floor, and the rest of the field, including the bunted strip, was harvested. This grain was placed next to the first load, and then a further 25 t of another healthy crop was added, to give a total of about 33 t.

Seed was sampled in each year by a trained sampler using a single chamber Neate spear apparatus capable of holding about 80g of seed. In each year, 20 primary samples were taken from each of the two trailer loads (about 4-5 t each) on the barn floor and then a further 40 samples were taken from the bulk after addition of healthy material by walking over the seed and inserting the sampler at different depths and moving over the entire surface of the bulk between 1 and 2 m at a time. An additional 20 samples were then taken in the same way to provide sufficient sample numbers to investigate the effect of either a) a total of 20 whole bulk samples only, or b) a total of 60 whole bulk samples. For each of the sampling points, three separate samples were taken to provide sufficient seed for primary testing and subsequent generation of a composite sample.

On farm bulks

To investigate the distribution of both *M. nivale* and *T.tritici* a number of certified seed producers and ware growers were contacted and permission requested to sample seed bulks at their premises. Seed bulks of varying sizes were sampled on farms by a trained seed sampler. Bulks were either intended for certification as C2 seed, or were farm-saved. Ware growers were selected on the basis of advisory test results for *T. tritici* that had been carried out at NIAB, in addition a number of home-saved bulks at the NIAB, Cambridge farm were also sampled. A trained seed sampler visited the farms during August and September in 1999, 2000, 2001 and 2002 and took varying numbers of primary samples with a single chamber Neate spear sampler, with a 3m extension or a four-chambered "walking stick" sampler where bulk depth was 1 m or less. Sampled bulks sizes were either estimated visually or provided by the grower. Three separate samples were taken at each sampling point when using the Neate sampler. With the multi-chambered sampler, two insertions were made at each sampling point. After each, the seed was emptied into a long shallow container, so that seed from equivalent compartments at the second sampling was added to that of the first. Each of the four individual piles of seed was placed in separate bags to constitute primary samples. Sampling equipment was not rigorously cleaned between sample points, but was washed with water and detergent and wiped thoroughly between different bulks.

From both the field experiments and on-farm bulks, composite samples were made from the primary samples after they had been tested for disease by mixing and dividing down using a Gamet and Pascal divider. In three lots sampled in Scotland the composite sample was made up from primary samples sampled in the same position as the individual primary samples taken for disease testing.

Seed tests on primary and composite samples

Primary or composite samples were prepared and tested for *T. tritici* as previously described. For *M. nivale*, samples were prepared by mixing and dividing down as before. Two hundred seeds were surface sterilised for 7-10 minutes in NaOCL (approximately 1% available chlorine), then plated onto potato dextrose agar (PDA), incubated for between 5 and 7 days at 20°C with 12h uv light, 12h dark, and then counting the number of seeds giving rise to colonies of *M. nivale*. Seed tests for three bulks were tested at SASA and incubation was in the dark.

RESULTS

Advisory tests for *T. tritici*

The results from commercial *T. tritici* testing from 1997 – 2002 were cumulated into annual sets representing observed infection levels. Infection levels were variable both within a year and particularly between years. For example 1997 had generally higher observed infection levels, while 2002 was a more moderate year. Seasons 1999-2002 showed a greater level of agreement i.e. less variation between seasons. Taking each annual set of observed infection data, Table 1 shows the computed percentage rejection rate for a range of potential threshold decision levels. It can thus be seen what the rejection rates would be in terms of the observed infection levels if seed contamination threshold levels had been set at x spores per seed. Annual effects can be seen in Table 1 where the potential threshold (x) ranges from 0 to 10 spores per seed. Marked seasonal variation, in terms of the expected rejection rates, occurred at low seed contamination threshold values. For example, with a threshold 0.5 spores per seed there is a range in annual rejection rate of 5.2% to 32.7% (6 fold difference) and a 5 fold difference at a threshold of 2.0 spores/grain (1.8% -10.7%). The seasonal variation can be seen graphically in figure 1a where the simulated threshold range is restricted to the core range 0 – 3 spores per seed. Pooled data over the period 1997-2002 gives observed rejection rates for potential threshold values of 0.5, 1.0, 1.5 and 2.0 spores per seed of 11.37%, 6.48%, 4.30% and 3.13% respectively.

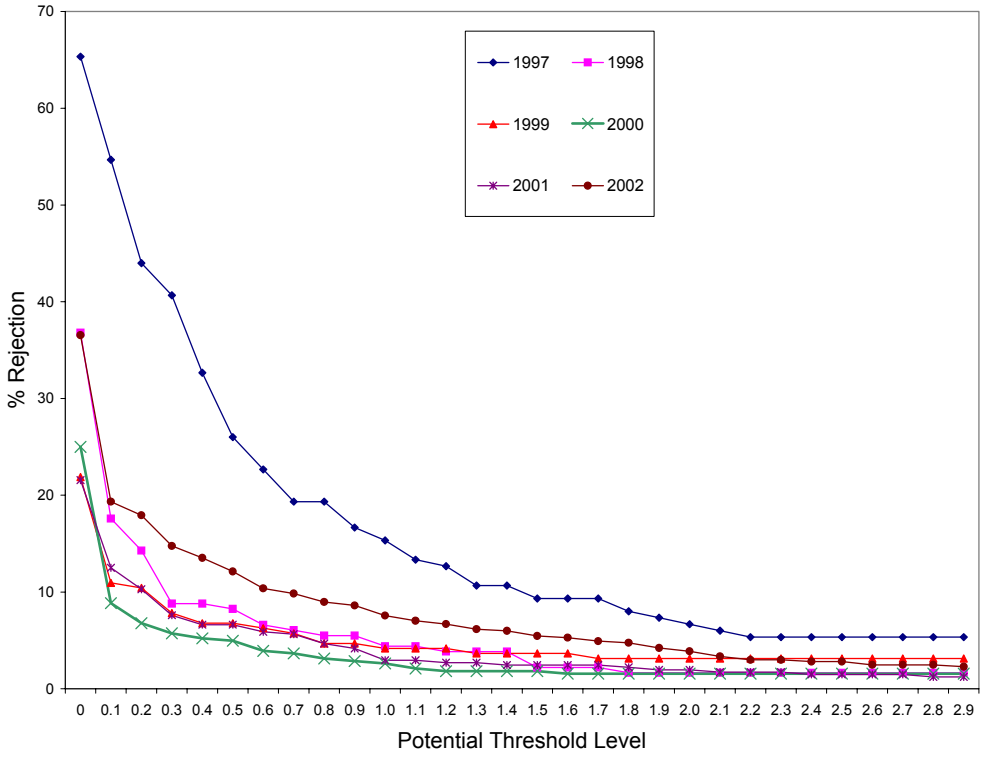
Table 1 Observed annual rejection rates (%) for a wide range of assumed threshold values of *T. tritici* spores per seed.

Threshold	1997	1998	1999	2000	2001	2002
0.1	65.33	36.81	21.88	25.00	21.57	36.56
0.2	54.67	17.58	10.94	8.85	12.50	19.33
0.3	44.00	14.29	10.42	6.77	10.29	17.93
0.4	40.67	8.79	7.81	5.73	7.60	14.76
0.5	32.67	8.79	6.77	5.21	6.62	13.53
0.6	26.00	8.24	6.77	4.95	6.62	12.13
0.7	22.67	6.59	6.25	3.91	5.88	10.37
0.8	19.33	6.04	5.73	3.65	5.64	9.84
0.9	19.33	5.49	4.69	3.13	4.66	8.96
1.0	16.67	5.49	4.69	2.86	4.17	8.61
1.1	15.33	4.40	4.17	2.60	2.94	7.56
1.2	13.33	4.40	4.17	2.08	2.94	7.03
1.3	12.67	3.85	4.17	1.82	2.70	6.68
1.4	10.67	3.85	3.65	1.82	2.70	6.15
1.5	10.67	3.85	3.65	1.82	2.45	5.98
1.6	9.33	2.20	3.65	1.82	2.45	5.45
1.7	9.33	2.20	3.65	1.56	2.45	5.27
1.8	9.33	2.20	3.13	1.56	2.45	4.92
1.9	8.00	1.65	3.13	1.56	2.21	4.75
2.0	7.33	1.65	3.13	1.56	1.96	4.22
2.1	6.67	1.65	3.13	1.56	1.96	3.87
2.2	6.00	1.65	3.13	1.56	1.72	3.34
2.3	5.33	1.65	3.13	1.56	1.72	2.99
2.4	5.33	1.65	3.13	1.56	1.72	2.99
2.5	5.33	1.65	3.13	1.56	1.47	2.81
2.6	5.33	1.65	3.13	1.56	1.47	2.81
2.7	5.33	1.65	3.13	1.56	1.47	2.46
2.8	5.33	1.65	3.13	1.56	1.47	2.46
2.9	5.33	1.65	3.13	1.56	1.23	2.46
3.0	5.33	1.65	3.13	1.56	1.23	2.28
4.0	3.33	1.10	2.08	1.56	0.98	1.93
5.0	3.33	0.55	2.08	1.30	0.98	1.76
6.0	2.00	0.00	1.56	0.78	0.98	1.58
7.0	1.33	0.00	1.56	0.78	0.98	1.23
8.0	1.33	0.00	1.56	0.52	0.98	0.88
9.0	0.67	0.00	0.52	0.52	0.98	0.70
10.0	0.67	0.00	0.52	0.52	0.98	0.70

Figure 1a and 1b.

(1a) Annual 'rejection' rates for assumed threshold values 0-3 spores per seed of *T.tritici* . (1b) Same data showing variation and potential outliers.

1a



1b

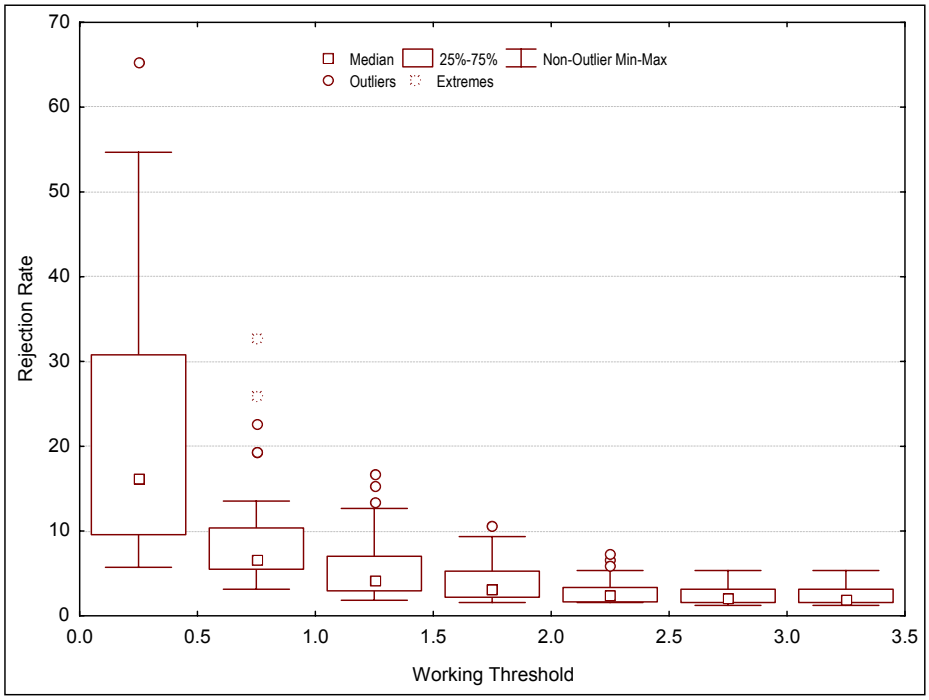


Fig 1b is a standard Box-Whisker plot which, for supplied data, shows the distributional properties in terms of the 25%, 50% and 75% percentile points (50% percentile is median); the non-outlier maximum to minimum limits and any potential outliers and or extreme values. Outliers and extreme values are defined as values that exceed 1.5 times and 2.5 times the inter-quartile range respectively. Values so flagged are data values that are inconsistent with the bulk of the data and if retained will have a significant affect on any further analyses. In this case, observed rejection rates have been grouped from threshold values 0.1-0.4; 0.5-0.9; 1.0-1.4; 1.5-1.9; 2.0-2.4; 2.5-2.9 and 3.0-3.5. The variation in rejection rates is large for very low threshold values (<1 spore per seed). Even for potential thresholds between 1 and 2.5 spores per seed, the observed variation in reject rate percentages is large with outliers indicative of the influence of seasonal effects.

The distribution of observed infection levels (spores per seed) is heavily influenced by the large number of zero or effectively zero values. Taking an arbitrary 'trace' or effectively zero value of 0.1

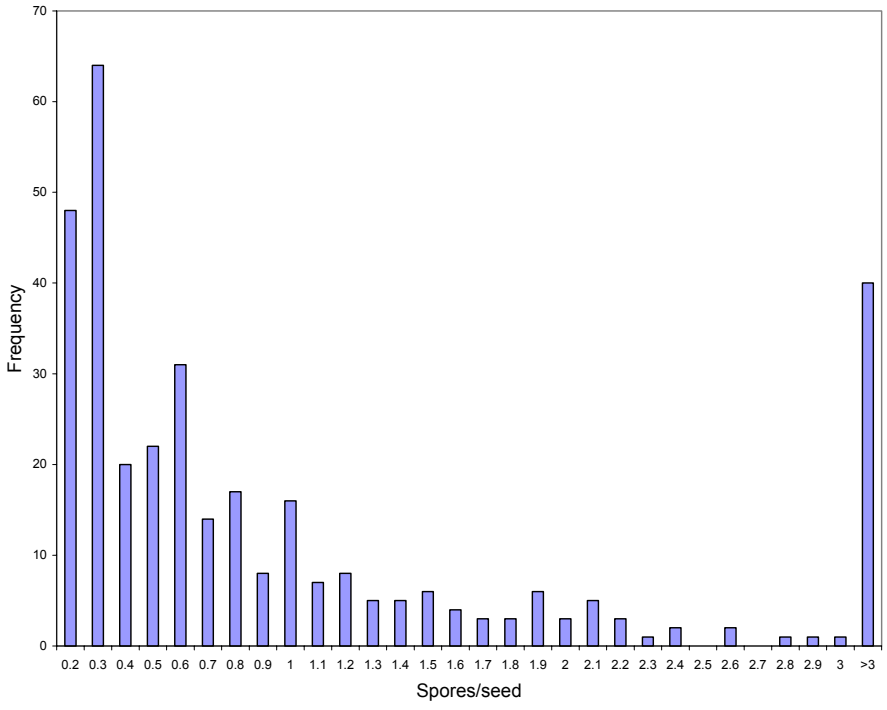


Figure 2 The observed % of commercial samples from 1997-2002 with greater than 0.1 spores per seed of *T. tritici*

spores/grain, annual percentages of samples tested during 1998 – 2002 were 17.6%, 11.0%, 8.8%, 12.5% and 19.4% respectively. In 1997 65% of the samples exceed 0.1 spores/grain. For any potential threshold level the variation due to seasonal effects is marked. However, for any potential threshold

above 1.5 spores per seed, the expected rejection rate is less than 10% even in a season with high infection levels such as 1997 based on the observed commercial samples from1997-2002. For thresholds between 2.5 and 3 spores per seed the rejection rate reaches an asymptote of 6 - 7% even in the worst case scenarios of heavy infection years. Figure 2 shows the frequency of observed infection in over 1880 commercial samples from 1997-2002. Infection levels over 3 spores per seed have been pooled.

Figure 3 shows individual annual rejection rates from thresholds between 0 and 3 spores per seed with logarithmic fitting. The fit with a logarithmic model is effective between 0 and about 2 spores per seed but is consistently underestimated for observed infections over 2.5 spores per seed. Using an exponential fitting model (not shown), predictions between 0 and 2 spores per seed are significantly underestimated with an acceptable fit for infection exceeding 2 spores per seed. Selecting an adequate model which is effective in the range of potential thresholds; is far from straightforward.

While statistical regression models can be fitted to the individual years separately over the range of thresholds 0.5 to 3 spores per seed; these fitted relationships are weak for thresholds less that 0.5 spores per seed and lead to an underestimation of rejection rates.

Descriptive statistics from each year 1997 – 2002 are shown in Appendix III in terms of spores per seed. While the mean of the data values for each year are non-zero, ranging from 0.19 to 3.26 spores per seed, the key statistics of the data are the mode and the median. Both of these statistics indicate that the data for each year consists of many zero values. In fact, the modal values (most common value) is zero for all years and, with the exception of 1997, the median is also zero. The median is the 50 percentile (middle observational value when the data are ranked or ordered) and when zero in data with all positive values; confirms that at least 50% of the values are zero. Further examination shows that, with the exception of 1997, 63% to 78% of the data are at zero. In 1997 81.3% of the observed data are non-zero, mostly due to modest infection levels; with a mean of 0.83 spores/seed and the median 0.21 spores per seed.

From the median and the mode, it is clear that the data are not normally distributed in the traditional symmetrical bell shaped distribution. This is confirmed in the skewness (“symmetry”) and kurtosis (“shape”) statistics each significantly different from that of a normal distribution. For a true Poisson

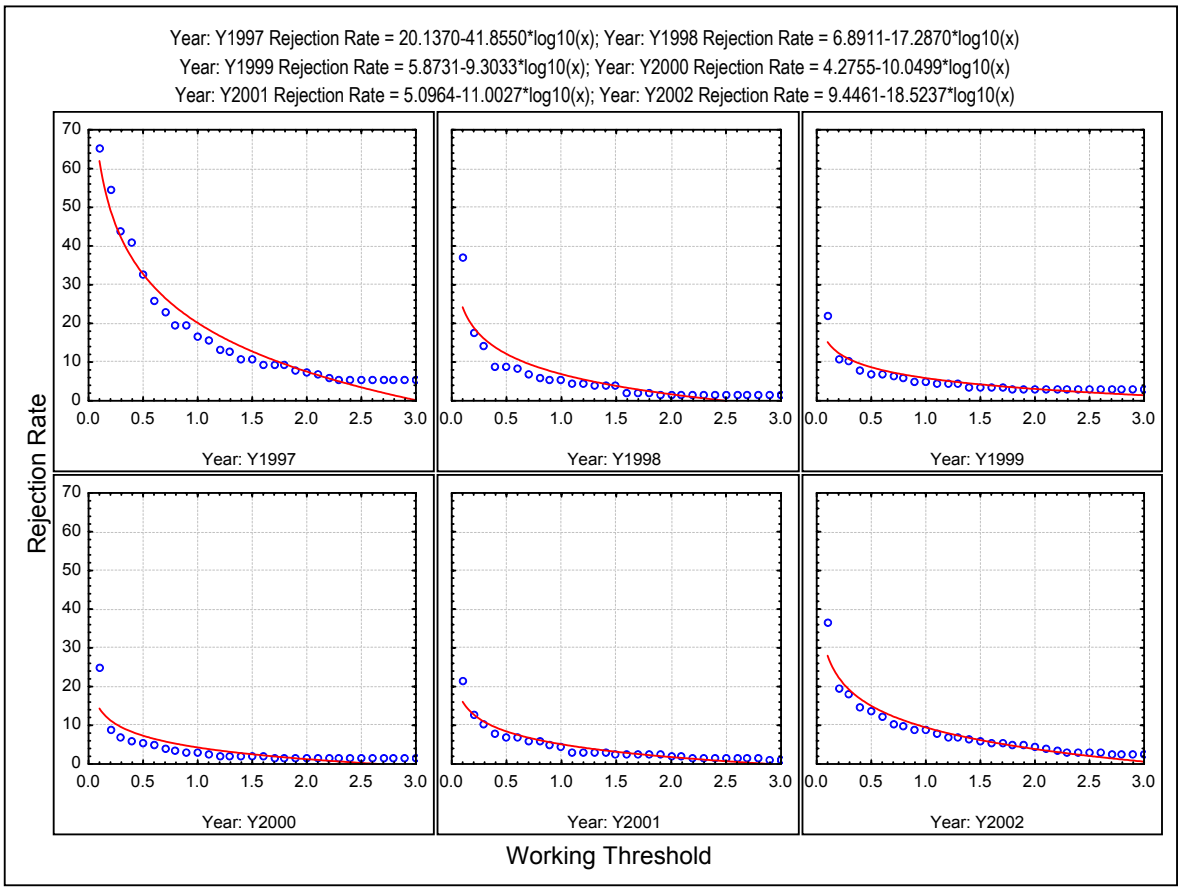


Figure 3 Scatter plot of annual computed values for *T. tritici* infection levels (spores per seed) based on advisory tests 1997-2002 with potential thresholds between 0 and 3 spores per seed with logarithmic fit.

distribution, the mean and variance parameters are equal. As shown above the variance greatly exceeds the mean. The issue is not so much the distribution over the full range of observed infection, although if this was defined robustly this feature could be utilised, as much as the variation that exists in the zone of infection about any proposed threshold value. Specifically, the variation influences the risk of type I (rejecting when should be accepted) and type II errors (accepting when should be rejected). Using a discrete Poisson distribution with the single parameter that of the observed average infection level (spores/grain) for that year, it is possible to assess type I error. Table 2 gives these type I errors for exceeding 1, 2, 3,…, 9, 10 spores/grain. The highlighted cells equate to risks circa 10%. Note that there is a substantial seasonal component. As noted before the median values are, with the exception of 1997, all zero and inappropriate for use in the Poisson model. Taking the true infection rate of 0.6 spores/grain; type I errors for exceeding threshold of 1 and 2 spores/grain are 12.2% and 2.3% respectively.

Table 2 Annual probability of exceeding the threshold (spores per seed) given observed average infection levels (row 1) and Poisson distribution (probabilities are cumulative ie 1997 probability of 3 or more given Poisson parameter 0f 0.8317 is 0.0104)

	0.831733	0.185714	0.408377	0.46875	3.260934	1.216901
Threshold	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**
0	0.5647	0.1695	0.3353	0.3742	0.9616	0.7039
1	0.2027	**0.0153**	0.0638	0.0809	0.8366	0.3435
2	0.0521	0.0009	**0.0084**	**0.0121**	0.6327	0.1242
3	**0.0104**	0.0000	0.0008	0.0014	0.4110	0.0353
4	0.0017	0.0000	0.0001	0.0001	0.2303	**0.0082**
5	0.0002	0.0000	0.0000	0.0000	0.1125	0.0016
6	0.0000	0.0000	0.0000	0.0000	0.0484	0.0003
7	0.0000	0.0000	0.0000	0.0000	0.0186	0.0000
8	0.0000	0.0000	0.0000	0.0000	**0.0064**	0.0000
9	0.0000	0.0000	0.0000	0.0000	0.0020	0.0000
10	0.0000	0.0000	0.0000	0.0000	0.0006	0.0000

Of major concern with this data set which comprises of a high proportion of zero values, is the influence of an occasional very high observed value in terms of infection rate. Between 1997 and 1999 the maximum observed infection rate was about 40 spores/seed. This maximum observed infection rose to over 100 spores/seed in 2000, 445 spores/seed in 2002 and over 1000 spores/seed in 2001. Infection rates exceeding 25 spores/seed occurred in 0.6% of all observed samples or at most 3 samples per year. Only 3 samples (out of a total of 1882) were observed with more than 100 spores/seed, one in each of the years 2000, 2001 and 2002.

The sample variance (sum of squared differences from the mean value) and the square root of the variance (standard deviation) show high values in 2001 and 2002 where a small number of high infection observations inflate the statistics.

The fact, based on empirical evidence, that an occasional high bunt infection level could occur, resulted in a defined sampling plan to study a small number of samples in more detail by analysing the distribution of infection in seed bulks, derived from inoculated field experiments and on-farm commercial seed bulks

Field experiments

The harvested seed from both field experiments showed a high level of bunt in the composite samples from the large bulks (Table 3), though many primary samples had little infection (Appendix I). However, it was possible to detect the pockets of high bunt infection which existed by taking 40 primary samples and using a sampling pattern which involved systematically accessing all parts of the bulk at different depths and in different areas. It is noted that the mean of the primaries (from Appendix I) is always greater than the corresponding composite sample determination. This is likely to occur in non-random (ie clumped or clustered) infection sites in the midst of a majority of clean seed. Samples from a mixed bulk for the composite, result in either diluting some high primaries, or missing some heavily bunted pockets within a primary. Non-scientifically this comes down to a pragmatic approach summarised as "… you can't sample all the seed all the time, just some of the seed some of the time".

Of the 28 tons harvested in the first year, and the 33 t in the second year, it was estimated that approximately 30 kg of severely bunted crop was harvested (ie where all tillers were infected, and all seed was replaced by bunt balls on about 40% of plants in the strip). In each year, one of the smaller bulks had lower levels of infection in the composite, though individual primaries had higher infection as the seed was contaminated by spores from the bunted strip within the combine, or by spores blown from shattered bunt balls. The other small bulk had much higher infection as it contained the grain harvested from the strip.

Table 3 Composite sample results for *T. tritici* from experimental bulks

Year	Bulk size (estimated t)	Composite sample spores/seed	Number of primary samples in composite
2000	4-5	201.4	20
	4-5	3.6	20
	28	40.0	40
	28	33.0	60
2001	4	2.7	20
	4	32.3	20
	33	6.3	60

On farm bulks

The descriptions of sampled bulks and sampling methods are summarised in Table 4. Composite sample results and original farmer submitted sample result, where available, are summarised in Table 5. No composite tests were carried out in 1999 on the bulks tested for *M. nivale* only. Primary sample results are tabulated in Appendix II. A variety of situations were sampled, ranging from seed in large bins, spread on drying floors, and loaded into bags ready for drilling. In general, all parts of the seed bulk were accessible, except for some instances of very deep bins.

The distribution of *M. nivale* was relatively uniform (see Table 6 for examples). There were no instances of very high infection levels well above the recommended treatment threshold of 10% occurring in a few primary samples when the majority had low levels. Only two comparisons of composite result with farmer submitted sample were available, and both showed very close agreement.

In the case of *T. tritici*, bulks fell into three categories Firstly, those where levels in all samples were zero or very low numbers of spores per seed; secondly those where all samples were well above the treatment threshold of 1 spore per seed, and thirdly, those where one or a few primary samples showed infection levels above the threshold, but the composite sample result was lower, and in some cases would have lead to a decision not to treat the seed. The majority of bulks examined fell into the first two categories (20 out of a total of 23 examined). There was also close agreement between composite result, and the result from farmer submitted samples in most cases in terms of potential treatment decisions at the 1 spore per seed threshold.

Statistical analysis – on farm bulk data

Given the relative uniformity of data obtained on *M.nivale*, no further analyses were carried out for this pathogen. A relatively small number of bulks were selected for further study of results for *T. tritici.* These were chosen to cover the zone of any potential threshold but samples well above this likely zone of interest, were also included. Many commercial samples with zero or effectively zero spores per seed were also available, though these were always highly uniform, with all primary samples at or close to zero infection. The purpose was to investigate the variation between primary samples as preliminary work had indicated that the presence of a few very heavily bunted seeds could increase the assessment of bunt infection made on the bulked sample. An example of the potential problem is illustrated in Figure 4.

Table 4 Bulk sampling details 1999-2002

Sample number	Certified (C) or farm saved (FS) and variety	Seed storage	Estimated weight sampled (t)	Sampling equipment	Number of primary samples	Disease test (Mn orTt)
99/S1	C Shamrock	Floor	200	Neate	40	Mn
99/S2	C Abbott	Floor	800	Neate	40	Mn
99/S3	C Rialto	Floor	250	Neate	40	Mn
99/S4	C Consort	Floor	400	Neate	40	Mn
99/S5	C Chaucer	Floor	250	Neate	40	Mn
99/S6	FS Equinox	Floor	30	Neate	40	Mn
99/S7	FS Equinox	Floor	30	Neate	40	Mn
00/S1	FS Savannah	Floor	150	Neate	40	Tt and Mn
00/S2	FS Chaucer	Floor	200	Neate	40	Tt and Mn
00/S3	FS Riband	Floor	200	Neate	40	Tt
00/S4	FS Claire	Floor	200	Neate	40	Tt and Mn
00/S5	FS Consort	Floor	200	Neate	40	Tt
00/S6	C Rialto	Floor	100	Neate	40	Tt and Mn
00/S7	C Consort	Floor	200	Neate	40	Tt
00/S8	C Rialto	Floor	150	Neate	40	Tt
01/S1	C Claire	Floor	160	Neate	40	Tt and Mn
01/S2	C Consort	Floor	500	Neate	80	Tt and Mn
01/S3	C Riband	Floor	175	Neate	40	Tt and Mn
01/S4	C Consort	Floor	500	Neate	80	Tt and Mn
01/S5	FS Malacca	bin (top)	40	Stick	40	Tt
01/S6a	FS Consort	Bin	60	Neate	40	Tt
01/S6b	FS Consort	bin (top)	7	Stick	40	Tt
01/S7	FS Claire	Bags	8	Neate	40	Tt
01/S8	FS Axona	Floor	15	Neate	40	Tt
01/S9	FS Claire	Floor	140	Neate	40	Tt
01/S10	FS Savannah	Floor	100	Neate	40	Tt
01/S11	FS Consort	Floor	25	Stick	40	Tt
01/S12	FS Equinox	Floor	120	Neate	40	Tt
01/S13	FS Claire	Floor	15	Stick	40	Tt
02/S1	FS Consort	Floor	30	Neate	40	Tt
02/S2	FS Oxbow	Floor	50	Stick	40	Tt
02/S3	FS Malacca	Floor	50	Neate	40	Tt

Table 5 Composite sample results and original farmer submitted sample test results for seed bulks sampled 1999-2002.

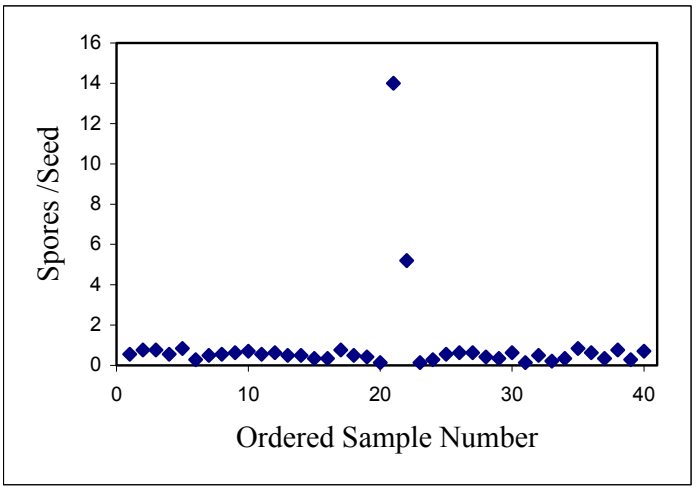
Sample no	Composite result for *T. tritici* (spores per seed)	Original farmer sample result for *T. tritici*	Composite sample result for *M. nivale* (% of seed infected)	Original farmer sample result for *M. nivale*
00/S1	47	103	1.5	*
00/S2	2.4	5.3	9.5	9.5
00/S3	0.1	0.1	*	*
00/S4	0.6	*	13	15.5
00/S5	0.0	*	*	*
00/S6	*	*	*	*
00/S7	*	*	*	*
00/S8	*	*	*	*
01/S1	0.08	*	16	*
01/S2	0.08	*	11	*
01/S3	0.25	*	2	*
01/S4	0.08	*	11	*
01/S5	19.0	58.0	*	*
01/S6a	1.2	2.8	*	*
01/S6b	2.7	2.8	*	*
01/S7	0.1	0.3	*	*
01/S8	317.0	1162.0	*	*
01/S9	0.6	0.9	*	*
01/S10	78.0	55.0	*	*
01/S11	1.0	0.8	*	*
01/S12	1.2	0.2	*	*
01/S13	0.5	0.3	*	*
02/S1	3.1	1.9	*	*
02/S2	1.5	0.9	*	*
02/S3	1.0	5.4	*	*

* is either no result available, or composite test not done

Table 6 Distribution of infection ranges (% seed infected) in primary samples tested for *M. nivale* for three bulks of seed with different composite sample results

Sample no.	Composite %	Infection ranges (%)		
		0-5	6-10	11-25
00/S2	9.5	0	37	3
01/S1	16	0	0	40
01/S3	2	40	0	0

Figure 4. Spores per seed for individual primary samples from sample 00/S4



Results in Figure 4 are shown in the order the samples were taken. Infection is between zero and one spore per seed with the exception of two higher values. Such values indicate the probable occurrence of a heavily infected area within the bulk, which has been detected from a single spearing at several depths.

The summary statistics for the case in Figure 4 are as follows: mean (of 40 samples) 0.96 spores per seed, median 0.56, and standard deviation 2.249. Both the mean and the standard deviation are influenced by the two high values. Without these, the mean and standard deviation statistics reduce to

0.505 and 0.197 respectively. As expected, the median, being more robust in the presence of potential outliers, was 0.525 without the high value points..

Work was undertaken to assess the effect of a small number of heavily bunted primary samples within a bulk, which was overall effectively at zero infection as judged from the composite sample result. The data comparing selected commercial bulks with detailed assessment results, is shown in Table 7. The table shows that there is good agreement between a decision based on the initial (farmer) test and the composite result derived from bulked primary samples. Different decisions would have been taken in three cases (based on a one spore per seed threshold). Of these, two of the initial tests were close to the threshold value. A paired 2-tail t-test comparing the initial bunt assessment carried out on the farmer sample, with the composite sample derived from mixing forty primary samples showed no statistical differences (at p=0.29) between the results. Seed bulks that were heavily infected varied quantitatively between the initial and composite sample, but the qualitative effect was unaltered.

DISCUSSION

Of the two major seed-borne diseases of wheat, *T. tritici* causes the greatest concern in terms of seed sampling for health tests. If areas of high levels of *M. nivale* infection remained undetected in a seed bulk, a subsequent crop could suffer significantly reduced establishment, and loss of yield, though complete crop failure is likely to be rare. However, undetected high levels *of T. tritici* would be likely to lead to serious losses.

The level of variation within some bulks, for *T. tritici* infection, based on 40 samples tested, remains a cause for concern. Eleven bulks of the nineteen examined in Table 7 have a standard deviation of two spores per seed, with seven below one spore per seed. High levels of variation, while they are quantified in this experimental situation, would be unknown in any diagnostic test on a single composite sample based on a blended and divided down set of primary samples. The effect of standard deviation can be seen in terms of the probability that, with the observed mean infection level below one spore per seed, there is a probability that the true bulk infection level is above the threshold. For example, a sample with a composite value of 0.5 spores per seed and observed standard deviation of 0.545 has a 17.5% risk that the true mean exceeds 1 spore per seed. Confidence intervals can be constructed for each of the bulks investigated (see Table 8), but with large standard errors, the resulting intervals are generally too large to have any practical application. Observed variation indicates a confidence interval of 0.7 to 1.5 spores per seed at 95% probability around a threshold of 1 spore per seed. Large standard error compared to composite infection level emphasises the critical importance of sampling the farm bulk effectively to obtain a working sample that is truly

representative of the bulk. Increasing sampling frequency to 80 primary samples has relatively little effect on predicted confidence interval (Table 8), and limiting bulk size provides a more effective way of ensuring that potentially damaging pockets of *T. tritici* infection are detected.

Table 7 Probabilities of composite sample results being greater than specified infection levels (figures in bold show when composite sample is likely to give a different decision to initial farmer sample assuming one spore per seed threshold)

Sample no	Initial test	Composite test	Standard deviation	Probability >0.7	Probability >0.8	Probability >0.9	Probability >1.0
00/S3	0.1	0.1	0.077	0.000	0.000	0.000	0.000
01/S12	**0.2**	**1.2**	**0.334**	**0.925**	**0.872**	**0.799**	**0.705**
01/S7	0.3	0.1	0.125	0.000	0.000	0.000	0.000
01/S13	0.3	0.5	0.545	0.350	0.285	0.226	0.175
01/S11	**0.8**	**1.0**	**2.084**	**0.565**	**0.546**	**0.527**	**0.508**
02/S2	**0.9**	**1.5**	**1.562**	**0.702**	**0.680**	**0.657**	**0.633**
01/S9	0.9	0.6	0.494	0.444	0.365	0.292	0.227
02/S1	1.9	3.1	15.94	0.559	0.556	0.554	0.551
01/S6a	2.8	1.2	1.126	0.665	0.632	0598	0.564
01/S6b	2.8	2.7	0.942	0.984	0.979	0.973	0.965
01/S6a/b+	2.8	2.1	1.035	0.909	0.892	0.873	0.852
00/S2	5.3	2.4	1.068	0.947	0.937	0.924	0.910
02/S3	5.4	1.0	2.144	0.563	0.545	0.526	0.507
01/S10	55	78	107.9	0.762	0.762	0.762	0.761
01/S5	58	19	42.1	0.665	0.664	0.664	0.663
00/S1	103	47	18.93	0.992	0.992	0.992	0.992
01/S8	1162	317	282.8	0.868	0.868	0.868	0.868
00/S4	*	0.6	2.249	0.475	0.440	0.440	0.422
00/S5	*	0.0	0.027	0.000	0.000	0.000	0.000

+ analysis carried out on combined results (80 samples) of seed from same crop in different bins

Table 8 Influence of sample size (40 and 80) on 95% confidence interval.

Sample no	Initial test (farmer sample)	Composite	SD	Prob >1	L95% CI(40)	U95% CI(40)	**L95% CI(80)**	**U95% CI(80)**
00/S3	0.1	0.1	0.08	0.00	0.05	0.09	**0.05**	**0.09**
01/S7	0.3	0.1	0.13	0.00	0.10	0.18	**0.11**	**0.17**
01/S13	0.3	0.5	0.54	**0.17**	0.32	0.66	**0.37**	**0.61**
00/S4	*	0.6	2.25	**0.42**	-0.14	1.26	**0.07**	**1.05**
01/S9	0.9	0.6	0.49	**0.23**	0.48	0.78	**0.52**	**0.74**
02/S3	5.4	1.0	2.14	0.51	0.38	1.70	**0.57**	**1.51**
01/S11	0.8	1.0	2.08	0.51	0.39	1.69	**0.58**	**1.50**
01/S6a	2.8	1.2	1.13	0.56	0.83	1.53	**0.93**	**1.43**
01/S12	0.2	1.2	0.33	0.70	1.08	1.28	**1.11**	**1.25**
02/S2	0.9	1.5	1.56	0.63	1.05	2.01	**1.19**	**1.87**
01/S6a/b	2.8	2.1	1.04	0.85	1.76	2.40	**1.85**	**2.31**
00/S2	5.3	2.4	1.07	0.91	2.10	2.76	**2.20**	**2.66**
01/S6b	2.8	2.7	0.94	0.97	2.42	3.00	**2.50**	**2.92**

The experimental bulks which were generated to provide "worst case" variability in bunt infection, showed that it was possible to detect a pocket of about 30 kg of severely infected seed by taking 40 primary samples over about 28 t. Even though many of the samples had low levels of infection, the composite clearly indicated that the seed would have needed treatment. When smaller bulks were sampled it was possible to detect a severe infection in about 4-5 t of seed with 20 primary samples. Cereal seed is certified in 25 t lots, and this volume of seed is thus well known to growers and to seed producers and merchants. Limiting the size of bulks for farm-saved seed sampling to about this weight would therefore appear to coincide with existing knowledge of seed bulk classification, as well as have experimental validity. Smaller bulks which may be typical of many farm-saved seed situations, could be sampled effectively and rapidly in practice. Growers using multi-chambered sticks could sample a medium sized bulk of around 25 t thoroughly by taking 10 or 15 "spearings" depending on chamber number and effectively creating 40 primary samples for mixing and dividing.

Appendix

Summary statistics for advisory samples tested for bunt from 1997-2002

	1997	*1998*	*1999*	*2000*	*2001*	*2002*
Mean	0.83	0.19	0.41	0.47	3.26	1.22
Median	0.21	0.00	0.00	0.00	0.00	0.00
Mode	0.00	0.00	0.00	0.00	0.00	0.00
Standard Error	0.22	0.05	0.21	0.28	2.86	0.79
Standard Deviation	2.75	0.62	2.90	5.53	57.74	18.84
Sample Variance	7.56	0.38	8.39	30.63	3334.01	355.11
Kurtosis	99.37	42.57	147.08	313.54	403.10	545.31
Skewness	9.29	6.05	11.63	17.24	20.03	23.15
Range	31.04	5.50	37.70	103.10	1162.50	445.00
Minimum	0.00	0.00	0.00	0.00	0.00	0.00
Maximum	31.04	5.50	37.70	103.10	1162.50	445.00
Count	150	182	191	384	407	568