PROJECT REPORT No. 204

OPTIMISING INPUT AND PERFORMANCE OF TRIAZOLE FUNGICIDES IN WINTER WHEAT BY MONITORING RESIDUAL ACTIVITY

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OPTIMISING INPUT AND PERFORMANCE OF TRIAZOLE FUNGICIDES IN WINTER WHEAT BY MONITORING RESIDUAL ACTIVITY

by

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This is the final report of a three year project which started in July 1995. The work was co-ordinated by Dr S J P Oxley, SAC Edinburgh in collaboration with Dr J Banks and Mr C Danks, Central Science Laboratory, York, Dr S Holmes and Miss Lynn Parker, ADGEN Ltd, Ayr, Mrs J Thomas, ADAS Cardiff, Dr N Paveley, ADAS High Mowthorpe, Dr D R Jones, ADAS Rosemaund and Dr N D Havis, SAC Edinburgh. The work was funded by a grant of £415,801 from HGCA (Project No. 1936).

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ABSTRACT

The aim of this research project was to develop a cost effective method of detecting fungicide levels in wheat plants, and to use the results to decide if there was sufficient fungicide to provide effective control of *Septoria tritici*.

Antibodies were developed which could detect the fungicide tebuconazole. A laboratory method was then produced which used these antibodies in an ELISA test to detect tebuconazole residues in plant sap. The laboratory method was compared with conventional (but expensive) analytical procedures, and they correlated well. The test was accurate in the range of 0.01 ppm up to 20 ppm, and this range corresponded to tebuconazole residues present in plant material which had an effect on the control of diseases.

Tests of the ELISA based laboratory kit, using samples from field experiments, demonstrated that:

i) the fungicide residue levels quantified by the test were consistent with the doses applied to the crop.

ii) decay of the active ingredient over time could be accurately measured.

iii) within an experiment, residue levels related well to the level of disease control obtained. However, some variation in the relationship was found when making comparisons between experiments carried out at different sites and different years.

A prototype kit was produced using the antibodies. During its development, consideration was given to the techniques and reagents used to ensure it would be robust enough to be used in a field situation. Results from the prototype field kit corresponded well to the laboratory kit.

SUMMARY

The aim of this research project was to develop a cost effective method of detecting fungicide levels in wheat plants, and to use the results to decide if there was sufficient fungicide to provide effective control of *Septoria tritici.*

An effective method of measuring triazole levels in wheat combined with an understanding of the effect of different residue levels on disease development would produce a number of benefits. A grower would be able to understand and monitor the persistence of fungicide within his crop. This in turn would allow a more accurate calculation of later fungicide inputs, in terms of number of sprays applied and dose rates used. There is a current trend away from prophylactic sprays and towards measured inputs in disease control. This approach has the added advantages of being beneficial in terms of cost, planning an anti-resistance strategy and protecting the environment from unnecessary pesticide sprays.

The fungicide group chosen for the project was the triazole group of fungicides, and the specific fungicide within the group was tebuconazole (trade name Folicur®). Triazole fungicides are systemic, broad spectrum fungicides which have eradicant and protective activity against a wide range of cereal fungal diseases. Despite the recent introduction of new fungicides (i.e. strobilurin fungicides), triazole fungicides remain a key part in disease control strategies in wheat, and they are used in combination with new fungicides as part of an anti resistance strategy and to widen the spectrum of control.

The project was carried out in a series of steps

- Develop a test to detect the fungicide tebuconazole
- Make sure the test only detects tebuconazole, and no other pesticide
- Compare the test with a sophisticated laboratory method
- Extract tebuconazole from plant material and quantify fungicide levels.
- Produce a method which is suitable for use in a field situation
- Develop a prototype kit which can be used in the field
- Interpret the test by linking test results to a specific fungicide dose
- Interpret the test by indicating if the fungicide dose detected in a leaf will provide effective disease control

Develop a test to detect the fungicide tebuconazole

Individual fungicide molecules are very small, so conventional methods used to produce an antibody test for larger organisms (i.e. fungi or viruses), were not suitable. It was necessary to make the fungicide molecule larger. This was achieved by attaching a large protein molecule to the small fungicide molecule using a spacer arm. This method produced a larger target to which antibodies could be produced. This technique was not successful because the antibodies produced could not recognise the

fungicide. It was assumed that the process of making the fungicide larger affected the immune response reaction.

Two other methods were tried. In the first, the large protein was attached directly to the fungicide without a spacer arm, and in the second, derivatives of tebuconazole were used instead of the tebuconazole molecule. Larger proteins could be added to the derivatives without affecting the active part of the fungicide. Production of antibodies using the derivative method was the most successful. A method was developed to allow the antibodies to be used in the laboratory (known as ELISA). The antibodies reacted well in the presence of the fungicide tebuconazole, and they provided different readings at different doses of fungicide. It had been calculated that the test had to be sensitive to tebuconazole at fungicide concentrations in the range of 0.1 parts per million (ppm) up to 10 ppm. Experiments showed that the antibodies used for the test were most active in the range of 0.01 ppm up to 100 ppm, and was therefore ideal for detecting the fungicide.

Make sure the test only detects tebuconazole, and no other pesticide

The next step was to see if the new antibody test reacted to other fungicides. Eight other triazole fungicides were tested at a range of concentrations from 0.01 ppm up to 1000 ppm. It was found that none of these fungicides could be detected at the lower concentrations, and for most a reaction only occurred at the very high concentrations of fungicide (1000 ppm). This was good news as it indicated that the test was specific to tebuconazole, and it would not give false readings if other fungicides were present.

Compare the test with a sophisticated laboratory method

The next step was to find out how sensitive the new test was at detecting tebuconazole from plant material, and how comparable the results were with existing laboratory methods.

Wheat plants were sprayed with a range of doses of tebuconazole from 1/64 of the field dose up to two times the field dose. After 24 hours, samples were harvested. Half of the samples were sent to a commercial laboratory, CEMAS, for conventional tebuconazole residue analysis using Gas Chromatography Mass Spectrometry (GCMS). The rest were stored at -20 °C and tested using the new test. The results correlated well, indicating that the new test could detect tebuconazole as well as the more expensive laboratory methods. Fortunately both methods showed that where higher doses of fungicides had been applied to plants, higher levels of fungicide could be detected.

Extract tebuconazole from plant material and quantify fungicide levels, and produce a method which is suitable for use in a field situation

During these steps of the project, the emphasis was to use techniques which could eventually be used for a field kit, but it was important that any extraction procedures were accurate. The fungicide was tested first in pure methanol (without any plant tissue). Later plants were treated with the fungicide, methanol was added, and the plant tissue was ground. Methanol appeared to provide the best results, but different solvents were tested, and found to be unsuitable. Different ratios of plant material to methanol were also tested to get the right balance of plant material to solvent.

Once dissolved in methanol, tests were carried out to determine the length of time required to extract the fungicide from the tissue. This was an important step, as it was important not to lose accuracy, but also make sure the test could be done in a reasonably short period of time.

It was discovered that the standards required to compare the test results must contain plant sap, as readings were different for tebuconazole alone compared to tebuconazole and plant sap. The different solvents gave poor results, and were also harsh on plastic plates, making them unsuitable for both a laboratory and a field kit. Tests using different concentrations of methanol showed that initial concentrations had to be high for the test to work properly. The time taken to carry out the test was critical, so it appeared that anyone taking short cuts in the procedures could affect the accuracy of the test.

Results using these modified methods were compared with the laboratory CEMAS method. There was a close correlation between the two.

Develop a prototype kit which can be used in the field

The aim was to make any kit as user friendly as possible. Lessons learnt from the modified extraction processes meant a protocol could be developed to allow anyone without any prior experience to carry out the test, but results also indicate that taking short cuts from the protocol could result in inaccurate results. It was also important to make sure that the kits had a reasonable shelf life, and did not require special storage conditions. All the reagents used in a field kit were performing well after long term storage, so it is anticipated that any test could have a shelf life of 12 months.

A field kit also has to be accurate, and tests showed that there was very little variation in the test results using a standard batch of plant material.

Determine variety differences and interpret the test by linking a result to a specific fungicide dose

Trials were carried out in the glasshouse and the field to produce plant material with different levels of fungicide. The working limit of the test using plant material from field crops was found to be between 20 parts per million down to 0.01 parts per million.

For most of the work, the variety Riband was used. It was important to see if the test worked with other varieties. The results of field trials showed that tebuconazole could be detected in all varieties suggesting that the test could be adapted for any wheat variety. There were however differences in the concentrations detected from field crops. This may be due more to differences in the canopy structure, which will affect the amount of fungicide which falls on specific leaves in the crop canopy.

Interpret the test by indicating if the fungicide dose detected in a leaf will provide effective disease control

Field trials were carried out in different seasons and at a range of sites for this part of the project. The assessments concentrated on the relationship between the dose applied, fungicide residues and the levell of disease control obtained. *Septoria tritici* was used as the test pathogen.

There were two main types of field trial. The first was aimed at measuring the effect of residues remaining from an early spray application on the appropriate dose for a later spray and tested the extent to which residue measurements might aid the choice of dose for the later spray. The second used sequential spray treatments to test how the residue data related to biological activity for (i) different doses applied at the same time, and (ii) the decline in active ingredient over time after application.

The data on disease development on the plants and residue levels in the plants were analysed to assess the precision and accuracy of the test, and relate the triazole test to disease control.

Tebuconazole residues in leaf two were measured using the test, and the results were analysed in a similar way to the disease data to produce Area Under Residue Progress Curves. (AURPC).

The graph below is taken from the same trial, but it describes the relationship between combinations of tebuconazole doses applied at GS 32 and GS 33, and the fungicide residue levels in leaf 2. Note the lowest residues are found where no fungicide was applied at GS32 and GS33, and the highest residues found where a double dose was applied at GS32 followed by a double dose at GS33.

The disease levels seen on the leaf were then linked to the residue levels detected in the leaf, and are demonstrated in the graph below. Note that at high disease levels (AUDPC), residue levels are low (AURPC). Alternatively at lower disease levels, residue levels are high. This graph, and others produced from the results of the

project were analysed statistically to demonstrate that there is a relationship between disease severity and fungicide residue.

Some of the observations from the field trials include the following:

Tebuconazole residues related logically to different fungicide doses applied.

Residues were detected in leaves even before their emergence. This suggests the fungicide moved into the leaf via the stem, or leaf sheath.

The half life of the fungicide (time taken for the dose to drop from full dose to half dose, or from half dose to a quarter dose) was calculated at different trials. The time taken varied from site to site, but was on average, 10 days.

Variation in residue from the early spray treatment had a relatively small effect on the appropriate dose for the later spray, probably because successive sprays control disease on different leaf layers within the crop canopy.

Although the general pattern of the relationship between dose applied and residue detected was similar for different experiments, the slope of the relationship was substantially different. These differences in slope may reflect variation in spray capture by the crop canopy, differences in the rate of decay of the fungicide, or inaccuracy in the ELISA test.

It would be unrealistic to expect a given residue level to relate to a given amount of disease severity, considering the potential for variation in disease pressure between experiments, but, as hoped, a given residue level gave a broadly similar proportional amount of control across sites and seasons.

**Folicur is a registered trade mark of Bayer AG*

1. PROJECT INTRODUCTION AND AIMS

Fungicides based on the active ingredients of the triazole group were first introduced in the 1970's and have been widely used on a range of crops since then. They are particularly important for the protection of autumn-sown cereals against major diseases such as *Septoria tritici,* brown rust and yellow rust.

Fungicides based on eleven different triazoles are marketed in the United Kingdom. New triazoles are still being developed and registered for the UK market. The costs of product development and registration are such that the newer products are likely to remain key components of the cereal fungicide market for at least the next ten years. Although agrochemical companies are actively seeking and introducing fungicides with novel modes of action (e.g. strobilurins), the triazoles are likely to be used in combination with these new active ingredients as part of an anti-resistance strategy. This in turn will help extend the useful lifespan of these new compounds.

In the past, farmers routinely applied sprays of fungicides to their cereal crops. However there has been a major shift away in recent years away from full manufacturers recommended doses and towards reduced-rate applications. This has been in some cases simply for economic reasons, but also because extensive field trial studies have clearly shown that well timed reduced-rate programmes can achieve disease control and yield responses which at least match those achieved with routine full-rate dose programmes.

The performance of reduced-rate programmes has been the subject of intensive research funded by HGCA. The ideal of applying the appropriate doses to meet the assessed risk situations is complex indeed. However it is further complicated by uncertainty about the period of protection provided by fungicides applied at reduced rates.

The triazoles are systemic in their activity. That is, when applied as a spray, the chemical is absorbed into the plant and moves upwards in the transpiration stream. This movement introduces a diluting effect, the rate of which may vary depending on cultivar, characteristics, climatic conditions and the rate of crop growth. With full rate treatments, the protection provided may be 4-6 weeks. With reduced rate treatments the period of protection could vary depending on the rate of fungicide used.

The primary mode of action for this group of fungicides (known as sterol biosynthesis inhibitors) is inhibition of sterol biosynthesis caused by binding of the fungicides to cytochrome P-450 leading to inhibition of the oxidative demethylation of the 14-methyl groups of the lanosterol level substrate.

The chosen triazole in this project is tebuconazole. Tebuconazole is a broad spectrum systemic foliar fungicide used to control diseases on a wide range of crops, most notably wheat, barley, oilseed rape, peanut and grasses grown for seed. It is marketed in a range of solid and liquid formulations. Trade names include Folicur, Raxil, Elite and Lynx. Tebuconazole is one of the newer triazoles of medium speed of uptake and

highly active against *Septoria tritici* and brown rust, which are the selected targeted pathogens.

Diagnostic tests based on specific antibody technology (immunoassays) are used widely in the medical and non medical areas. The basic principals of the test procedures were established some 20 years ago. They have been used for the detection of plant pathogens since around 1980. Kit formats for the detection of many plant pathogens are now commercially available. One of the most important features of these kits is their user - friendly nature.

Immunoassays for the detection of pesticide residues are a natural extension of the technology application and have been widely accepted in the agricultural, water and food industries. Standard methods of measuring tebuconazole involved expensive GCMS techniques. More recently a method using capillary chromatography has been developed (Harbin, 1997). Work in Italy has shown that antibody based tests can be developed for the detection and quantition of a triazole fungicide, tetraconazole in fruit and fruit juices (Cairoli *et al.*, 1996). The ELISA test developed was found to be sensitive down to 2 parts per billion (ppb). Immunoassays for the detection of three triazole fungicides, hexaconazole, myclobutanil and penconazole in apple leaf tissue have also been reported (Murray and Clark, 1996).

Aims

The main aim of this project is to develop an on-site test to determine the residual activity of tebuconazole against target pathogens in cereals.

The successful achievement of this objective would then lead onto the incorporation of the triazole test into appropriate fungicide dose programmes to optimise fungicide inputs, maintain maximum disease control and minimise unit cost of production.

2. ANTIBODY PRODUCTION

J. Banks and C. Danks (Central Science Laboratory, York)

2.1 INTRODUCTION

The antibody production phase of this project was carried out by CSL and it can be summarised in the following;

(i) Conjugation of several triazole analogues and production of polyclonal antibodies
(ii) Evaluation of antibody performance and preliminary formatting
(iii) Confirmation of tebuconazole specificity and cross reaction studies with other triazoles
(iv) Competition assay performance and sample extraction methods
(v) Comparison with GCMS samples

2.1.1 Conjugation of several triazole analogues and production of polyclonal antibodies

Pesticide molecules are not large enough to induce an immune response in animals. To stimulate this, the pesticide molecule must first be bound (conjugated) to a carrier protein which is large enough to elicit antibody production. Using this approach, two types of antibodies were produced, i.e. polyclonal and monoclonal.

To improve the potential for a good immune response, the analyte was attached to the carrier protein via a spacer arm (amino acid) which 'projected' the analyte away from the carrier molecule. This served to enhance the possibility of generating an immune response to the analyte which may be masked by the carrier molecule if it is too close.

Although a good immune response occurred (i.e. antibodies were produced), neither the polyclonal or the monoclonal antibody recognised the pesticide when evaluated in a standard inhibition assay.

There then followed an extensive evaluation of alternative conjugations. This was done in consultation with specialist chemists introduced to the project for this purpose.

None of the approaches proved satisfactory and it was concluded that the OH group on the pesticide targeted for conjugation with carrier is extremely inactive, most probably due to steric hindrance of the three large groups surrounding it.

Two different approaches were subsequently adopted.

Direct conjugation

Using what is known as the Mannichs reaction, it is possible to conjugate the analyte to the carrier without using a spacer arm. Four different carriers were conjugated and used to inoculate rabbits and mice.

The resulting rabbit polyclonal antibodies were evaluated. The indications were that tebuconazole-specific antibodies had been produced, and there was high binding of antibody to the fungicide tebuconazole. Encouragingly, negative rabbit sera and an antibody to the horticultural triazole, hexaconazole, gave only low reactions. Further, two of the monoclonal fusions resulted in tebuconazole-specific antibodies demonstrating the specificity of the tebuconazole antibody.

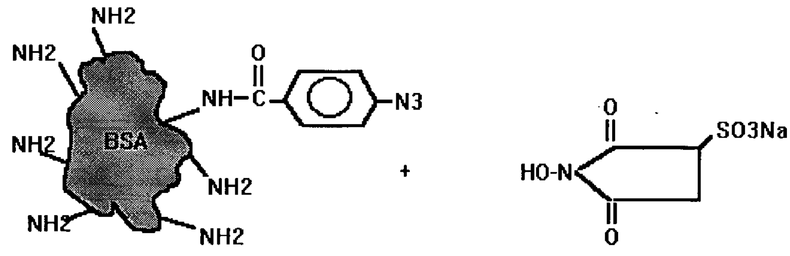
Derivatives

There are a number of derivatives of tebuconazole which have different molecular structures whilst retaining fungicide activity. Two of these derivatives were obtained in pure form from a commercial source. The selected derivatives do not cause steric hindrance. Thus the analyte can be attached to a carrier protein and retain presentation ideal for inducing an immune response. These derivatives were used in a third round of conjugations. Antibodies produced by this method detected the original tebuconazole molecule. They also detect derivatives thereby enhancing the sensitivity of the test, possibly beyond that of conventional analytical procedures.

The two derivatives obtained were conjugated as shown below:

Conjugation of OH Derivative with BSA using HSAB Linker

i:- Binding HSAB and BSA in the Dark



ii:- Binding Carrier to Pesticide irradiate with short wave UV light

NH2 NH2 O NH-C NH-CH2 CH2-CH2-C C-CH3 OH CH3 CH2 CH3 NH2 BSA NH2 NH2 NH2 N N N

Conjugation of NH Derivative with BSA
Using Succinyl Chloride Spacer

Figure 1. Conjugation methods of Tebuconazole derivatives

Conjugation Methods using the 'Bayer' Derivatives:-

- Conjugation of NH Derivative with BSA using Linker One
- Conjugation of OH Derivative with BSA using Linker Two

Although both derivatives are conjugated to the same protein, i.e. BSA (bovine serum albumen), due to it being easier to handle, the linker is distinct in each of the conjugations making them to a degree heterogeneous.

2.1.2 Evaluation of antibody performance and preliminary formatting

All of the available sera was tested, after appropriate boosting (supplementary tebuconazole injections) or finished immunisation schedules, against plates coated with the new derivative conjugates, one of the old conjugates and also BSA only. This constituted a large 60 plate indirect assay with a chequerboard titration of both conjugate and sera covering a total 88 possible coat/sera combinations for each sera.

The results obtained indicated Sera 59 and 62 produced the best response to the heterogeneous carrier, indicating better specificity. Both sera were chosen at two Coat/Sera concentrations and tested in an inhibition assay.

Before the inhibition assay was carried out both sera were tested for solvent effects using several common solvents and also the concentration of BSA required to cross absorb the sera. The conclusions being 20% Methanol and 2% BSA were the best.

Sera 59 tested at 1/8000 on 5ug/ml NH-BSA, 5ug/ml AI-BSA, and 5ug/ml BSA Only
Sera 62 tested at 1/8000 on 1ug/ml OH-BSA, 1ug/ml AI-BSA, and 1ug/ml BSA Only

The pesticide was diluted in 20% Methanol throughout from 1000ug/ml - 0.000001ug/ml.

The protocol used was as follows:-

1:- Pre-incubate sera for 1 hour at 4% BSA in PBST (Phosphate buffered Saline solution with added Tween 20) at 1/4000 (Double strength)

2:- Pesticide stocks added 50:50 with sera and incubated for 1 hour

3:- Samples added to the pre coated/ blocked plates with 8 replicates again 1 hour

4:- Add sheep anti- rabbit conjugate 1 hour

5:- TMB developed stopped after 15 mins

The results showed that for Sera 59 was inhibited by tebuconazole, see the figure below:-

Inhibition Curve for Tebuconazole using Sera 59

% Bo

100

50

0

0.000001 0.00001 0.0001 0.001 0.01 0.1 1 10 100 1000

Concentration of the Inhibitor ug/ml

Figure 2. Inhibition of sera 59 by tebuconazole

Sera 62 did show an inhibition response but was not as good.

The plates coated with a AI-BSA conjugate, treated the same way as the derivative conjugates, were not effective, again confirming that the active ingredient is not suitable for use in conjugates.

Even from this un-optimised format sera 59 gave a 50% inhibition of around 2-4 ppm (ug/ml) which was very promising for further development.

The OH conjugation was used for animal immunisations, and the NH conjugation is used for plate coating.

A competitive format was selected for the assay development. The basic methodology behind the assay is given in Appendix One.

The protocol adapted for the competition ELISA is detailed in Appendix Two

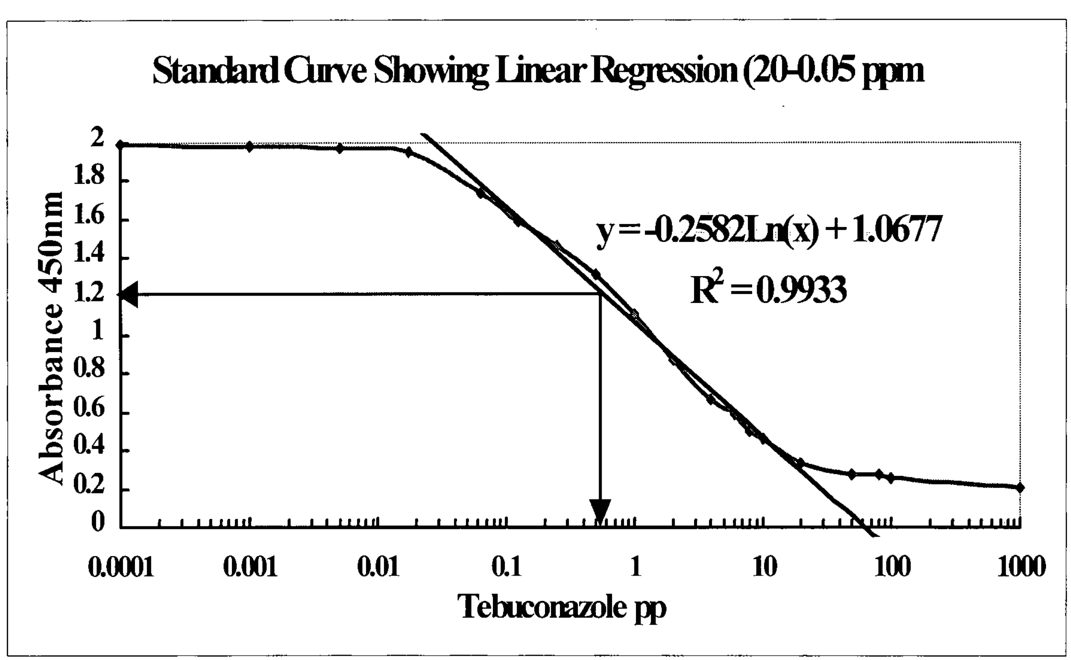
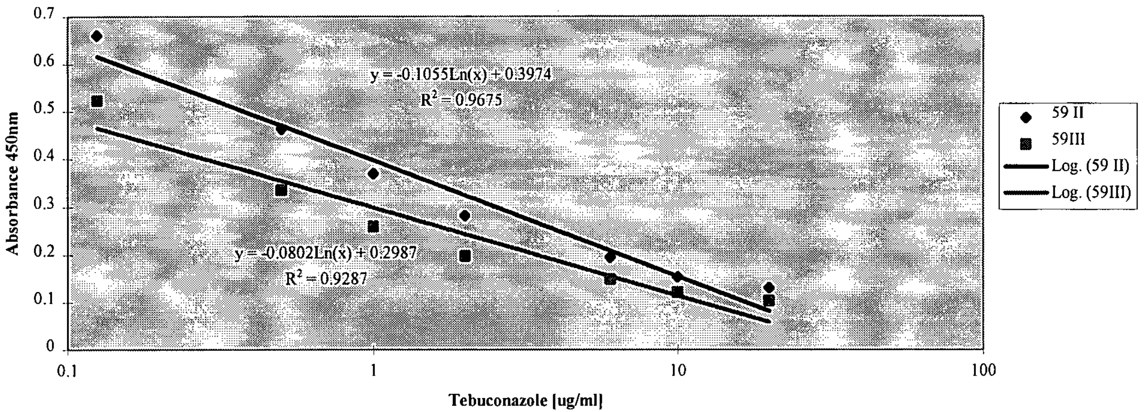
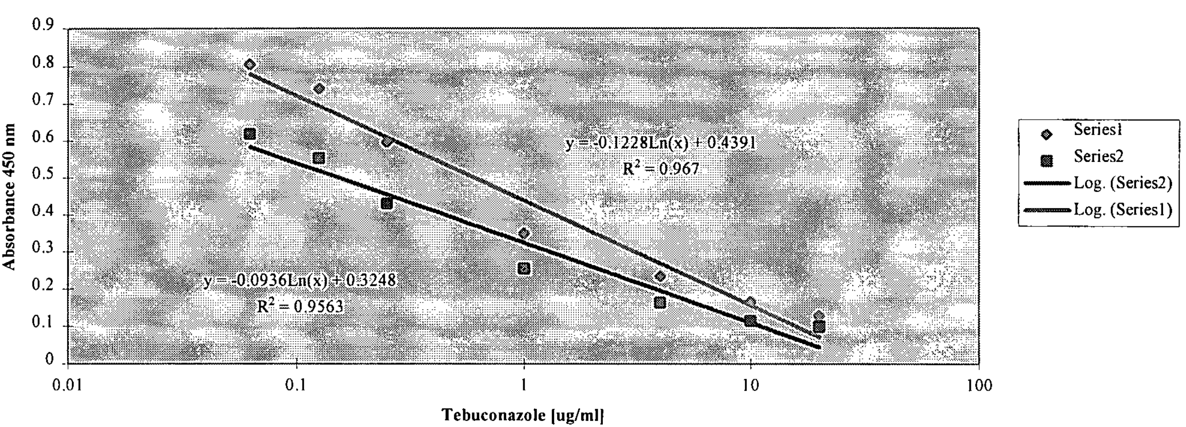


Figure 3. Standard curve showing linear range which could be used in analysis of samples

To obtain the standard curve as shown in Fig. 3, 24 individual standards were used with at least 12 covering the linear region. Obviously this would not be suitable in a 96 well plate format assay, so suitable combinations of 7 standards with a Methanol blank control were employed and compared. The results from three such 'sets' of standards are shown in Figure 4. Also shown in these results are two different sera samples. Sera bleeds are taken every month from Rabbit 59, it has been found that although sera 59II shows the greatest tebuconazole affinity subsequent monthly bleeds III, IV, and V are still performing within the same linear range.

Standard set three was found to give the best trendline fit with R^2 value of 0.9792. These standards have been tested in numerous assays (in excess of 30 individual plates on numerous different occasions and also by different operators) and although the overall absorbance figures can vary due to experimental fluctuations R^2 values of >0.9 have always been obtained. A typical example of the performance of such standards is given in Figure 4 later in this report.





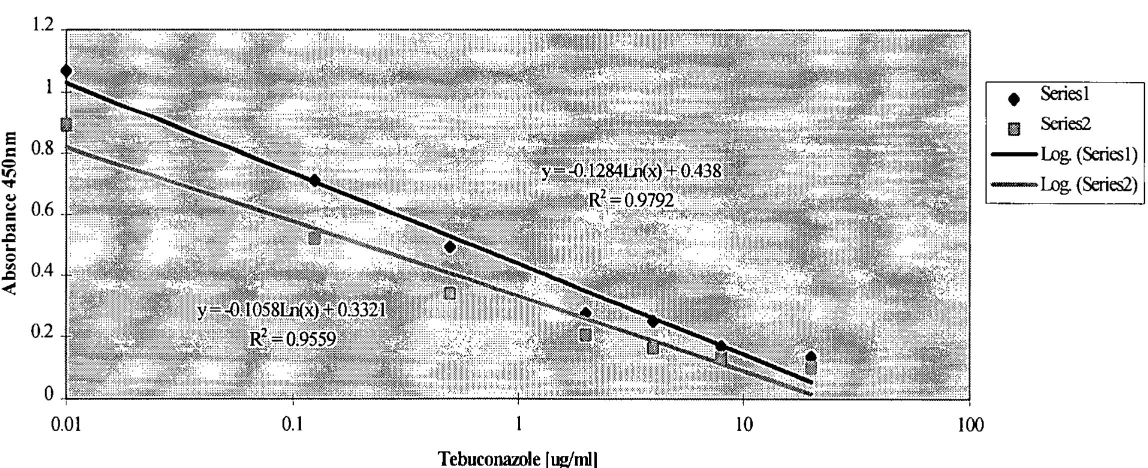


Figure 4. Standard curves generated by using a variety of sets of standard tebuconazole concentrations and sera.

2.1.3 Confirmation of tebuconazole specificity and cross reaction studies with other triazoles

The specificity of the antisera was investigated using cross reactivity studies with 8 other triazoles. Standards of all these triazoles were produced from 0.0001 ppm to 1000 ppm, it is difficult to produce standards above 1 mg/ml (1000 ppm) as the triazoles tested do not stay soluble when not in 100% solvent. At 40% Methanol all the triazoles compared were soluble at 1 mg/ml, except epoxiconazole.

The sera was found to be extremely specific towards tebuconazole and did not cross react significantly with any of the other triazoles tested, even those with similar structures. The results of which are shown in the table below and Figure 5. As levels in the plant after spraying appear to be approximately 1- 25 ppm (From CEMAS Data) there should be no problem with cross reactivity with other similar triazole compounds. However, breakdown derivatives of tebuconazole have not been tested although it is not apparent if these are chemically active.

Table 1. Cross Reactivity of Sera 59 Competition Assay Results

Pesticide	Structure	50 % Inhibition	Results
Tebuconazole	OH; Cl; $-CH_2-CH_2-C-C(CH_3)_3$; CH_2; N; N; N	1ppm-0.1ppm	1000 ppm 5% 100 ppm 7% 10 ppm 15% 1 ppm 47% 0.1 ppm 80% 0.01 ppm 97%
Triadimenol	OH; Cl; $-O-CH-CHC(CH_3)_3$; N; N; N	1000ppm - 100ppm	1000 ppm 27% 100 ppm 65% 10 ppm 84% 1 ppm 95 %
Prochloraz	Cl; $(CH_2)_2CH_3$; $CONCH_2CH_2O-$; Cl; N; Cl; N	1000 ppm	1000 ppm 50% 100 ppm 89 %
Penconazole	Cl; $-CH-(CH_2)_2CH_3$; CH_2; Cl; N; N; N	> 1000 ppm	1000 ppm 60% 100 ppm 92%

Myclobutanil		> 1000 ppm	1000 ppm 75% 100 ppm 96%
Propiconazole		> 1000 ppm	1000 ppm 79% 100 ppm 98%
Epoxiconazole		> 100 ppm *	100 ppm 83%
Hexaconazole		> 1000 ppm	1000 ppm 89% 100 ppm 96%
Flutriafol		> 1000 ppm	1000 ppm 91%

* not in 40% solvent

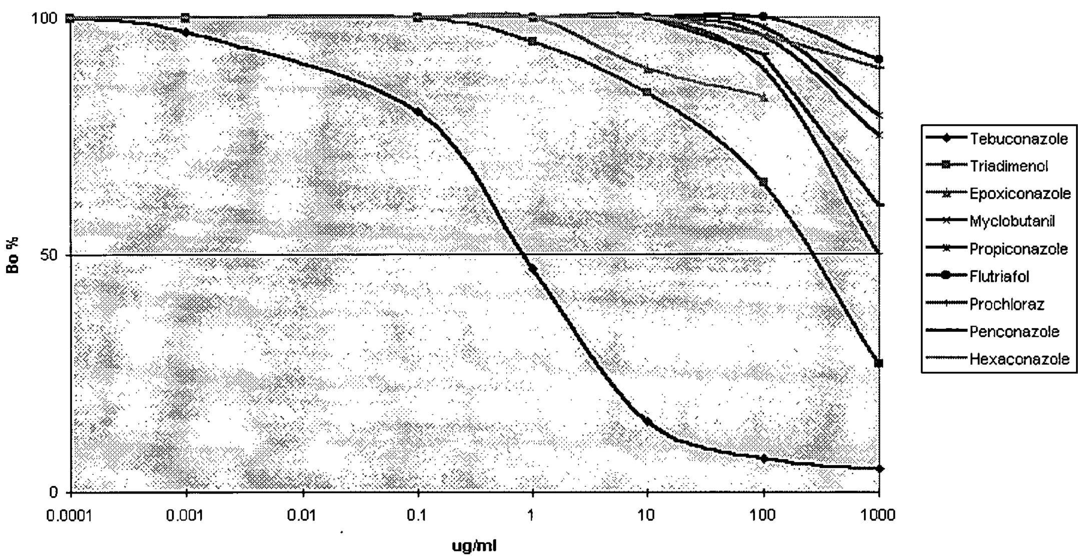


Figure 5 Cross reactivity studies with other triazoles

2.1.4 Competition assay performance and sample extraction methods

During the 1997 season small plots of winter wheat were sprayed with Tebuconazole at a range of concentrations. Concentrations ranged from two times the normal field rate to 1/64th of this rate. Bulk leaf samples were taken from these plots 24 hours later and then subdivided. 50 gram samples were sent off to a commercial company, CEM Analytical Services Ltd (CEMAS), for conventional tebuconazole residue analysis using GCMS (Gas Chromatography Mass Spectrometry). The remaining leaf samples were frozen at -20 °C. CEMAS results were then used in comparison studies with the antibody based assay.

The next part of the assay development was to establish sample extraction protocols. Simple grinding and methanol extraction methods were first investigated, using the samples which were originated for CEMAS analysis. 10g of leaf material was ground in 20mls Methanol then 30mls water added.

Initial results using this method looked promising, see figure 6, however when a number of samples were tested the extraction protocol appeared to be failing. This was shown by no significant correlation with the GCMS results, although absorbance did reduce with increased sample application. But, when the same sample were diluted to three different concentrations neat, 1:2, and 1:5 the absorbance figures did not correlate with this. During this time the overall assay was performing at lower A_{450} Maximums (1.0 c.f. 2.0) although the standards were giving R^2 values consistently over 0.95. In other words although the standard curves were still significant the Absorbance levels recorded on the plate were dropping.

Table 2. Absorbance values measured at 450 nm from dilution experiments on CEMAS extracts

Sample	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6
Methanol	0.870	0.753	0.819	0.753	0.790	0.829
20 ppm	0.186	0.132	0.186	0.128	0.133	0.156
8 ppm	0.189	0.183	0.167	0.180	0.170	0.186
4 ppm	0.214	0.171	0.191	0.206	0.182	0.255
2 ppm	0.219	0.187	0.221	0.241	0.221	0.246
0.5 ppm	0.296	0.291	0.312	0.301	0.320	0.323
0.125 ppm	0.431	0.392	0.447	0.468	0.426	0.502
0.01 ppm	0.687	0.602	0.673	0.693	0.676	0.667
Untreated N	0.715	0.576	0.706	0.558	0.577	0.696
Untreated 1/2	0.882	0.667	0.708	0.557	0.523	0.664
Untreated 1/5	0.855	0.766	0.761	0.614	0.610	0.725
64th Rate N	0.566	0.513	-	0.471	0.512	-
64th Rate 1/2	0.661	0.628	-	0.581	0.591	-
64th Rate 1/5	0.736	0.728	-	0.525	0.595	-
32nd Rate N	0.543	0.515	0.510	0.497	0.464	0.527
32nd Rate 1/2	0.692	0.606	0.588	0.577	0.562	0.578
32nd Rate 1/5	0.725	0.727	0.645	0.624	0.648	0.617
16th Rate N	0.559	0.483	-	0.529	0.511	-
16th Rate 1/2	0.581	0.553	-	0.498	0.505	-
16th Rate 1/5	0.665	0.655	-	0.557	0.573	-
8th Rate N	0.671	0.614	0.404	0.553	0.656	0.579
8th Rate 1/2	0.469	0.458	0.413	0.433	0.441	0.470
8th Rate 1/5	0.520	0.556	0.494	0.467	0.553	0.483
4th Rate N	0.455	0.500	-	0.361	0.412	-
4th Rate 1/2	0.436	0.529	-	0.416	0.471	-
4th Rate 1/5	0.495	0.594	-	0.424	0.496	-
Half Rate N	0.444	0.542	0.427	0.415	0.426	0.468
Half Rate 1/2	0.487	0.539	0.462	0.420	0.483	0.456
Half Rate 1/5	0.628	0.654	0.544	0.492	0.535	0.515
Normal N	0.448	-	0.460*	0.468	-	0.391
Normal 1/2	0.478	-	0.481*	0.458	-	0.428
Normal 1/5	0.510	-	0.554*	0.505	-	0.462
2xNormal N	-	0.261	0.332*	-	0.249	0.299
2xNormal 1/2	-	0.280	0.288*	-	0.270	0.262
2xNormal 1/5	-	0.396	0.342*	-	0.331	0.306

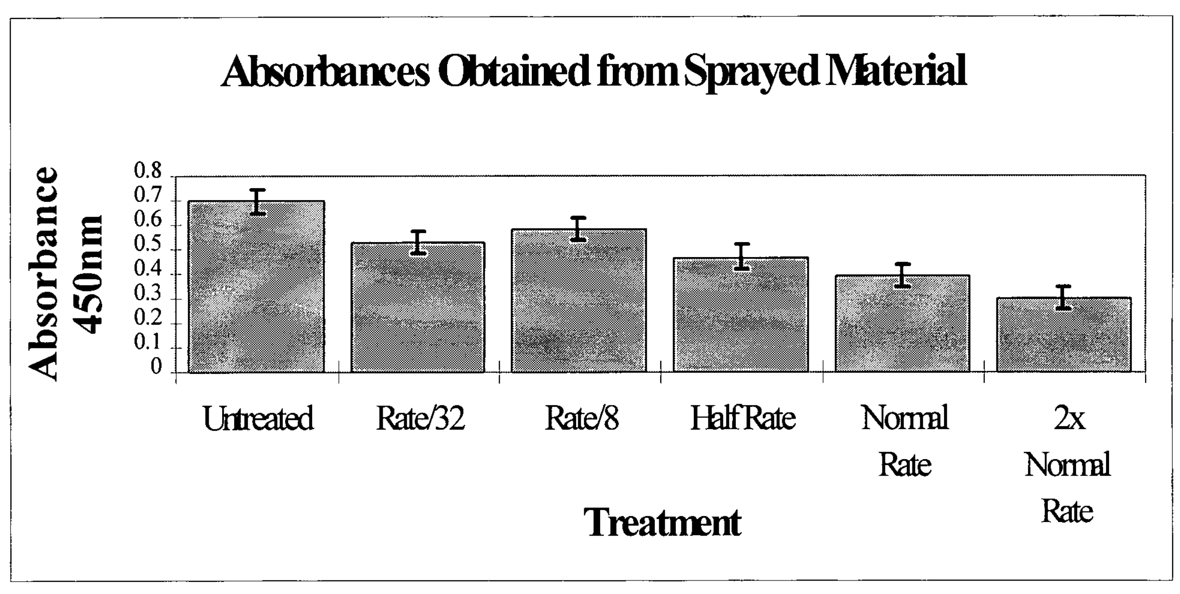


Figure 6. Initial analysis of field sprayed material using ELISA test.

2.1.5 Comparison with GCMS samples

The samples sent to CEMAS for GCMS analysis were extracted as outlined by Bayer, method published by W Maasfeld (1987). The protocol used is extremely labour intensive and the sample undergoes extensive clean up after extraction into dichloromethane. Aliquots of these extracts were sent to CSL. To enable these samples to be tested by the ELISA the solvent from each aliquot was passively evaporated, and re-suspended in 3 mls of 40% Methanol. Some of these samples were tested in the ELISA, figure 6 shows the Standards performance in this particular assay, figure 7 shows absorbance obtained related to spray application, and figure 8 correlates GCMS and ELISA data for exactly the same samples.

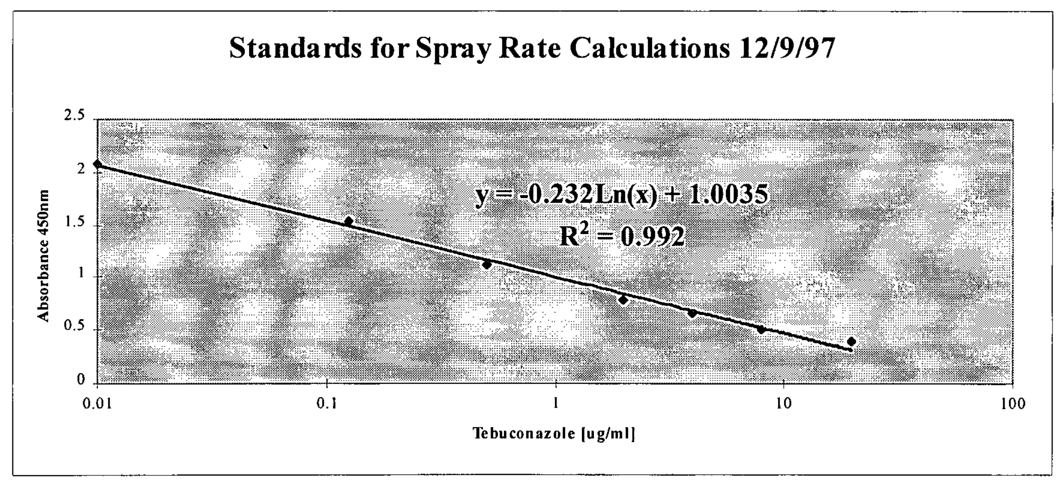


Figure 7. Performance of Standards in GCMS/ELISA comparison

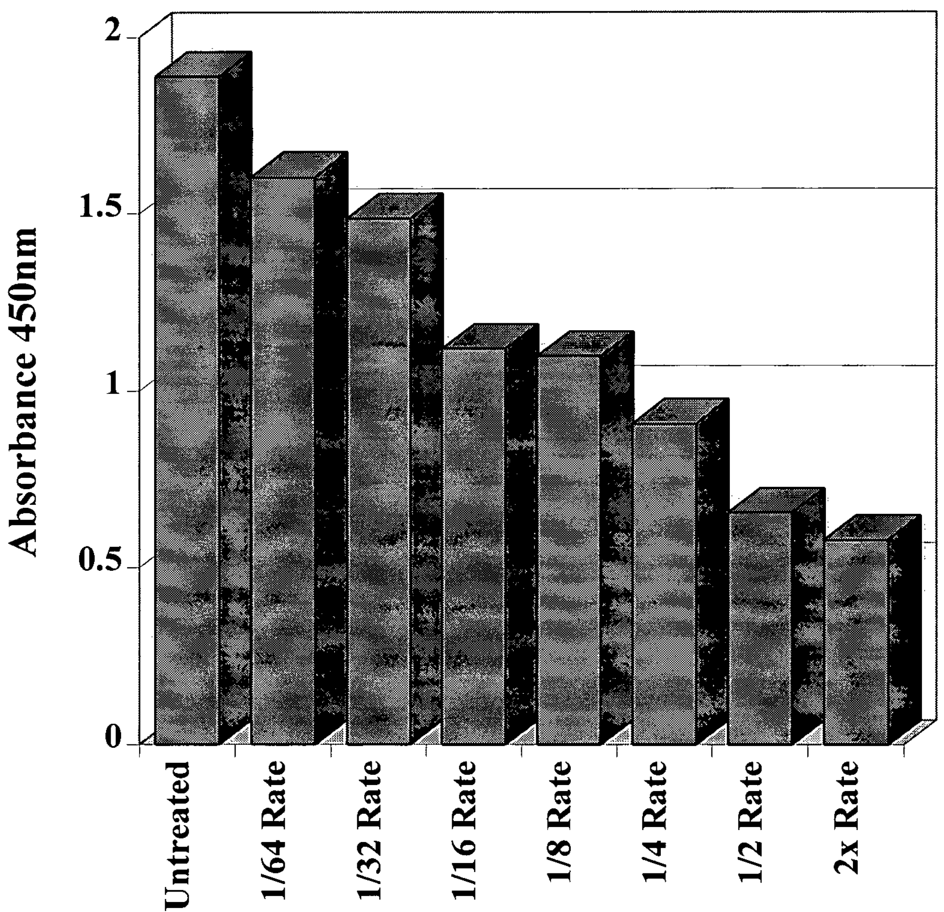


Figure 8. Quantification of tebuconazole residue from CEMAS samples using ELISA method.

The results show that as the spray application is increased the amount of tebuconazole detected in the leaf after 24 hours also increases, causing greater competition in solution and hence inhibiting/reducing binding to tebuconazole on the plate (A_{450}nm Untreated ~1.9, 2x Rate ~0.55).

It is shown that when the extraction procedure is efficient with good recoveries and extraction, the ELISA can be used to measure tebuconazole levels in the leaf. This can then be correlated to the activity of the fungicide in the field.

The results below in figure 9 show how well the data from the ELISA correlates with that obtained from the GCMS. Unfortunately we have been unable to quantify the value related to the absorbance figure, as we were not aware of the volumes and quantities used by CEMAS in preparing the extracts that we analysed. However, regardless of the actual amounts the ELISA and GCMS have a positive relationship.

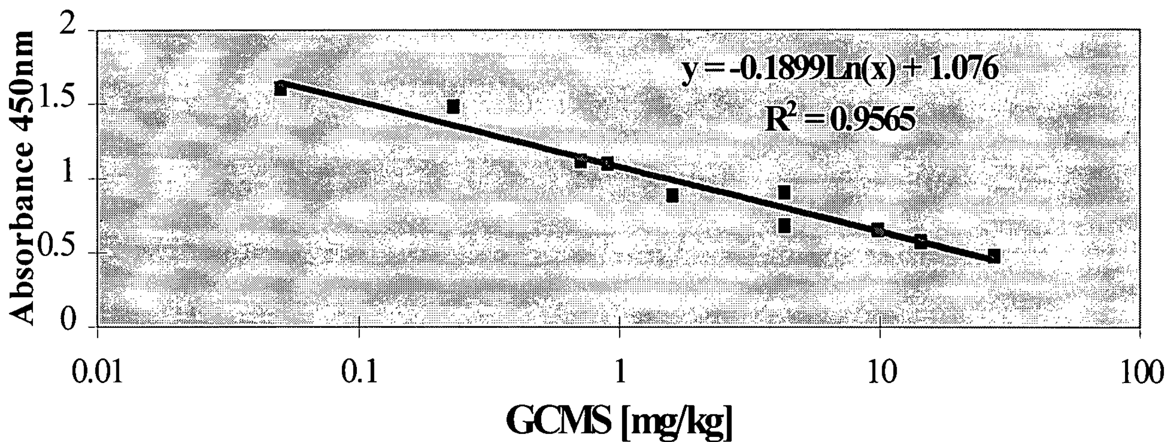


Figure 9. Comparison of tebuconazole residue analyses using GCMS and ELISA technique.

2.2 CONCLUSIONS

Antibodies have successfully been raised to the tebuconazole molecule by use of a derivative of the fungicide.

Antibodies are specific to tebuconazole and there were no cross reactions with other triazole and non triazole fungicides.

A competition assay format was selected as the best method to use in detecting tebuconazole.

The range at which the antibody shows maximum sensitivity was determined and found to be wide enough to encompass the expected range found in field conditions.

The assay has been shown to successfully detect tebuconazole in sprayed plant material.

The correlation between the ELISA and standard analytical systems was only significant when the appropriate extraction protocols were followed.

When these were adhered to the results compared very favourably and indicated that the ELISA format would allow for a large throughput of samples, with the only limiting factor being the extraction procedure.

3. ASSAY VALIDATION AND FIELD KIT DEVELOPMENT

S.J Holmes and L Parker (ADGEN Ltd, Ayr)

3.1 INTRODUCTION

The assay valiadtion and field kit development was carried out by Adgen and is summarised as follows:-

(i) Establishing the performance of the tebuconazole antibodies in a competitive enzyme linked immunosorbent assay (ELISA) format devised by CSL and using pure tebuconazole.

(ii) Develop and evaluate a system for the efficient and reproducible extraction of tebuconazole from plant material.

(iii) Establish a correlation between ELISA test results and GCMS analysis (conducted by CEMAS) of the same plant material using the methods developed in (i) and (ii) above.

(iv) Devise and validate an extraction and testing system for the quantitative/qualitative determination of tebuconazole extracted from plant material.

(v) Develop and validate a prototype test kit.

3.2 MATERIALS AND METHODS

3.2.1 Establishing the standard curve

(i) <u>Evaluating solvents for production of standard curves</u>

A range of solvents were tested for their efficiency in extracting tebuconazole from plant material and their compatibility with the assay system. The performance of the solvents (methanol, ethanol, dimethylsulphoxide and isopropanol) was ascertained by producing standard curves from a range of tebuconazole concentrations over a range of solvent dilutions. The tebuconazole concentrations ranged from 0.01 - 20 ppm and the solvent dilutions from neat to 40% (diluted in deionised water).

(ii) <u>Evaluation of anti-species conjugates for the tebuconazole assay</u>

While the most suitable solvent for use in the assay was being determined two different anti-species conjugates were tested. Anti-species conjugates are used in combination with a substrate to produce a measurable colour and allow quantitative and qualitative ELISA tests to be carried out. An anti-Rabbit IgG-Alkaline phosphatase (AP) and an anti-Rabbit IgG-Horseradish peroxidase (HRP) were used.

3.2.2 Physical extraction of tebuconazole from plant tissue

Several approaches were tried to determine the most efficient and reliable method for extraction of tebuconazole from plant material. The methods used were:-

(i) Homogenisation using a hand-held homogeniser, followed by immediate extraction into 40% methanol and filtration through a double layer of muslin.

(ii) Homogenisation followed by an overnight soak at 4^0C in 40% methanol then filtration through a double layer of muslin.

(iii) Homogenisation followed by an overnight soak in neat methanol which was diluted to 40% with the addition of deionised water to the extract before filtration through a double layer of muslin.

Various ratios of plant material and solvent were used.

3.2.3 Extraction of plant material into alternative solvents

The extraction method used for the GCMS analysis is too complex for the nature of the Triazole kit planned. However, solvents used for the GCMS were investigated. Tebuconazole can be solubilised in both acetone and toluene. Acetone is used in the initial extraction for GCMS and the end product is in toluene. However, both acetone and toluene are incompatible with microtitre plates and these volatile solvents would evaporate from the wells even in a dilute form. The extraction method used was as follows:-

The plant leaf material (1g) was homogenised in a heavy duty poly bag with either 4ml of acetone or 4ml of toluene. The bags were sealed and the samples incubated overnight at 4^0C. The samples were filtered through a double layer of muslin and allowed to evaporate to dryness (passive evaporation in a fume cupboard).

Residues were re-suspended in neat methanol and then diluted to 40% with deionised water prior to testing in the assay. Residues from acetone - extracted plant material were also re-suspended directly into 5% methanol.

3.2.4 Production of standard curves in lower concentrations of methanol

Although 40% methanol produces good standard curves when using 'pure' tebuconazole it may be interacting with the plant sap, which in turn could prevent the tebuconazole from being free for capture in the assay.

Tebuconazole standards were prepared from a 1mg/ml stock (in neat methanol) into 40, 20, 10, 5, 2.5, and 1.25% methanol solutions. These standard curves were tested using the standard competitive ELISA protocol determined by CSL. (See Appendix 1)

3.2.5 Extraction of plant material for testing in dilute methanol

Samples of plant material (1g) were homogenised and incubated in 4ml of 80% methanol overnight at 4^0C.

Deionised water (60 ml) was added to the extract and mixed well before filtration through a double layer of muslin. The final concentration of methanol in these samples was 5%. These extracts were tested using the standard protocol.

3.2.6 ELISA v GCMS analysis (CEMAS samples)

Sub- samples from the leaf material which had been sent to CEMAS for GCMS analysis were extracted as in **3.2.5** above and tested using the standard protocol.

3.2.7 Establishing reduced incubation times for the tebuconazole assay

Methods for reducing the time taken for the assay without losing any of the sensitivity were investigated.

First, we ran the normal ELISA in parallel with assays where the incubation times for antibody and conjugate were reduced both independently and together.

(i) Standard incubation protocol

The standard or sample was added to the plate with the cross adsorbed antibody and incubated for 2 hours at room temperature on a plate shaker (or at 37^0C without shaking).

The conjugate is added to the plate and incubated at 37^0C for 1 hour.

(i) Reduced incubation protocols

The two incubation steps described above were reduced to 30 minutes each. These reductions occurred both independently and together as follows:-

a. Antibody incubation time reduced to 30 minutes.
   Conjugate incubation time remains at 1 hour.

b. Antibody incubation time remains at 1 hour.
   Conjugate incubation time reduced to 30 minutes.

c. Antibody incubation time reduced to 30 minutes.
   Conjugate incubation time reduced to 30 minutes.

d. The conjugate and antibody/sample steps were combined and incubated for various times.

The time taken for an antibody-antigen interaction to reach equilibrium is dependent on the rate of diffusion. Thus, we ran the above reduced incubation protocols with the concentrations of antibody and conjugate used in the standard incubation protocol and with increased concentrations.

3.2.8 Development of a Field Kit

(i) Long-term storage of coated plates and reagents for a tebuconazole field kit

Batches of plates were coated with the tebuconaozle-BSA coating conjugate. These plates were subsequently blocked with one of two blocking/stabilising agents or left unblocked during storage.

A diluted, ready to use cross- adsorbed antibody was prepared as well as a concentrated solution at 5 times the normal strength. Batches of standards were prepared at working strength and also at 10 times normal working concentration.

Sufficient quantities of all of the above were prepared to ensure that they could be tested at various time points over a period of 12 months. When the reagents and coated plates were tested freshly coated plates and reagents were tested at the same time. All combinations of blocked plates and reagents were tested.

(ii) Method of filtration

An important part of developing the field kit for the detection of tebuconazole was to make it as user- friendly as possible. Different methods of filtering the extract after the overnight incubation were investigated. This included comparing the standard method of filtration (through a double layer of muslin) with two other commercially available methods as well as testing bags containing an integral filter.

(iii) Variation between extracted samples and strips for the field kit

Variation between microtitre well strips was determined by testing batches of strips with the same standards/samples. Strips are used as an alternative format for ELISA tests in more practical situations i.e. non laboratory. A small portable reader can then be used to measure absorbance at 450 nm.
Day to day variation was determined by testing the same extracted samples on different days.

3.3 RESULTS

3.3.1 Production of standard curves

Methanol, ethanol, dimethylsulphoxide and isopropanol were all used over a range of concentrations to produce standard curves with tebuconazole.

The neat and 80% solvents all caused problems with the assay. Some of them damaged the microtitre plates and all of them interfered with the immunochemical reaction.

The best standard curve, that is the curve that covered the largest range of O.D (Optical Density) values and produced a regression coefficient nearest to 1, was produced with 40% methanol and the anti-Rabbit IgG-HRP.

3.3.2 Physical extraction of tebuconazole from plant tissues

The initial results from the various extraction procedures looked promising, the ELISA O.D values seemed to be reducing with increased application of tebuconazole however, when dilutions of the extracts were tested the values obtained did not correlate. This suggests that there are plant components interfering in the assay. However, when a set a standards were spiked with untreated plant sap the standard curve obtained was comparable to the unspiked tebuconazole standards run on the same microtitre plate.

3.3.3 Samples extracted into acetone and toluene

Samples that had been resuspended in neat methanol and then diluted to 40% methanol gave similar ELISA values when the same extract was tested on different days. However, comparison of different extracts from the same batch of leaf material did not correlate.
Samples extracted in acetone and resuspended in 5% methanol gave extremely low values for all treatment groups. No difference between treatments was observed.

3.3.4 Standard curves in lower concentrations of methanol

The curves produced at all the concentrations of methanol were very similar. It is clear that lowering the concentration of methanol in the assay has no effect on the detection of tebuconazole which is able to stay in solution in 1.25% methanol.

From these results it seems clear that the problem when the samples were extracted in acetone and resuspended in 5% methanol is that although the tebuconazole stays in solution at 5% methanol it may not be able to go into solution at this level i.e. tebuconazole will have to be placed into solution in a higher concentration of methanol and then diluted down.

3.3.5 Testing plant extracts in dilute methanol

After obtaining the results of plant material extracted in acetone and resuspended in 5% methanol it was decided to return to extracting the samples in methanol but diluting the sample after extraction.

The concentration of methanol used was increased from the previous 40% to 80% in an attempt to improve the efficiency of extraction.
Separate extractions from the same treatment groups of plant material tested on the same day showed good correlation with treatments and good correlation between the extractions.

3.3.6 CEMAS Samples

A close relationship was found between the detection by ELISA and GCMS of tebuconazole in leaves treated with different amounts of the chemical (Fig 10).

Also, a close correlation was found between the values obtained by ELISA and GCMS analysis (Fig 11).

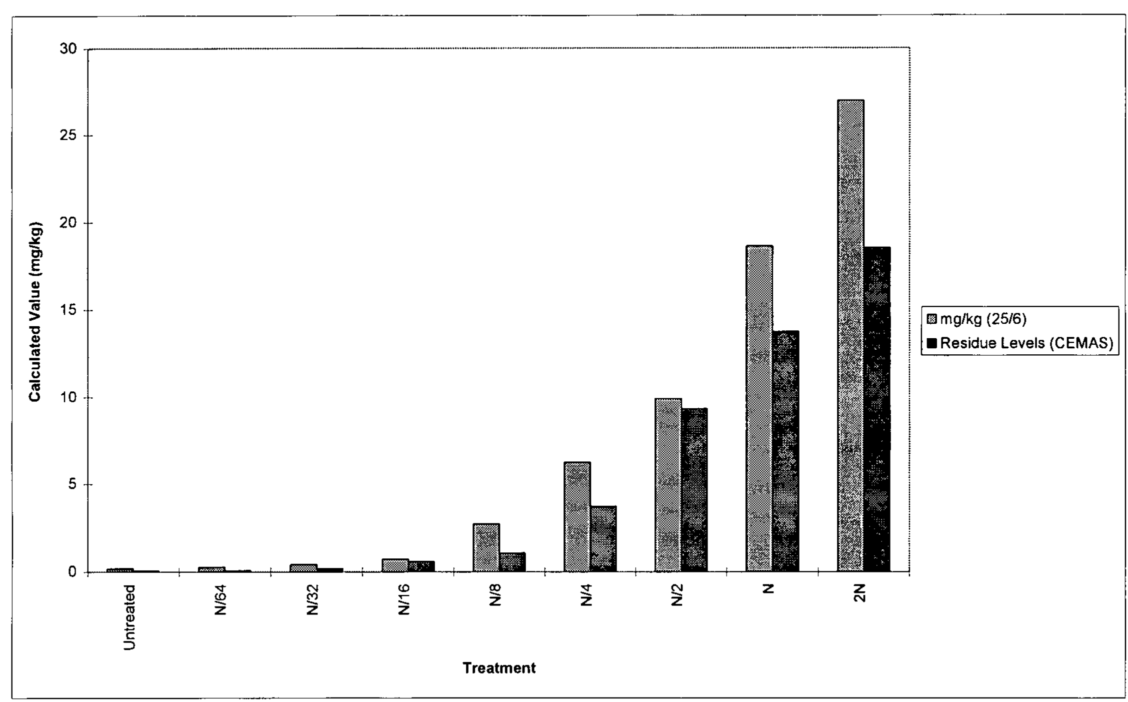


Figure 10. Comparison of values obtained by ELISA and by GCMS analysis of CEMAS samples.

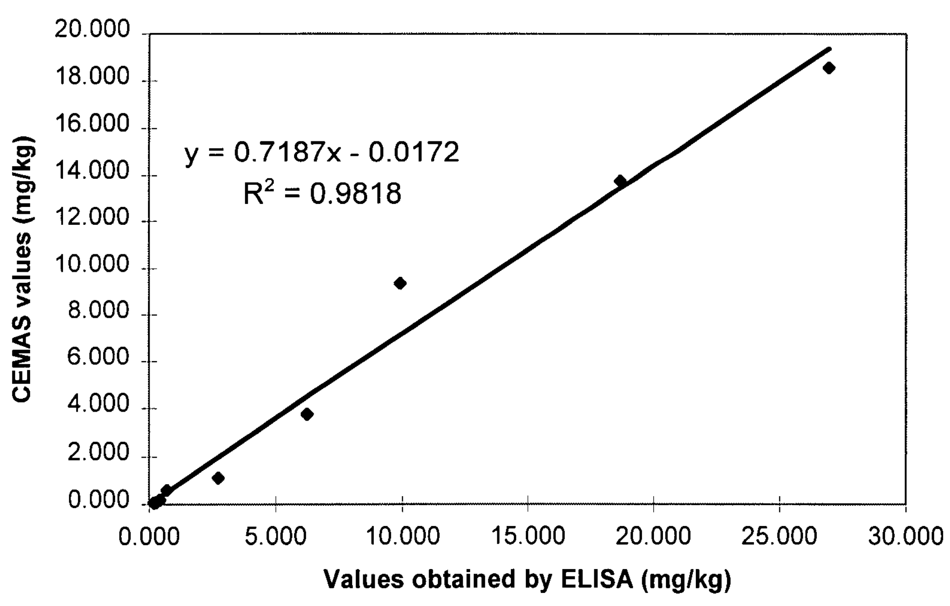


Figure 11. Correlation between the values obtained by ELISA and by CEMAS analysis.

3.3.7 Reducing the incubation times for the tebuconazole assay

Figures 12 and 13 show the curves obtained when the antibody and conjugate incubation times are reduced independently and within the same assay. These figures clearly demonstrate that there is a reduction in the gradient of the standard curve when the incubation times are reduced. A reduction in the gradient of the standard curve will lead to a reduction in the sensitivity of the assay.

In a competitive ELISA the amount of antibody is critical since if there is too much present both the coating antibody and the tebuconazole in the standard/sample will bind antibody and there will be no difference in O.D values irrespective of the amount of tebuconazole in the sample.

However, by titrating the antibody and conjugate over a range of dilutions we were able to overcome this potential problem and the loss of sensitivity by using increased amounts of antibody and conjugate.

Figure 14 shows that it was possible to produce standard curves under reduced time assay conditions which were comparable with the curve obtained using normal assay conditions. The dilutions normally used for the antibody and conjugate were 1/600 (purified antibody) and 1/8000 (anti-Rabbit IgG-HRP), the dilutions that gave the closest standard curve when the incubation times were reduced were 1/200 (antibody) and 1/6000 (conjugate).

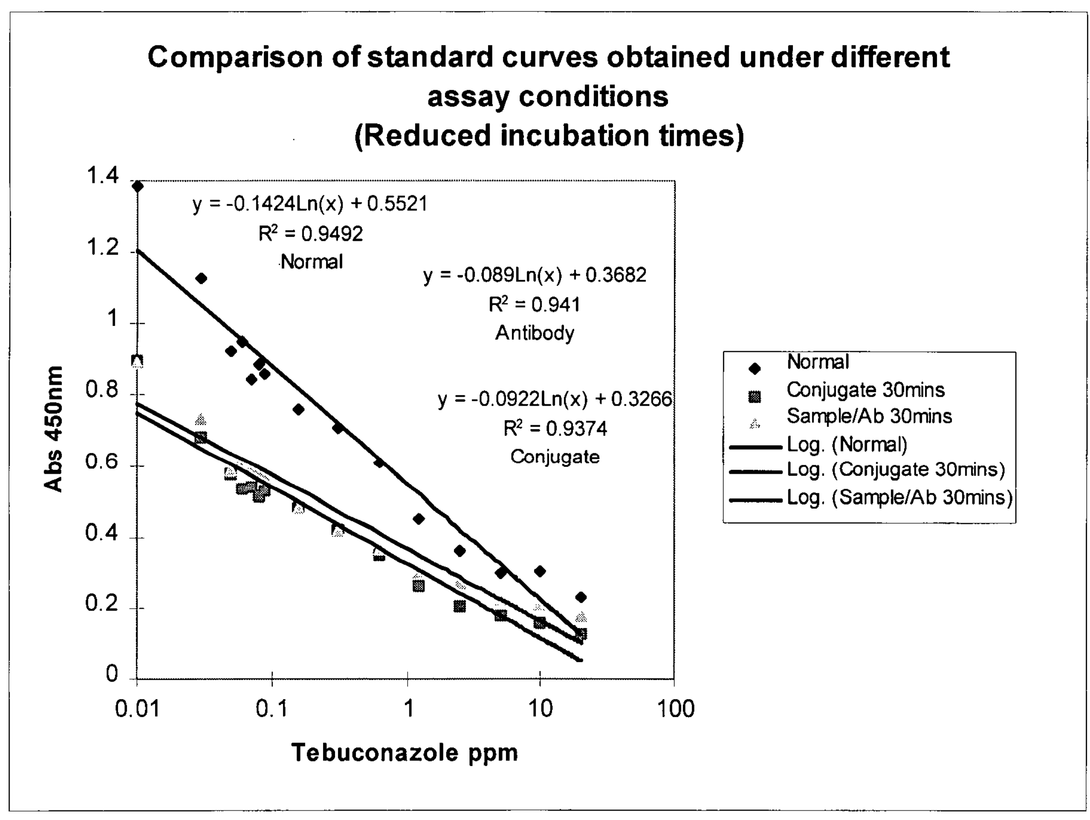


Figure 12. Standard curves obtained under normal and reduced incubation times.

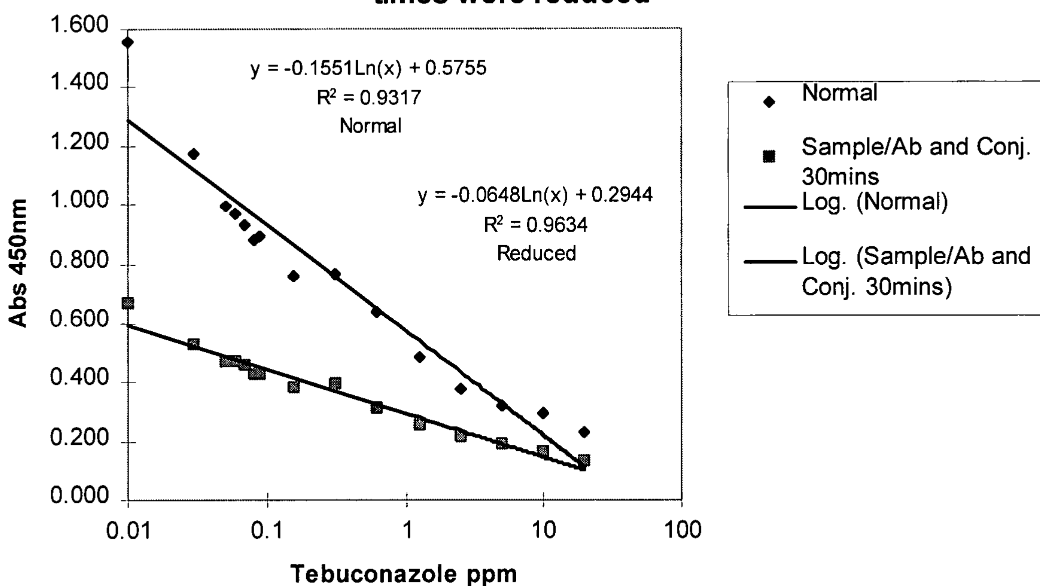


Figure 13. Standard curve produced when the incubation times for antibody and conjugate were reduced in the same assay.

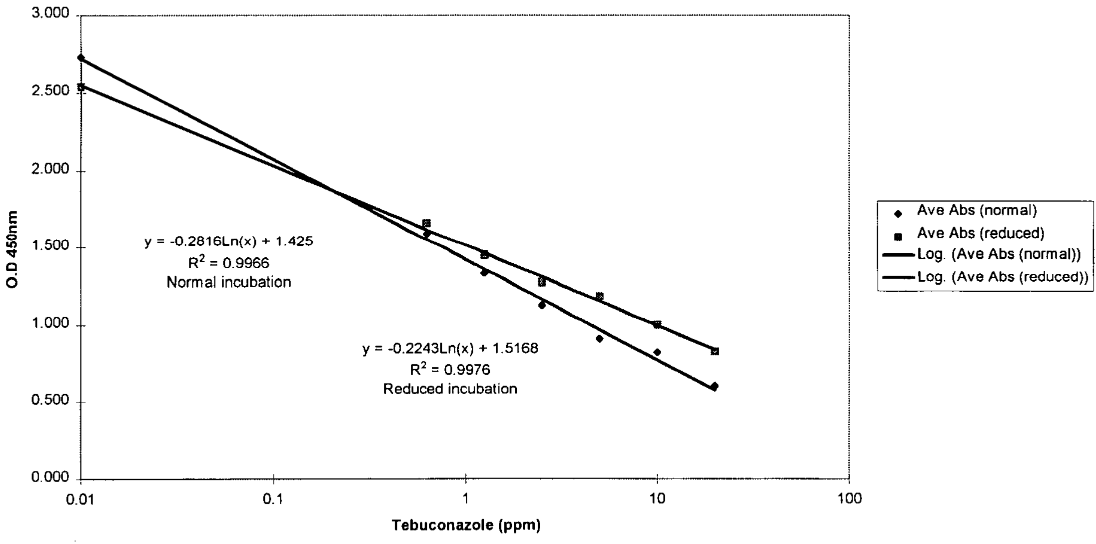


Figure 14. Comparison of standard curves produced under normal assay conditions and reduced time with increased antibody and conjugate concentrations.

The real test of the reduced incubation assay was its ability to detect tebuconazole in leaf samples. Samples of leaf material that had been sprayed with tebuconazole were tested in the 'standard' assay and the 'reduced' time assay. The material had been treated with 0, 0.0125, 1.25 or 125mg/l of tebuconazole. Table 3 shows the ability to detect tebuconazole was not reduced when the incubation times were reduced and the antibody and conjugate concentrations were increased to compensate. It is worth noting that the residue figures produced by the untreated plants are caused by the background reading on the ELISA plate.

Table 3. Comparison of values (mg/kg) obtained by testing the same extracts under both assay conditions (normal and reduced time)

Treatment (mg/l)	Standard assay (mg/kg)	Reduced time assay (mg/kg)
0	0.316	0.124
0.125	1.342	0.923
1.25	3.722	2.533
125	103.9	120.5

3.3.8 Development of a field kit

(i) Shelf - life

From the results obtained after six months storage of coated plates and reagents it indicated that the components of this kit were very stable. All of the plates and reagents at various stored concentrations appear to be performing as well as freshly prepared plates and reagents tested on the same day as the stored reagents. It is anticipated that the shelf – life of the kit will exceed 12 months.

(ii) Method of filtration

The two commercial filtration methods proved unsatisfactory, significantly reducing the amount of tebuconazole recovered (Table 4). However, when the standard filtration method was compared to extraction by maceration in polythene bags with an integral nylon filter, the values obtained were very similar (Table 5).

Table 4. Comparison of filtration methods on the level of tebuconazole detected by the assay.

Treatment (mg/l)	Muslin (mg/kg)	Filtration 1 (mg/kg)	Filtration 2 (mg/kg)
0	0.079	0.068	0.059
0.0125	2.065	0.843	0.250
1.25	3.421	0.720	0.360
125	91.346	29.339	10.075

Table 5. Comparison of the standard method of filtration and using bags with integral filters.

Treatment (mg/l)	Muslin (mg/kg)	'Filter' bags (mg/kg)
0	0.001	0.012
0.0125	0.043	0.071
1.25	0.507	0.736
125	97.30	127.5

(iii) Variation

The variation between strips was determined by testing the same standards across a range of strips. Day to day variation was determined by testing the same extracts over several days. The variation in sample extraction was determined by testing separate extracts of the same leaf material on the day of extraction and testing together on the same day.

Figure 15 clearly shows the reproducibility of the standards between individual strips when tested on the same day. The coefficient of variation between strips is very low.

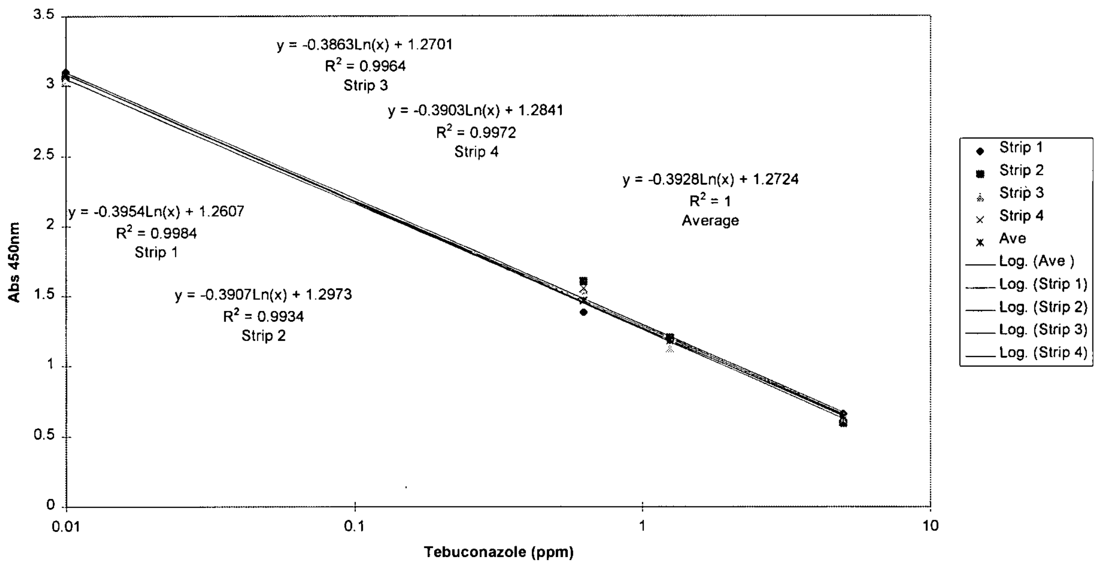


Figure 15. Comparison of standards on individual strips for use in the field kit.

Table 6 shows that the variation observed between different extracts of the same samples tested on the same day is also very low.

Table 6. Coefficients of variation for the same samples extracted on three days and tested on the same day.

Treatment (mg/l)	Average (mg/kg)	SD	%CV
0	0.135	0.048	35.9
0.0125	0.476	0.171	36.0
1.25	2.50	0.647	25.8
125	112.8	13.76	12.2

The variation seen when the same extract is tested on different days is also very low. This shows a stability of extracts over at least a three day period. This could be important when testing of extracted material is delayed or 'batching' of tests is more efficient

3.4 CONCLUSIONS

A sensitive and reproducible assay has been developed for the precise determination of tebuconazole in cereal leaves.

A simple extraction procedure that does not involve hazardous chemicals has been devised. This includes a cheap and easy to use leaf grinding, extraction and filtration system.

The sensitivity of the test is such that a quantitative test has been developed in addition to a qualitative one.

The quantitative test is designed for use by laboratories, it will also be of value to companies developing fungicides and for epidemiological studies. It is a powerful tool for incorporation into decision support systems. The quantitative kit is now available.

The qualitative test provides a yes or no (above or below a prescribed threshold). It could be used by consultants and farmers. This kit is in prototype format awaiting field evaluation.

4. BIOLOGICAL EVALUATION OF TEBUCONAZOLE AND ELISA TEST EVALUATION

N.D. Havis and S.J.P. Oxley (SAC), J. Thomas (ADAS, Cardiff), N. Paveley (ADAS, High Mowthorpe), D.R. Jones (ADAS, Rosemaund).

4.1 INTRODUCTION

There were a number of objectives set to relate the diagnostic to disease control;
i) validate the working protocol for the antibodies in the laboratory.
ii) produce an *in vivo* dose response curve for fungicide dose vs. antibody reading in the glasshouse.
iii) determine whether any varietal differences would affect the test
iv) relate antibody reading and fungicide dose to disease control in the field and also to compare antibody readings for known doses at different sites.
v) relate disease control in the field to final grain yields

4.2 MATERIALS AND METHODS

4.2.1 Validation of antibody protocol.

Some preliminary assays were carried out on the leaf samples obtained from the plots at 'Cereals 98' event to verify the protocol used at SAC Edinburgh. Plots of Winter wheat cv. Consort were sprayed at GS 31 with Folicur (250g/l tebuconazole). Treatments were as follows; Plot 1 0.25 l/ha, Plot 2 0.5 l/ha, Plot 3 0.5 l/ha followed by simulated rainfall and Plot 4 1.0 l/ha. Simulated rainfall treatment consisted of another pass of the sprayer with a tank of clean water. Leaf samples were taken from the plots 24 hours after treatment and stored at -20 °C.
Folicur is formulated as an oil in water emulsion commercial and contains 250 g of tebuconazole per litre. Normal rate (manufacturers recommended rate) of Folicur is 1 l/ha. It is usually applied in 200 litres of water per hectare giving an application rate of 1,250 mg tebuconazole per litre.

4.2.2 Production of the *in vivo* dose response curve under glasshouse conditions.

Wheat seeds (cv. Riband) were sown in compost in 5 inch pots and placed on an isolation plant propagator in a glasshouse. Ten seeds were sown per pot and thinned out to 7 plants per pot after emergence. Plants were grown under an environmental regime of 16 hour daylengths and a mean glasshouse temperature of 21 °C. At GS 13 plants were sprayed with tebuconazole at a range of concentrations to maximum leaf retention using the method of Shephard (1987). Leaf samples were harvested 24 hours later and frozen in black plastic bags at -20 °C until assays were performed using the test protocol. One modification made to the protocol at SAC Edinburgh was to use plastic bags containing integral nylon filters during leaf homogenisation in order to speed up the throughput of samples.
This method of producing sprayed leaf material was also used when preparing samples assay development work as detailed in Chapters 2 and 3.

4.2.3 Effect of variety on ELISA test.

Field trials were carried out on winter wheat at SAC Edinburgh in the 1996 growing season

using seven different varieties. A number of tebuconazole treatments were applied to the varieties. The treatments looked at the effects of timing and dose rate on control of *S. tritici*. Flag leaf samples were harvested after a spray at GS 47. Results from the ELISA analysis are presented in the following figure. A full description of the trial site and treatments used in given in Appendix 3.

4.2.4 Establishing the relationship between antibody reading, fungicide dose and disease control observed in the field and compare antibody readings for known doses at different sites.

Two field trial experiments were designed to study this relationship in 1997.

Experiment One: The primary aim of this trial was to establish the effect of earlier treatments on variation in disease control achieved by later treatments. The secondary aim was to obtain further information on the response of *S. tritici* to various fungicide doses. Leaf F2 (leaf directly below the flag leaf) was chosen as the target leaf. Tebuconazole treatments are summarised in the following table.

Treatment No	Dose at first spray (GS 32) (l/ha)	Dose at second spray (GS33) (l/ha)
1	0	0
2	0	0.25
3	0,	0.5
4	0	1.0
5	0	2.0
6	0.25	0
7	0.25	0.25
8	0.25	0.5
9	0.25	1.0
10	0.25	2.0
11	0.5	0
12	0.5	0.25
13	0.5	0.5
14	0.5	1.0
15	0.5	2.0
16	1.0	0
17	1.0	0.25
18	1.0	0.5
19	1.0	1.0
20	1.0	2.0
21	2.0	0
22	2.0	0.25
23	2.0	0.5
24	2.0	1.0
25	2.0	2.0

Table 7. Factorial trial design used at ADAS Rosemaund and SAC Edinburgh

Leaves were harvested at weekly intervals from the first spray date and *Septoria tritici* infection assessed visually. Twenty five leaves were taken from each plot at each assessment date for ELISA analysis. Ten leaves were used for each disease assessment. Samples were frozen for later ELISA analysis at -20 °C.

This trial was carried out at SAC Edinburgh and ADAS Rosemaund in 1997 and 1998. Details of sites used and other treatments applied to the crop can be found in Appendix 4.

Experiment Two: The primary aim of this trial was to discover the effect of sequential fungicide application on *S. tritici* development. In order to do this a series of full dose rate tebuconazole sprays were applied at weekly intervals.
The secondary aim of this trial was to measure triazole levels in the target leaf layer and relate this to the levels of disease control observed. As in experiment one, leaf F2 was chosen as the target leaf in the plant.
Twenty five leaves were taken from each plot 24 hours after spraying and then weekly intervals for ELISA analysis. Ten leaves were used for visual disease assessment. Samples were frozen for later ELISA analysis.
Early samples required dissection of leaf F2. This allowed measurement of tebuconazole levels in developing leaf tissue to be carried out. Sampling continued until leaf senescence. The site used was the same as for Experiment One in both 1997 and 1998. Further details are given in Appendix 4.

The full range of treatments are detailed in Table 8.

Treatment No.	Spray timing
1	4 weeks pre emergence of Leaf F2
2	3 weeks pre emergence of Leaf F2
3	2 weeks pre emergence of Leaf F2
4	1 week pre emergence of Leaf F2
5	leaf emergence of Leaf F2
6	1 week post emergence of Leaf F2
7	2 weeks post emergence of Leaf F2
8	3 weeks post emergence of Leaf F2
9	4 weeks post emergence of Leaf F2
10	Untreated

Table 8. Sequential treatments used in Experiment Two at SAC Edinburgh

4.2.5 Relating disease control in the field to final grain yields

Experiment One was taken to harvest at ADAS in both seasons and at SAC Edinburgh in 1998.
In addition experiment two was harvested at SAC Edinburgh in both years.
Yields were adjusted to 15 % moisture content to allow comparisons to be made.

4.3 RESULTS

4.3.1 Validation of protocol.

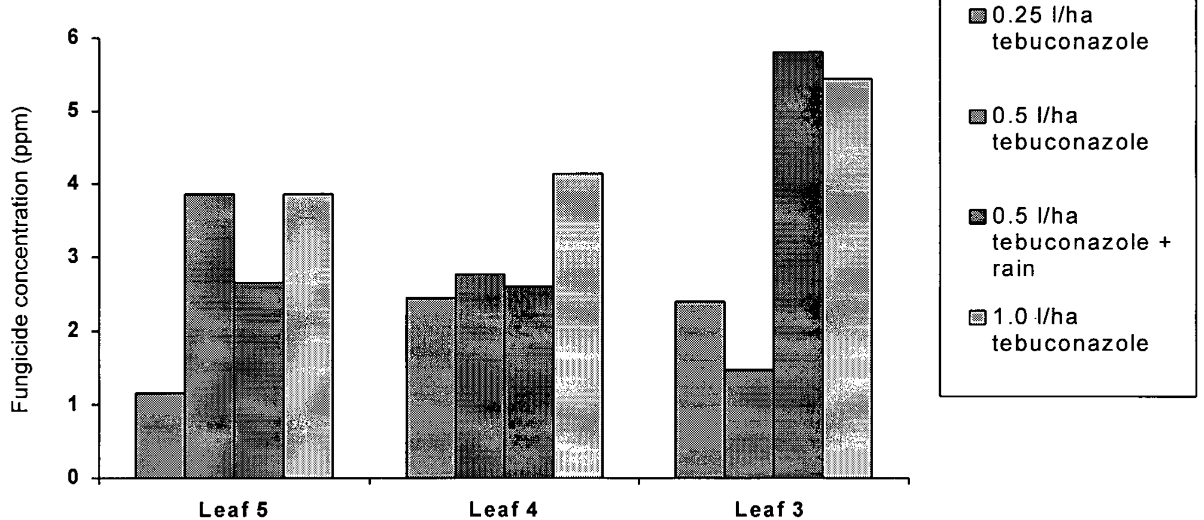


Figure 16. Tebuconazole detected in different leaf layers after 4 spray treatments

The results obtained indicated that the test was able to recognise tebuconazole in field sprayed samples and also to distinguish between different dose rates. The simulated rainfall treatment appeared to increase the amount of tebuconazole into the developing leaf (leaf 3). This could be the result of improved leaf cover by the spray or faster translocation within the plant. Standard curves were generated on ELISA plates using known concentrations of tebuconazole. Curves produced had Regression coefficient = 0.97

4.3.2 Production of *in vivo* dose response curve under glasshouse conditions.

A range of concentrations were sprayed and results expressed graphically. Results indicated that the test was sensitive enough to detect changes in fungicide dose applied to plants under laboratory conditions. A standard curve was produced, although a loss of sensitivity at lower concentrations lead to an R-squared value of 0.873 only. This loss of sensitivity reflected the sigmoidal shape of the original response curve for antibody and tebuconazole produced by CSL (Figure 2). This indicated an optimal range for detection of the fungicide of 20 to 0.01 parts per million. (ppm)

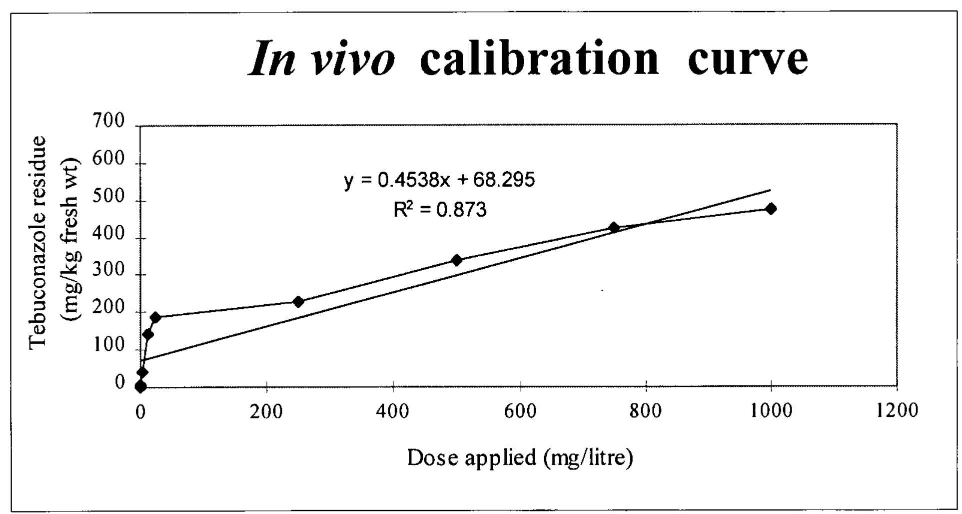


Figure 17. In vivo calibration curve produced with glasshouse sprayed leaf material.

4.3.3 Effect of variety on ELISA test.

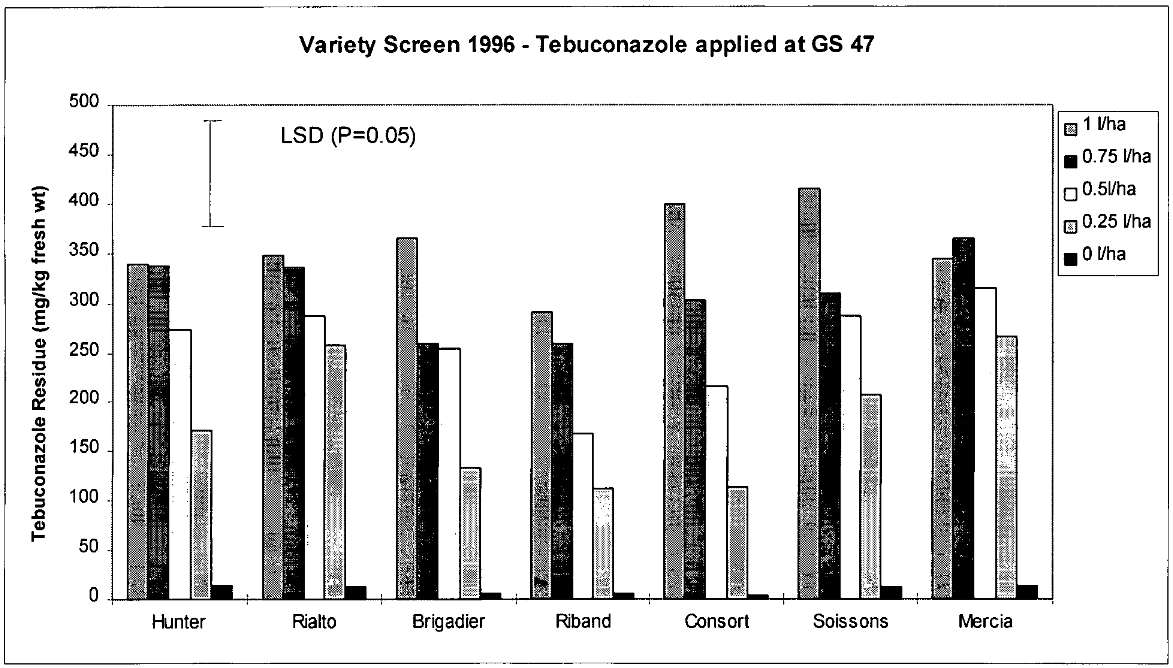


Figure 18. Tebuconazole detected in flag leaf of seven varieties of wheat 24 hours after application.

The results indicate that the ELISA test was capable of detecting tebuconazole in a number of different varieties and that there should be no varietal interaction to hinder the future development of the test. The observed differences were statistically significant. The differences which were observed probably reflect the differences in canopy structure between the varieties and the subsequent effect this will have on spray capture.

4.3.4 Establishing the relationship between antibody reading, fungicide dose and disease control observed in the field and compare antibody readings for known doses at different sites.

Experiment One

Data from Experiment One at both sites and over two seasons was analysed and reported in this section by Jan Thomas at ADAS Cardiff. Graphs showing the detailed disease development and tebuconazole residues found at SAC Edinburgh in 1997 are presented in Appendix 5.
The aim of the analysis was to define the relationship between the ELISA assay results and the epidemic development in order to (i) test the precision and accuracy of the assay, and (ii) relate the assay results to disease control

Experiment One : Epidemic development

At SAC Edinburgh and ADAS Rosemaund in 1997 and 1998, measurements of *Septoria tritici* as percentage area of eventual leaf two were recorded at 7 day intervals from the time of the first fungicide application until the crop was senescent. The percentage *S.tritici* measurements at each assessment date were subjected to analysis of variance, and normal plots, histograms and plots of residuals were used to assess the assumption of normality.

Plots of the percentage *S. tritici* over time were made for each treatment, and the area under the disease progress curve (AUDPC) calculated in Genstat by numerical integration using the trapezoidal rule (Fig. 19). The AUDPC values provide a measure of the damage caused by the disease integrated over the life of the leaf. AUDPC values were also subjected to analysis of variance.

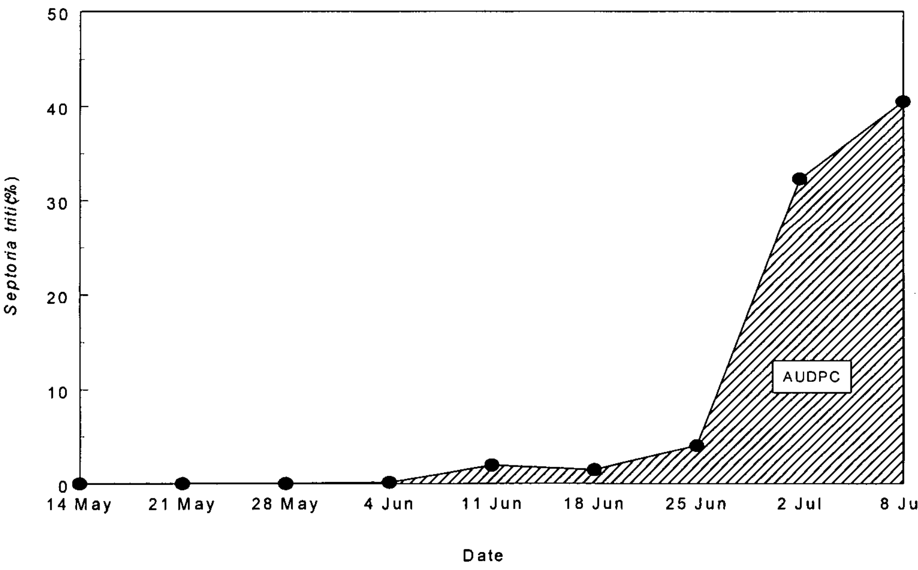


Figure 19. *Septoria tritici* epidemic development over time. The shaded area is the area under the disease progress curve (AUDPC). Data from ADAS Rosemaund 1997.

In order to examine the response of AUDPCs to different doses of fungicide, plots of AUDPC on dose applied at GS 33 were made for each dose applied at GS 32. Mathematical descriptions of these dose-response curves were then obtained by fitting the exponential function
$AUDPC = a+be^{k.dose}$ (Fig. 20)

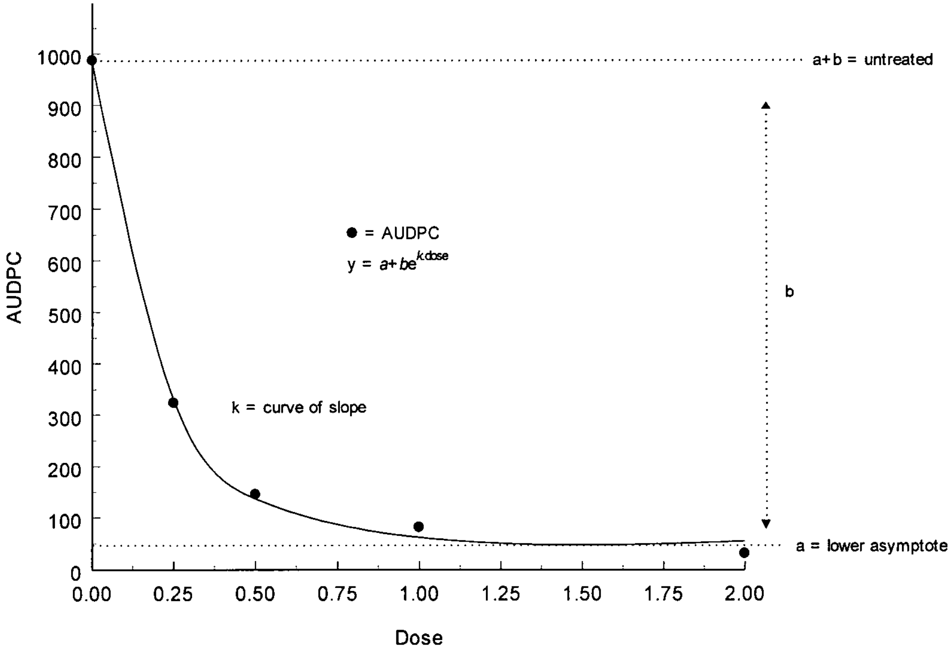


Figure 20. Example of dose-response curve and fitted exponential curve.

Parameter a is the lower asymptote which represents the lowest level of disease achievable. Parameter b is the difference between the untreated AUDPC and the lower asymptote, which represents the range of disease control which could potentially be achieved by the fungicide. Parameter k is a measure of the curvature of the slope. More negative values of k are associated with greater curvature which indicates high dose efficiency. An example of the effects of different doses of early sprays on the GS 33 dose-response curve is shown in Fig. 21 and parameter estimates for the fitted curves in Table 9.

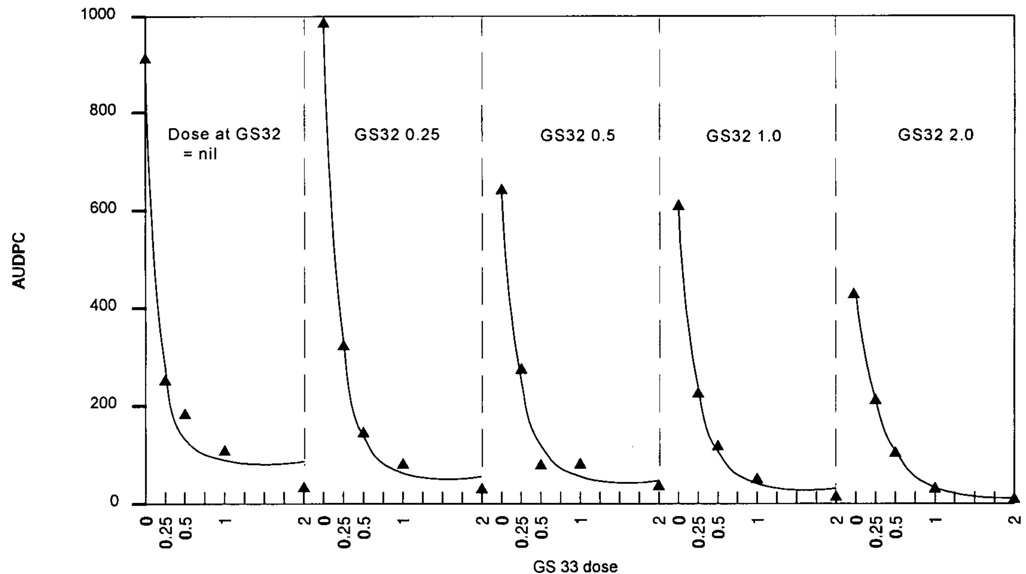


Figure 21. ADAS Rosemaund 1997: exponential curves fitted to AUDPC dose-response curves for leaf 2 for tebuconazole applied at GS 33, following differing doses applied at GS 32.

Dose applied at GS 32	*a*	*b*	*k*	F.prob.	R^2
0	80	691	-4.9	0.0013	0.99
0.25	47	744	-3.9	0.002	1.0
0.5	46	601	-4.2	0.011	1.0
1	31	577	-4.1	0.003	0.99
2	11	420	-2.9	0.001	1.0

Table 9. ADAS Rosemaund 1997: Parameter estimates for exponential fits to AUDPC dose-response curves for leaf 2 for tebuconazole applied at GS 33. following differing doses applied at GS 32.

Fitting curves in this way provided a set of parameters for each individual GS 32 and GS 33 dose-response curve, and in order to simplify this, a single set of parameters which describe the same data was required.

This was achieved by fitting a three-dimensional surface to the AUDPC values using the function AUDPC = $a + be^{k_{32}\text{dose}_{32} + k_{33}\text{dose}_{33}}$ which fits the data for both the GS 32 dose-response curves and the GS 33 dose-response curves simultaneously.

Parameter a is the lower asymptote which represents the lowest level of disease severity achievable.

Parameter b is the difference between the lower asymptote and the untreated AUDPC, and is an indicator of the potential control given by the fungicide.

Parameter k_{32} represents the degree of curvature of the surface given by the GS 32 dose-response, and k_{33} the curvature of the surface given by the GS 33 dose-response.

Experiment One : ELISA assay results

Analysis of variance of Rosemaund 1997 and SAC Edinburgh 1998 AUDPC data showed that only the late (GS 33) spray applications gave a significant reduction in disease severity. Analysis of residues focused on SAC Edinburgh 1997 and ADAS Rosemaund 1998. Plant samples taken from each plot at all assessment dates were analysed for tebuconazole residue. The residue values at each assessment date were subjected to analysis of variance, and normal plots, histograms and plots of residuals were used to assess the assumption of normality .

Plots of the tebuconazole residues over time were made for each treatment, and the area under the residue progress curves (AURPC) were calculated using integration by the trapezoidal rule. The AURPC values provide a measure of the tebuconazole residue found in the leaf during the life of the leaf. AURPC values were subjected to analysis of variance. It was found that the residue measurements for SAC Edinburgh for 10 July 1997 were abnormally high by comparison with measurements from previous and following weeks, so data for this date were omitted from the calculation of AURPC.

The relationship between dose applied and fungicide residue measured by ELISA assay was examined by fitting a regression plane to AURPC values using the function
$\text{AURPC} = a+b_{32}\text{dose}_{32}+b_{33}\text{dose}_{33}$.
Parameter a is the lower asymptote which represents the amount of tebuconazole residue detected in the leaf tissue samples when dose applied = zero.
Parameter b_{32} represents the rise in the amount of residual fungicide per unit dose applied at GS 32, and b_{33} the rise per unit of GS 33 dose.

Experiment One : Relating epidemic progress to ELISA assay results

The relationship between disease severity and tebuconazole residues was examined by fitting an exponential function of the form $\text{AUDPC} = a+be^{k.\text{AURPC}}$ to the AURPC and AUDPC data.

The relationship between ELISA test results and AUDPC was quantified by fitting the exponential function $\text{AUDPC} = a+be^{k.\text{AURPC}}$ for each ELISA test date in turn.

The half-life of tebuconazole was calculated as time = $\ln 2/k$, where k represents a decay constant which differs for different materials. The decay constant for tebuconazole was obtained by fitting the function $y = a+be^{k.\text{time}}$ to the decline of residual fungicide over time.
Parameter k in the exponential function being the decay constant.

At SAC Edinburgh 1997 and ADAS Rosemaund 1998 significant reduction in *Septoria tritici* levels were given by GS 32 and GS 33 applications, and there was a significant reaction between the applications.

Disease epidemics were of similar severity at ADAS Rosemaund 1998 (Fig. 22) and SAC Edinburgh in 1997 (Fig. 23), where the untreated AUDPCs were in the range 876-1006 ($a+b$). At these sites, the potential reduction of disease over time was between 761 and 887 (b). The two-spray treatment of 2.0 litres/ha Folicur at GS 32 and GS 33 was the most effective, and gave AUDPCs in the range 50-197 (a).

At all sites a 2-spray programme gave greater control of disease on leaf 2 than a late spray alone, but treatments applied at GS 33 were more efficient than those applied at GS 32. This was demonstrated by the greater curvature of the surfaces in the GS 33 dose-response direction (k_{33}) than the curvature in the GS 32 does-response direction (k_{32}). GS 33 sprays were most efficient at ADAS Rosemaund 1997 (k_{33} = -4.62).

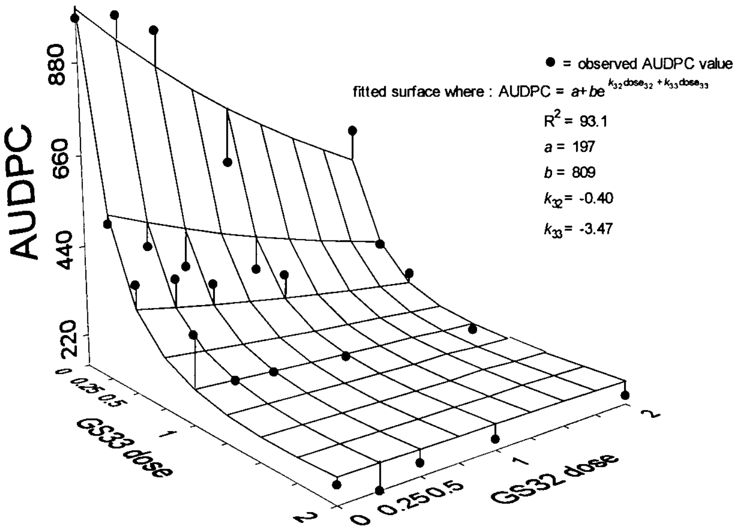


Figure 22. Fitted dose-response surface describing the effect of combinations of tebuconazole doses at GS 32 and GS 33, on AUDPC on leaf 2 at SAC Edinburgh, 1997.

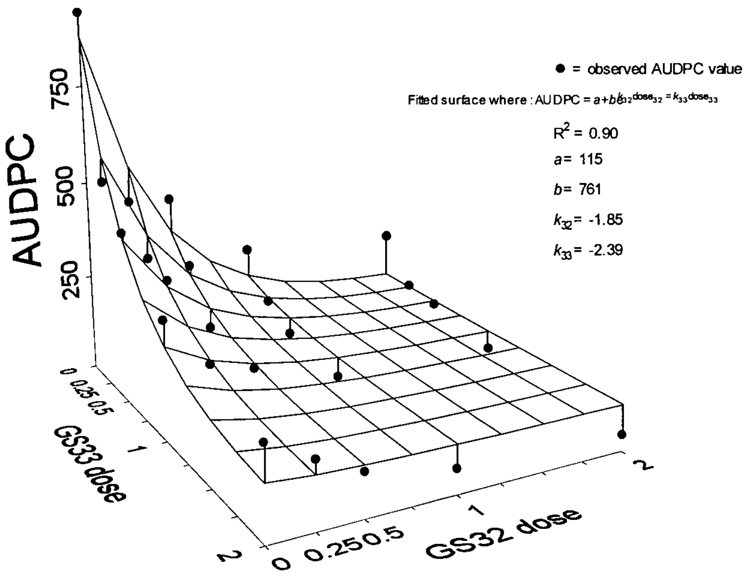


Figure 23. Fitted dose-response surface describing the effect of combinations of tebuconazole doses at GS 32 and GS 33, on AUDPC on leaf 2 at ADAS Rosemaund, 1998.

Analysis of variance of AURPC values for SAC Edinburgh 1997 showed that tebuconazole residue levels were significantly different ($P>0.05$) within GS 32 doses and GS 33 doses, and that there was not a significant interaction.

Tebuconazole residues were detected in samples from untreated plots at both sites, and overall residue levels in treated plots at the ADAS Rosemaund site were greater than those at SAC Edinburgh.

Residual fungicide was detected in leaf 2 of plots which had only been treated at GS 32, suggesting that some fungicide had been translocated within the plant. The GS 32 treatment was applied one to three days before the emergence of leaf 2 at both sites.

The three-dimensional plane was a good fit to the data for SAC Edinburgh 1997 (Fig. 24), as shown by the R^2 of 0.93. The untreated AURPC was 699 (a) which was small in relation to the treated values. Each dose unit of Folicur at GS 33 increased the AURPC by 1682 (b_{33}). Each dose unit of Folicur at GS 32 gave a smaller increase of 579 (b_{32}).

At ADAS Rosemaund 1998, the analysis of variance of AURPC values showed that there were significant differences ($P>0.5$) between doses applied at GS 33 and within the interaction of early and late doses. There were no significant differences between doses applied at GS 32.

The three-dimensional plane (Fig. 25) was a reasonably good fit to the data, as shown by the R^2 of 0.73. The untreated AURPC was 941 (a) which was small in relation to the treated values.

Each dose unit of Folicur at GS 33 increased the AURPC by 4744 (b_{33}). Each dose unit of Folicur at GS 32 gave a smaller increase of 646 (b_{33}).

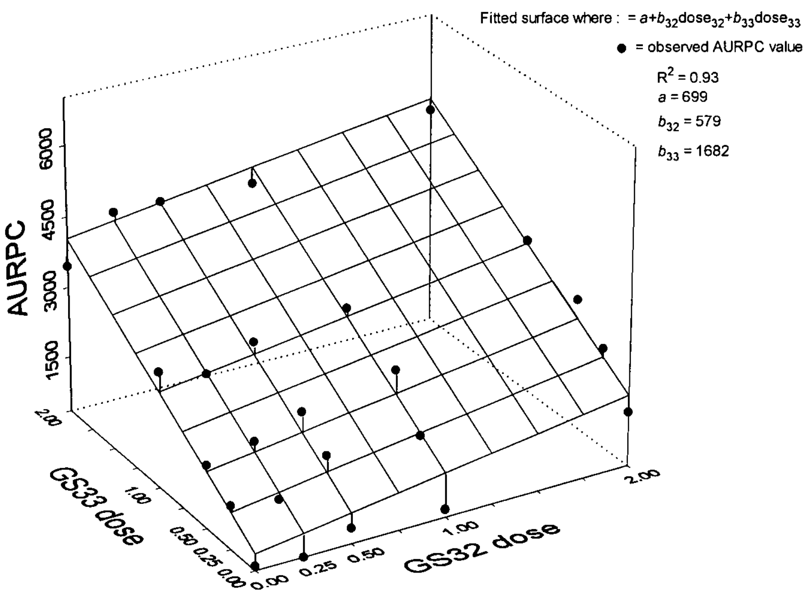


Figure 24. Fitted plane describing the relationship between combinations of tebuconazole doses applied at GS 32 and GS 33, and ELISA assay of fungicide residues (AURPC) in leaf 2 at SAC Edinburgh 1997.

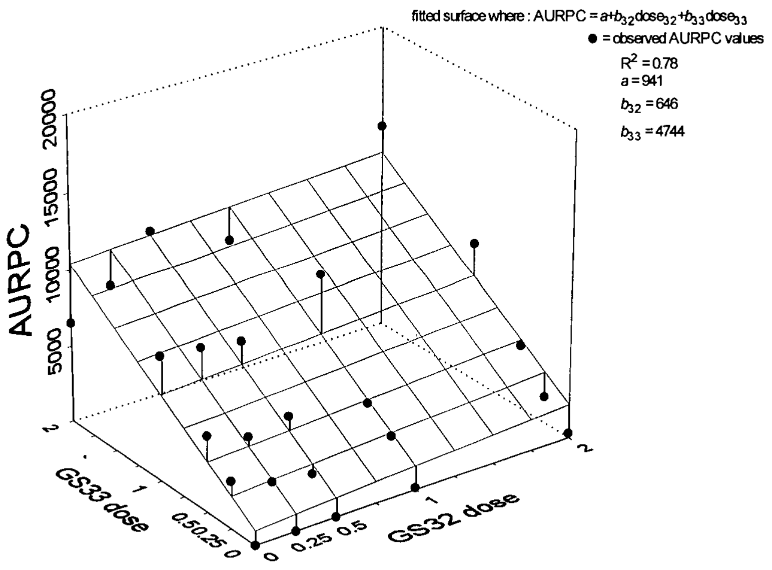


Figure 25. Fitted plane describing the relationship between combinations of tebuconazole doses applied at GS 32 and GS 33, and ELISA assay of fungicide residues (AURPC) in leaf 2 at ADAS Rosemaund 1998.

Exponential curves fitted to AURPC v AUDPC showed that there was a relationship between disease severity and fungicide residue. The relationship was closer at SAC Edinburgh (R^2 = 0.86, Fig. 26) than at ADAS Rosemaund (R^2 = 0.43, Fig. 27). The *b* and *k* values varied between experiments.

The proportion of disease control by a given amount of AURPC was similar at both sites. An AURPC of 5000 reduced AUDPC by 82% at SAC Edinburgh, and 84% at ADAS Rosemaund. A 75% reduction in AUDPC was given by an AURPC of 2939 at SAC Edinburgh, and 2941 at ADAS Rosemaund.

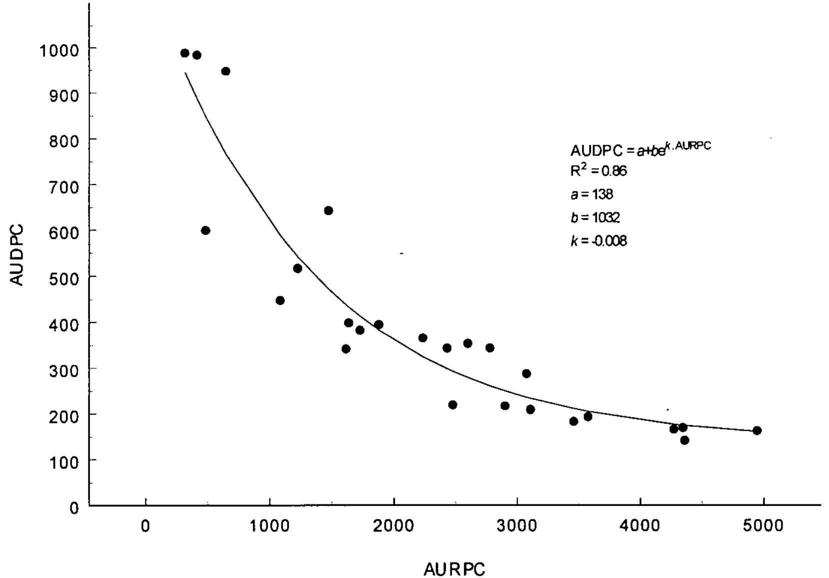


Figure 26. Fitted exponential curve describing the relationship between *S.tritici* severity measured by AUDPC, and tebuconazole residue measured by AURPC at SAC Edinburgh 1997

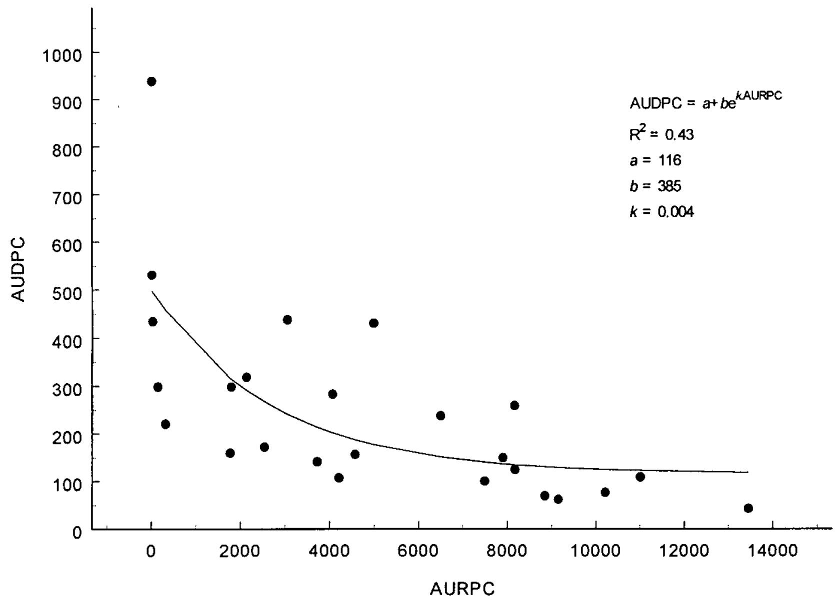


Figure 27. Fitted exponential curve describing the relationship between *S.tritici* severity measured by AUDPC, and tebuconazole residue measured by AURPC at ADAS Rosemaund, 1998

The highest residue levels at SAC Edinburgh 1997 were detected at 7 and 14 days after the application GS 33 treatments, and at ADAS Rosemaund 16 days after the GS 33 treatments. Examples of ELISA residue measurements from the time of the GS 33 fungicide treatment for both experiments are shown in Fig. 28

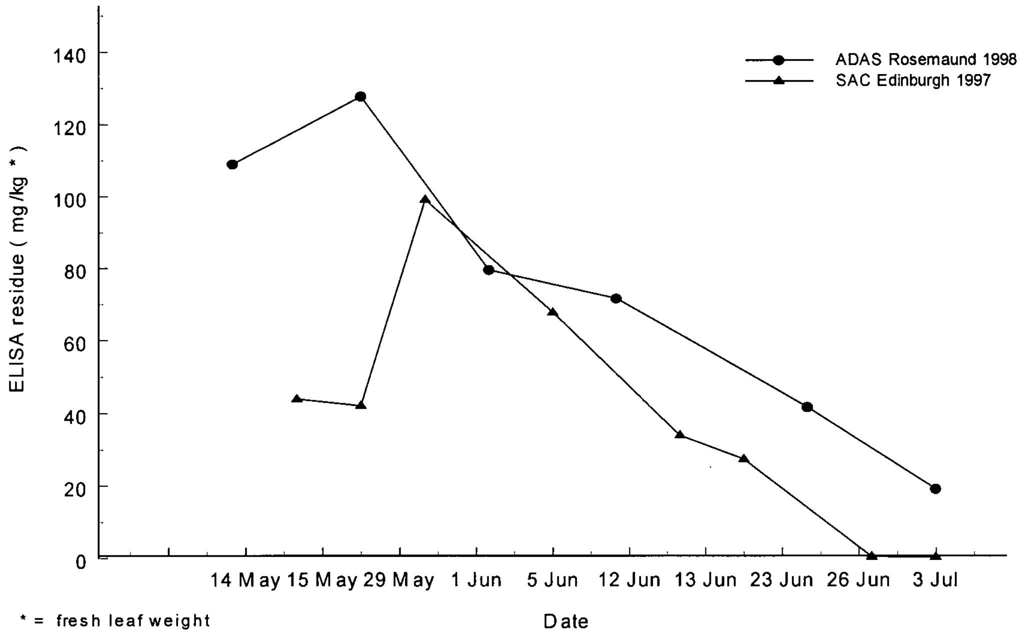


Figure 28. Example of ELISA residue measurements (mg/kg fresh leaf weight) for SAC Edinburgh 1997 and ADAS Rosemaund 1998 from the time of the GS 33 spray applications.

The average half-life of the tebuconazole at SAC Edinburgh was 10 days, for a range of 2 to 15 days. At ADAS Rosemaund, the average half-life was 23 days for a range of 8 to 39 days.

It would be unrealistic to expect a grower to test for residues at weekly intervals, therefore it is important to identify the optimum time to sample for the ELISA test to achieve reliable results. Where AUDPC was related to fungicide residue at each ELISA test-date, the R^2 relationships ranged from 0.42 to 0.92. In general, the ELISA test results were a more precise prediction of disease severity two to three weeks after the GS 33 treatment, which coincides with the peak residue levels.

Experiment Two

Plots were sampled at weekly intervals. A visual assessment of *S. tritici* infection was carried out and samples frozen for residue analysis. Samples for the experiment in 1997 were run through the ELISA protocol and residue and disease data was sent to Jan Thomas for statistical analysis. Graphs showing the detailed disease development and tebuconazole residues found at SAC Edinburgh in 1997 are presented in Appendix 6. See table 8 in materials and methods for treatment timings.

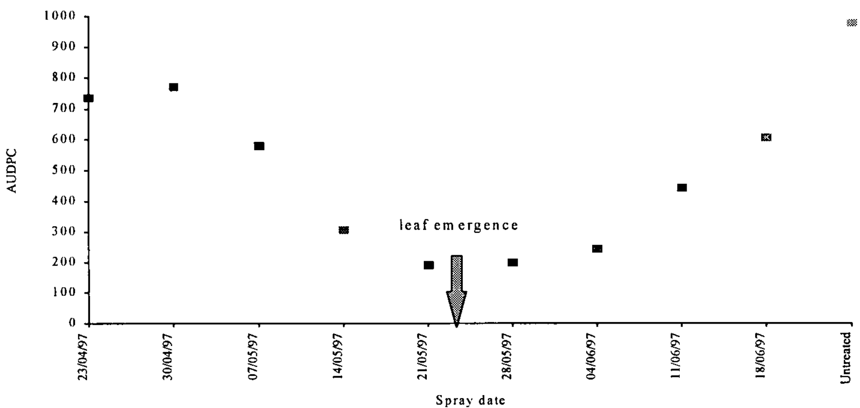


Figure 29. Figure showing the total AUDPC values calculated for each treatment in Experiment Two, SAC Edinburgh 1997.

Figure 29 shows clearly that the best control of *S.tritici* (lowest AUDPC) was given by the treatments which coincided with the full emergence of leaf F2 and before the flag leaf unfurled completely (June 4th onwards). The lowest AUDPC was given by spray applied on the 21st of May (190.8). Treatment 10 (Untreated) gave an indication of disease severity in the trial. It produced an AUDPC value of 974.

Residue levels in the second leaf were also determined and the graphs plotted against time (see Appendix 5). AURPC levels were calculated and shown graphically in Fig. 30

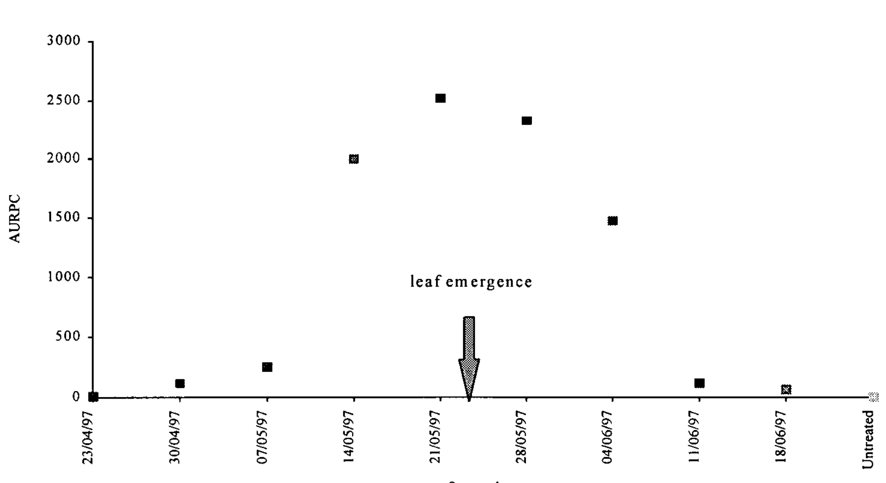


Figure 30. Figure showing the total AURPC values calculated for each treatment in Experiment Two, SAC Edinburgh 1997.

These results show that the highest AURPC value was given by Treatment 5. This would be expected since at this spray date leaf F2 would be fully emerged and not shaded by any upper leaves. It would therefore be capable of maximum spray interception. It is clear that earlier sprays also produced measurable levels of tebuconazole in the developing leaf. Treatment 3 gave an AURPC value of 248. This is higher than AURPC produced by sprays applied 3 weeks after full leaf emergence.

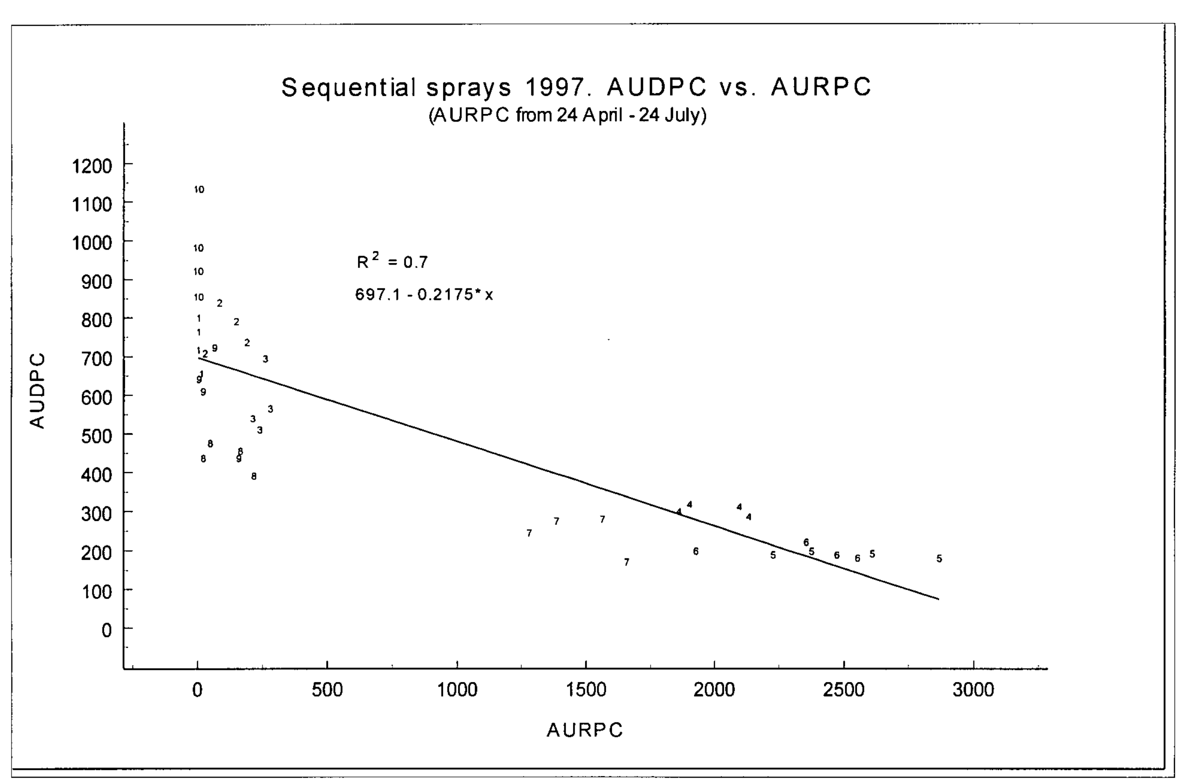


Figure 31. Fitted line describing the relationship between *S.tritici* severity measured by AUDPC, and tebuconazole residue measured by AURPC at SAC Edinburgh, 1997

A scatter point graph was produced in order to demonstrate the relationship between AURPC and AUDPC in this trial. Points on the graph were plotted for each individual plot in the experiment. Each point was given its corresponding treatment number when being plotted. This allowed a further indication of the variation produced within each treatment in the experiment. The graph indicates that there is a clear relationship between the AUDPC values and the AURPC values in this experiment. This relationship was defined in the equation

AUDPC = 697.1 - 0.2175(AURPC)

This equation had an R^2 value of 0.7, indicating a good fit for the data.

As in experiment one, a relationship was clearly demonstrated between the residues recorded in the leaf and the disease levels observed.

4.3.5 Relating disease control in the field to final grain yields

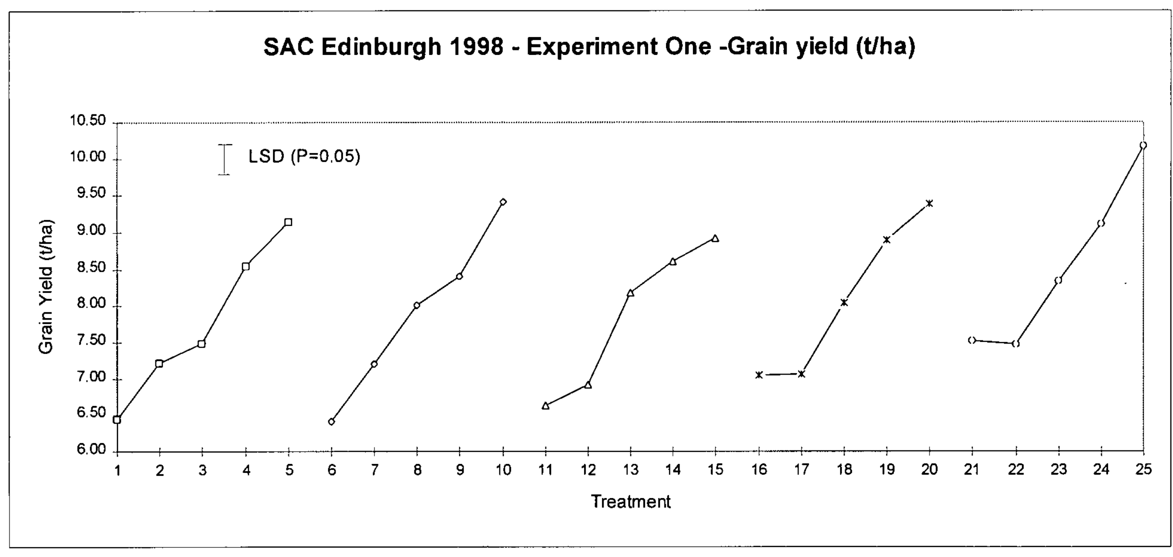


Figure 31. Yield response curves produced in Experiment One in 1998 at SAC Edinburgh.

Yield response curves were produced for Experiment One. The results for the two sites and the two years followed the pattern shown in Fig. 31. Treatments were grouped in blocks of five depending on the GS 32 Treatment. e.g. Treatments 1-5 had zero fungicide at GS 32. Results show that maximum yield was obtained by Treatment 25 (10.17 t/ha), which had two sprays of twice the Normal rate. However, the graphs show that, in general, increasing fungicide doses produce increases in yield. However it is worth pointing out that there is no assessment of cost in these graphs.

These curves fit well with the detailed analysis carried out by Jan Thomas and also the dose response curves for disease control and tebuconazole residue produced by the treatments (see Appendix 5)

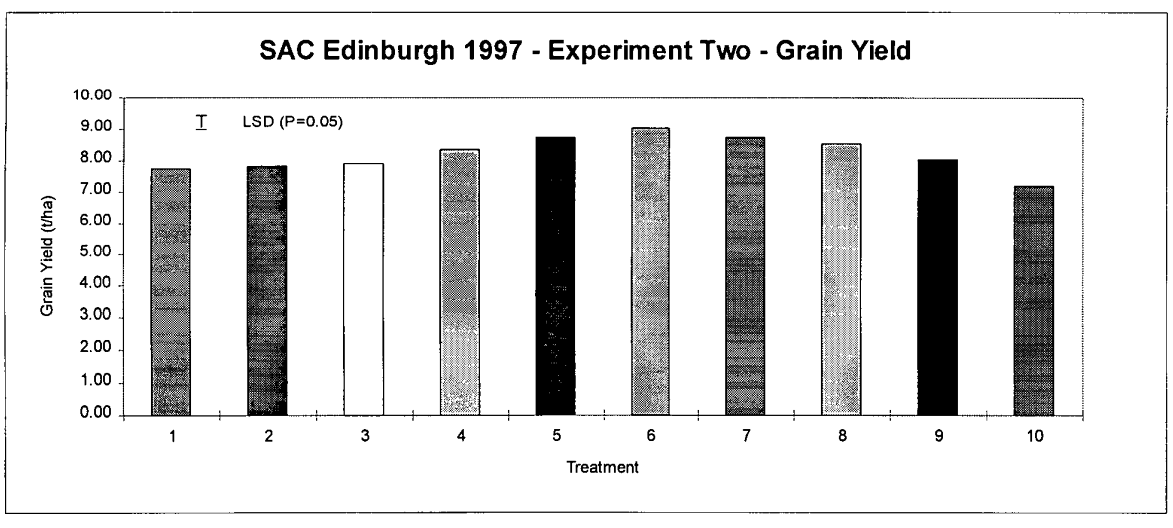


Figure 32. Yield results for Experiment Two carried out SAC Edinburgh in 1997

Yield results for experiment two indicate the importance of timing tebuconazole applications to give maximum leaf protection and optimise yield. These results clearly show that the treatments aimed at leaf F2 emergence and the timings close to it gave the largest yield benefit. However all of the treatments have shown a response over the untreated. These increases are well within the range of percentage increase in grain yield achieved by tebuconazole in published trials. Statistical analyses indicated a good correlation between grain yield and the recorded disease in each plot.

The figures for disease control indicate that the greatest control of disease on leaf two was achieved by Treatment 5 (application at leaf F2 emergence). However the yields from Treatment Six show a small but significant increase over Treatment 5. This may indicate that this timing is associated with greatest capture and retention of tebuconazole by emerged leaf F2 and the emerging flag leaf.
Detailed curves for disease development and tebuconazole residues for this trial are given in Appendix 5.

4.4 CONCLUSIONS

The ELISA protocol was successfully validated in the laboratory in Edinburgh. This is encouraging as the test has now been shown to be robust and work successfully in three different laboratories.

Production of an *in vivo* dose response curve demonstrated that the test can successfully distinguish between a wide range of tebuconazole doses applied to plants in the glasshouse. This suggests that the test will be able to detect small changes in residue levels in wheat plants.

Results from residue analysis on a variety trial carried out in 1996 indicate that the antibody is not affected by varietal differences in the plant material.

In experiment one, carried out at SAC Edinburgh and ADAS Rosemaund, tebuconazole residue related well to fungicide dose within each site. In the target leaf, leaf 2, residue levels were lower for a unit of tebuconazole applied at GS 32 than for a unit applied at GS 33. This was reflected in the relationship between dose applied and AUDPC, in that a unit applied at GS 32 was less effective at suppressing disease on leaf 2 than a unit applied at GS 33

Although the general pattern of relationship between dose and residue at the two sites was similar, the slope of the relationship was substantially different. These differences in slope represent variation in spray capture by the crop canopy, differences in the rate of decay of the fungicide or inaccuracy in the ELISA test.

It would be unrealistic to expect a given AURPC to relate to a given amount of disease severity, considering the potential for variation in disease pressure for each site and season, but, as hoped, a given residue level gave a broadly similar proportional amount of control across sites and seasons.

The results from Experiment two indicated the optimal time for tebuconazole application, in terms of disease control, is at full leaf emergence. However data also showed that earlier and later applications of fungicide will provide a measurable level of residue and therefore protection within the crop canopy.

Yield response curves indicated that higher tebuconazole doses lead to improved disease control, which in turn resulted in improved yields. Nearly all applications of fungicide produced yield increases compared to the control. However, no accounting for fungicide cost was made in these experiments.

In conclusion, these results indicate that the antibody based test has been shown to be able to detect residual levels of tebuconazole in winter wheat. These levels have been shown to be directly related to the timing and dose rate of previous spray applications. The effect of different crop residue levels on future disease development and hence yield has been demonstrated.

5. GLOSSARY

The following is a brief glossary of some of the scientific terms and abbreviations used in this project.

BSA -	Bovine serum albumin
a.i. or AI -	active ingredient
PBST -	Phosphate buffered saline with Tween 20
TMB -	Tetramethylbenzidine
H_2SO_4 -	Sulphuric acid
A_{450} -	Absorbance at 450 nm wavelength
CEMAS -	CEM Analytical Services Ltd
GCMS -	Gas Chromatography Mass Spectrometry
% Bo -	Inhibition of the tebuconazole by sera (expressed as a percentage)
ELISA -	Enzyme Linked Immunosorbent Assay
O.D. -	Optical Density (read at 450 nm)
AUDPC -	Area under disease progress curve
AURPC -	Area under residue progress curve

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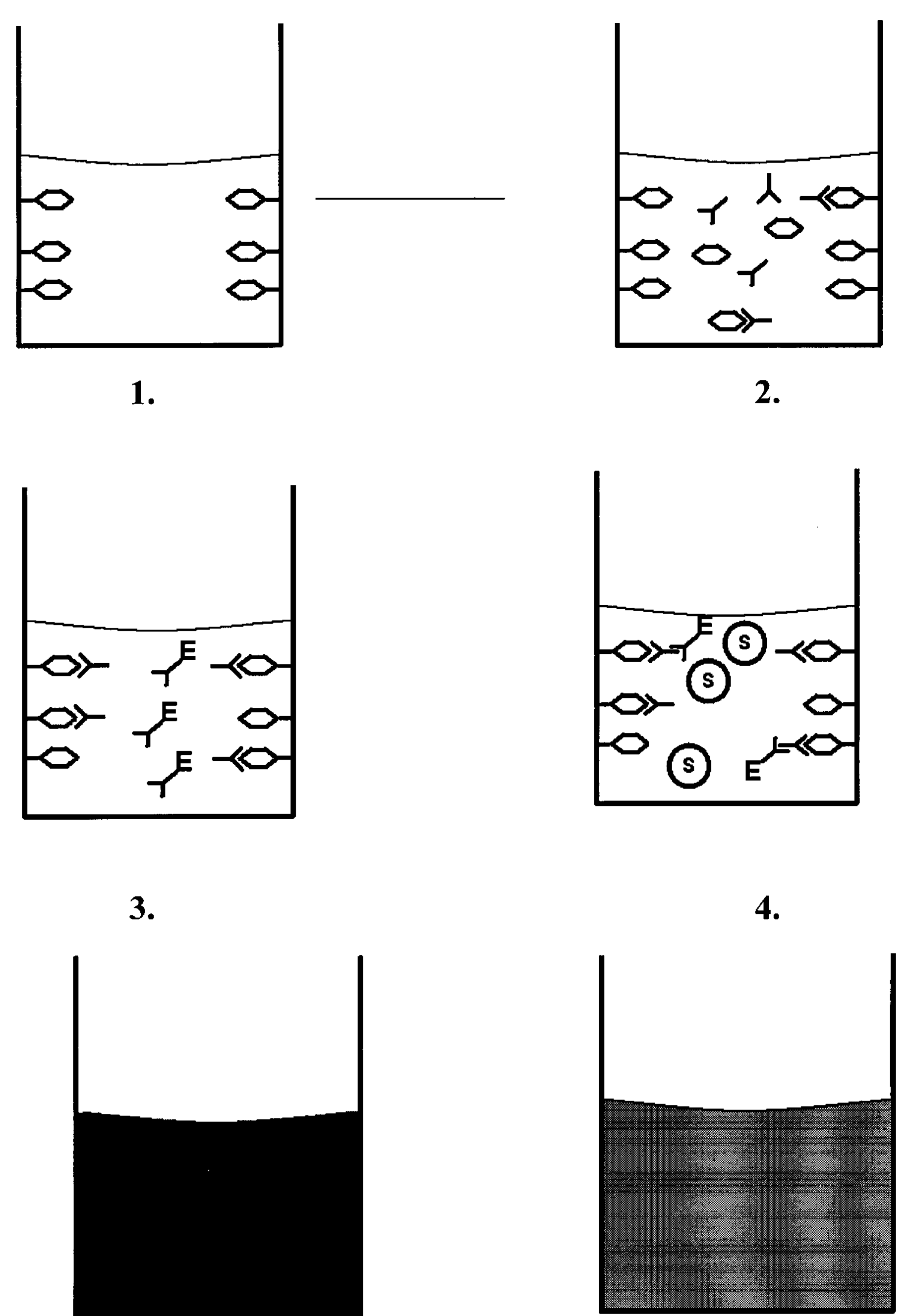
Shephard, M.C. (1987) Screening for fungicides. ***Annual Review of Plant Pathology 25***: 189 - 206.

APPENDIX ONE -TEBUCONAZOLE COMPETITIVE ASSAY



LIGHTER COLOUR = MORE TEBUCONAZOLE IN THE SAMPLE

SUMMARY OF COMPETITIVE ELISA

1. Coat wells with a known concentration of tebuconazole. In the kit the plate is supplied pre-coated.

2. Add sample and specific antibody. If the sample contains tebuconazole it will COMPETE with the tebuconazole coated onto the wells to bind the antibody. Some antibody molecules will bind to the tebuconazole on the well and some to the tebuconazole in the sample. Antibody bound to tebuconazole in the sample is removed by washing.

3. A conjugate, which in this case is an antibody labelled with an enzyme, is added to the wells. The conjugate is able to recognise and bind to the specific antibody that is bound to the tebuconazole in the well.

4. Unbound conjugate is removed by washing. A substrate for the enzyme is added. The product of the enzyme-substrate reaction is coloured and can be visualised or the absorbance measured with a spectrophotometer.

The 'free' tebuconazole and the bound tebuconazole COMPETE for the antibody. If there is a high level of tebuconazole in the sample most of the antibody will bind to the 'free' tebuconazole and be removed by washing. Thus, when the conjugate is added there is no, or very little, antibody bound to the tebuconazole in the wells. Therefore there is nothing for the conjugate to bind to and the colour production is very low.

High level of tebuconazole in the sample = low colour.

Low level of tebuconazole in the sample = high colour

APPENDIX TWO - SAMPLE PREPARATION AND COMPETITION ELISA PROTOCOL

A. Preparation of extract

1. Place 1g of leaf material in a heavy duty polythene bag. Add 4ml of 80% methanol and homogenise using a hand held homogeniser (Bioreba) for 3 - 4 minutes.
2. Heat seal the bag and incubate at 4^0C overnight (for at least 16 hours).
3. Add 60ml distilled H_2O to the extract in the bag and shake gently to mix.
4. Filter the sample through a double layer of muslin.
5. The sample(s) is/are then ready for use in the tebuconazole assay.

B. Competition ELISA

1. Coat plates with Tebuconazole-NH-BSA conjugate 1µg/ml in coating buffer (9µl of stock in 100mls). Add 100µl per well. Incubate overnight at 4^0C.

2. Wash 4 times with PBST and pat dry on paper towels.

3. Block with PBSTM (5% non-fat milk powder) at 200µl/well. Incubate at 37^0C for 2 hours.

4. Meanwhile cross-adsorb the sera in PBST-4%BSA for 30 - 60 minutes at 37^0C.

5. Wash plate 4 times with PBST and pat dry on paper towels.

6. Add to plate (i) 50µl of cross-adsorbed sera diluted at 1/5000
(ii) 50µl of standard/samples in solvent.
Cover plate with plastic film and place on shaker at room temperature for 2 hours.

7. Wash 4 times with PBST and pat dry on paper towels.

8. Add 100µl/well of goat anti-Rabbit IgG-HRP conjugate in PBST and incubate at 37^0C for 1 hour.

9. Wash plate 4 times with PBST and pat dry on paper towels.

10. Add 100µl/well of K-blue substrate (or alternative TMB substrate) to the wells. Incubate in the dark at room temperature for 15 minutes.

11. Stop the reaction by adding 50µl/well H_2SO_4 (10%). Gently shake the plate to mix the substrate and stop solution and read at 450nm.

APPENDIX THREE - SITE DATA AND TREATMENTS - VARIETY TRIAL SAC EDINBURGH 1996

Grid Reference:	NT 452 742	
Elevation:	70 m	
Soil Series:	Winton	
Soil Type:	Clay Loam	
Soil Analysis:	pH 6.5	
P	16 mg/l	(High)
K	158 mg/l	(Moderate)
Mg	210 mg/l	(High)
S	5.8 mg/l	(Low)
Mn	4.8 mg/l	(Moderate)
Cu	8.2 mg/l	(Moderate)

Previous Cropping:

1995	Winter Wheat
1994	Winter OSR
1993	Winter Wheat
1992	Winter Wheat

Sowing Date: 06/10/95

Seed Rate: 190 kg/ha

Fertiliser (kg/ha):

seed bed:	60 kg/ha P	17/10/95
	60 kg/ha K	17/10/95

Product:	Rate/ha		Date Applied
Trace Elements:	None		
Herbicides:	Merit	2.0 l/ha	GS 11
	Duplosan	1.0 l/ha	GS 11

Treatments

Treatment Number	Tebuconazole applied
One	1.0 l/ha at GS 47
Two	0.75 l/ha at GS 47
Three	0.5 l/ha at GS 47
Four	0.25 l/ha at GS 47
Five	Untreated
Six	1.0 l/ha at GS 32 and GS 58
Seven	0.75 l/ha at GS 32 and GS 58
Eight	0.5 l/ha at GS 32 and GS 58
Nine	0.25 l/ha at GS 32 and GS 58
Ten	Untreated

APPENDIX FOUR - SITE DATA
ADAS ROSEMUAND AND SAC EDINBURGH

ADAS ROSEMAUND

Grid Reference: SO560478
Field Name: Flatfield
Soil Series: Bromyard
Soil Type: Silty Clay Loam
Drainage: Good
Soil Analysis: pH 6.9

P	20 mg/l	(Index2)
K	135 mg/l	(Index 2)
Mg	84 mg/l	(Index 2)

Previous Cropping:

1996	Winter Beans
1995	Winter Wheat
1994	Winter OSR
1993	Winter Wheat

Previous Crop Residue: Chopped and incorporated
Cultivations:
Ploughed (15-20 cm) 07-08/10/96
Power Harrow 09/10/96
Sowing Date: 10/10/96
Seed Treatment: Beret Gold
Crop 50% emerged 22/10/96

Fertiliser (kg/ha):
seed bed: nil
top dressings:

40 kg/ha N	18/03/97
104 kg/ha N	01/05/97
52 kg/ha N	07/05/97

Product:	Rate/ha	Date Applied
Trace Elements:	None	
Herbicides:	Optical 0.1 l/ha	15/11/96
	IPU 2.2 l/ha	15/11/96
	Panther 0.8 l/ha	15/11/96
	Optical 5 l/ha	07/04/97
Insecticide	Cyperkill 0.25 l/ha	15/11/96
Molluscicide	None	

Plant Growth regulator:	Holdup 2.25 l/ha	01/04/97

SAC EDINBURGH- MARKLE MAINS, EAST LOTHIAN 1997

Soil Analysis: pH 6.4

P	Moderate
K	Moderate
Mg	High
S	Moderate

Previous Cropping:

1996	WinterWheat
1995	Winter Oilseed Rape
1994	Winter Wheat

Previous Crop Residue: Chopped and incorporated
Sowing Date: 08/10/96

Fertiliser (kg/ha):

seed bed:	13 kg/ha N	14/10/96
	65 kg/ha P	14/10/96
	65 kg/ha K	14/10/96
top dressings:	70 kg/ha N	01/03/97
	110 kg/ha N	15/03/97

Product:	Rate/ha	Date Applied
Trace Elements:	None	
Herbicides:	Jolt 3 l/ha	05/11/96
	Duplosan	05/11/96
	Ally 15 g/ha	12/04/97
Insecticide	None	
Molluscicide	None	
Plant Growth regulator:	5C Cycocel 2.5 l/ha	12/04/97

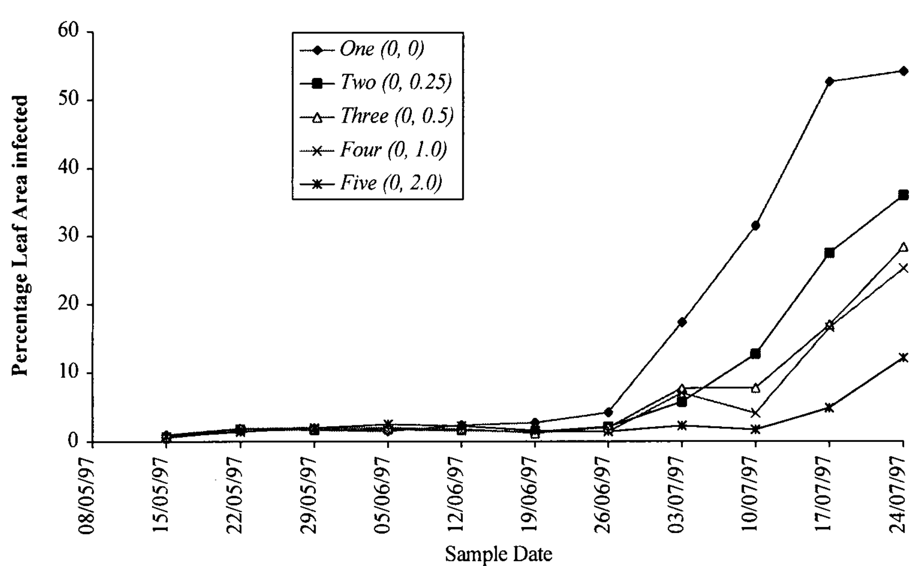
SAC EDINBURGH - MARKLE MAINS 1998

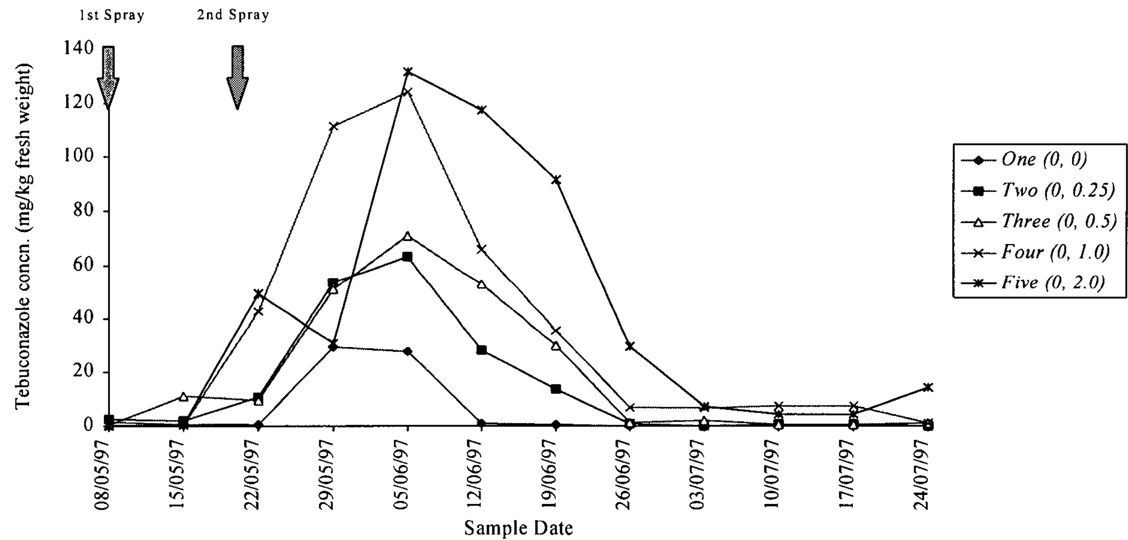
Field Name:	Braeface
Soil Analysis:	pH 6.4

Previous Cropping:		
1997	Winter Oilseed Rape	
Previous Crop Residue:	Chopped and incorporated	
Sowing Date:	27/9/97	
Fertiliser (kg/ha):		
seed bed:	40 kg/ha P	27/9/97
	40 kg/ha K	27/9/97
top dressings:	52 kg/ha N	09/02/97
	28 kg/ha N	23/03/97
	69 kg/ha N	14/04/97
	27 kg/ha N	09/05/97

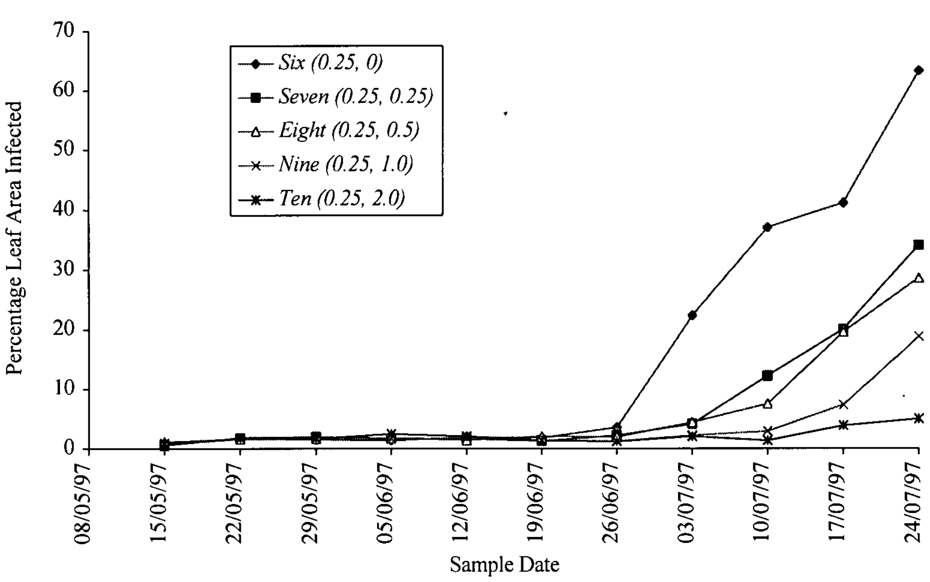
Product:	Rate/ha	Date Applied
Trace Elements:	None	
Herbicides:	Javelin 1.0 l/ha	10/10/97
	CMPP 3.0 l/ha	22/04/98
	Sportak Delta 0.8 l/ha	22/04/98
Insecticide	Cyperkill 0.25 l/ha	15/11/96
Molluscicide	None	
Plant Growth regulator:	5C Cycocel 2.0 l/ha	22/04/98

APPENDIX FIVE
Experiment One- SAC Edinburgh 1997

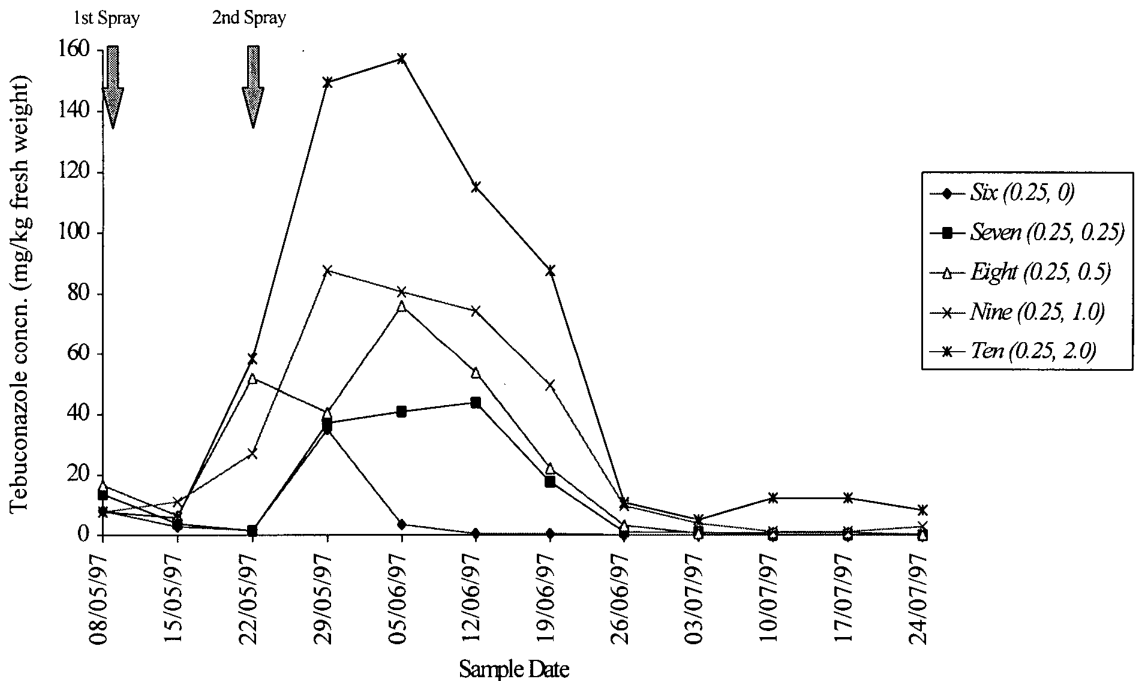




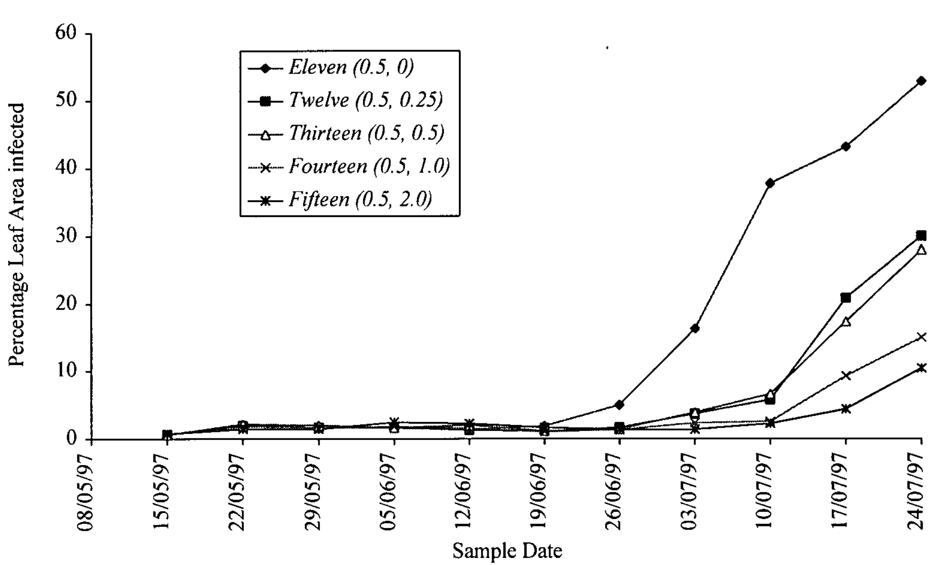
S. tritici infection - Leaf F2 - Trial 1 -1997



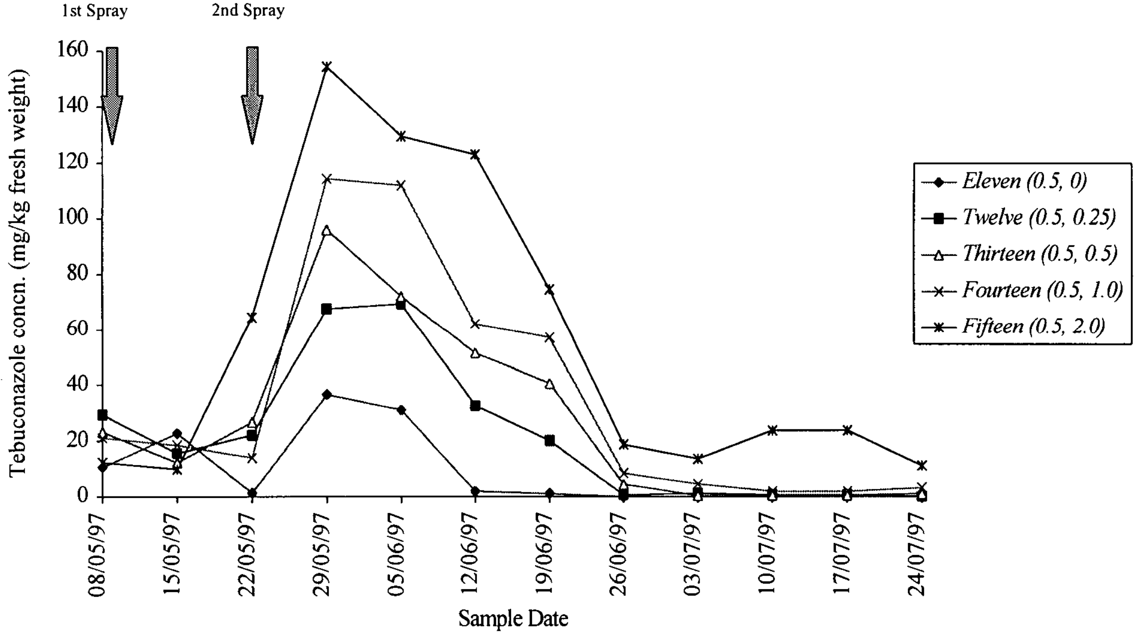
Tebuconazole concentration - Leaf F2 - Trial 1 -1997



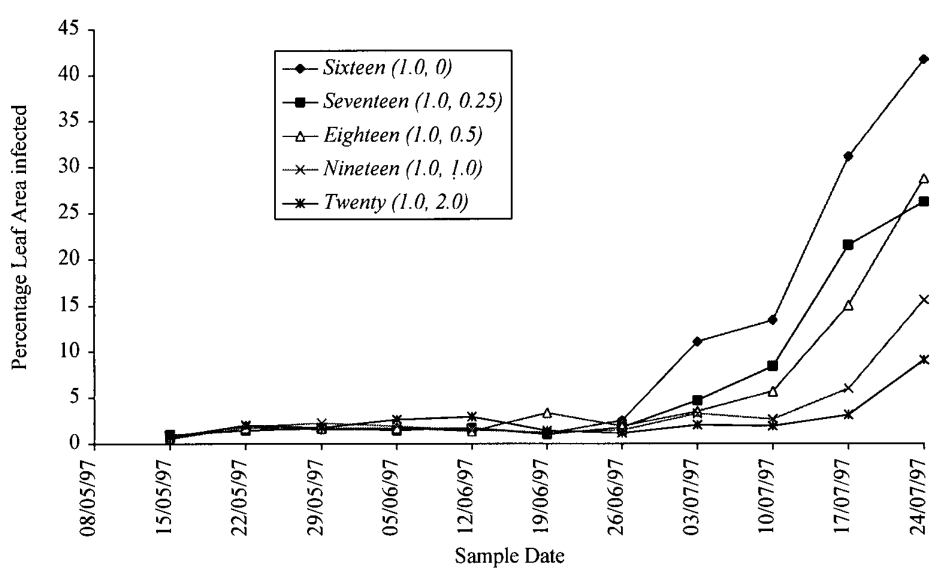
S. tritici infection - Leaf F2 - Trial 1 -1997



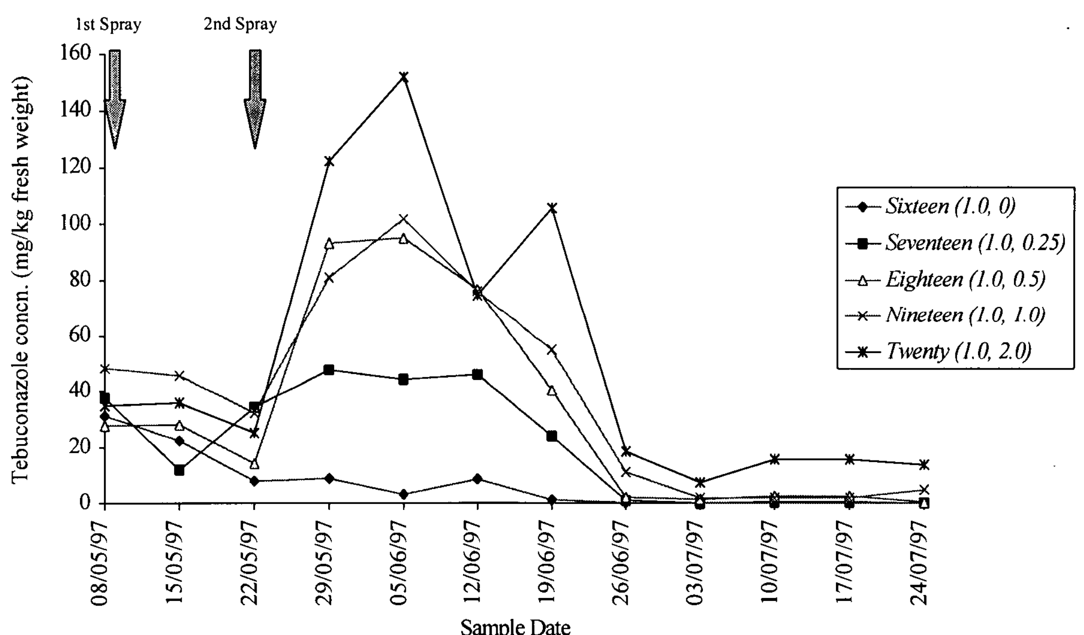
Tebuconazole concentration - Leaf F2 - Trial 1 -1997

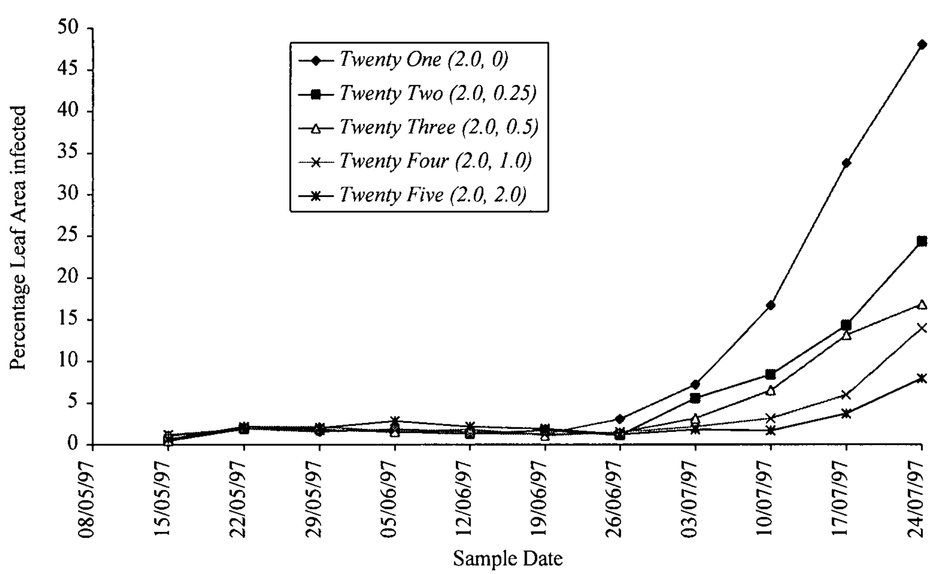


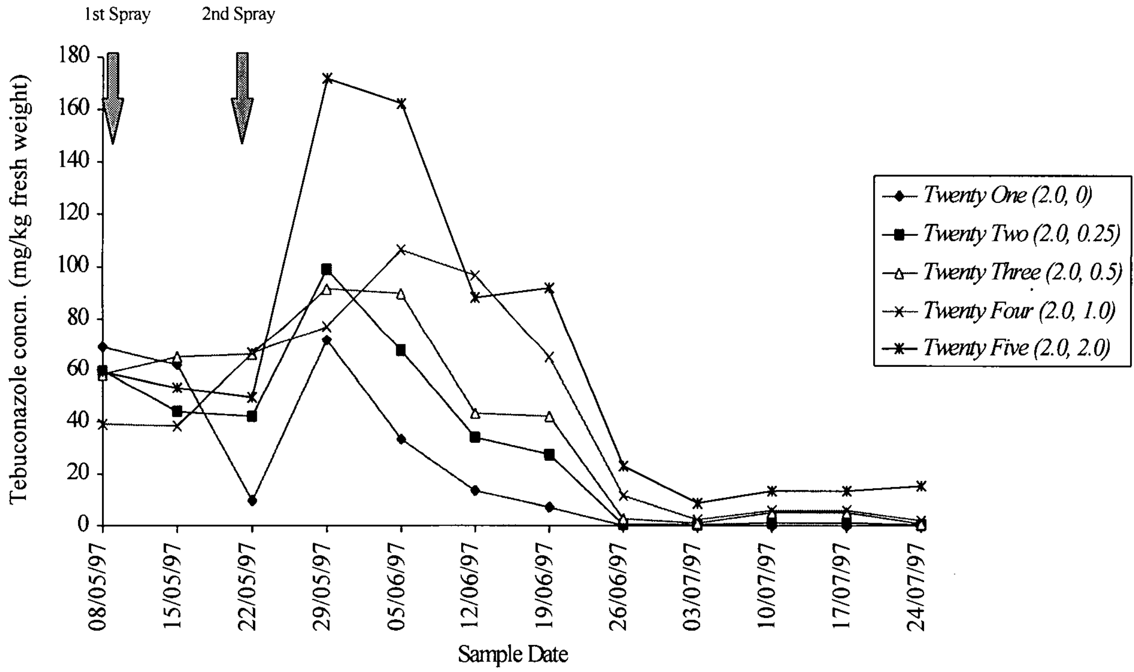
S. tritici infection - Leaf F2 - Trial 1 -1997



Tebuconazole concentration - Leaf F2 - Trial 1 -1997

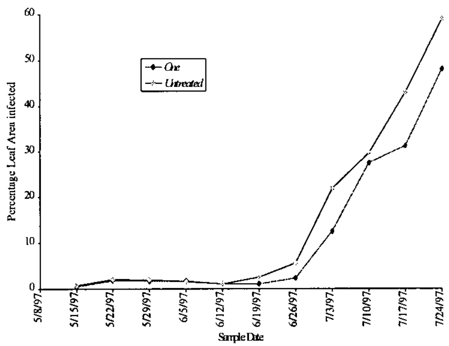



S. tritici infection - Leaf F2 - Trial 1 -1997
Percentage Leaf Area infected
50
45
40
35
30
25
20
15
10
5
0
Twenty One (2.0, 0)
Twenty Two (2.0, 0.25)
Twenty Three (2.0, 0.5)
Twenty Four (2.0, 1.0)
Twenty Five (2.0, 2.0)
08/05/97
15/05/97
22/05/97
29/05/97
05/06/97
12/06/97
19/06/97
26/06/97
03/07/97
10/07/97
17/07/97
24/07/97
Sample Date


Tebuconazole concentration - Leaf F2 - Trial 1 -1997
1st Spray
2nd Spray
Tebuconazole concn. (mg/kg fresh weight)
180
160
140
120
100
80
60
40
20
0
Twenty One (2.0, 0)
Twenty Two (2.0, 0.25)
Twenty Three (2.0, 0.5)
Twenty Four (2.0, 1.0)
Twenty Five (2.0, 2.0)
08/05/97
15/05/97
22/05/97
29/05/97
05/06/97
12/06/97
19/06/97
26/06/97
03/07/97
10/07/97
17/07/97
24/07/97
Sample Date

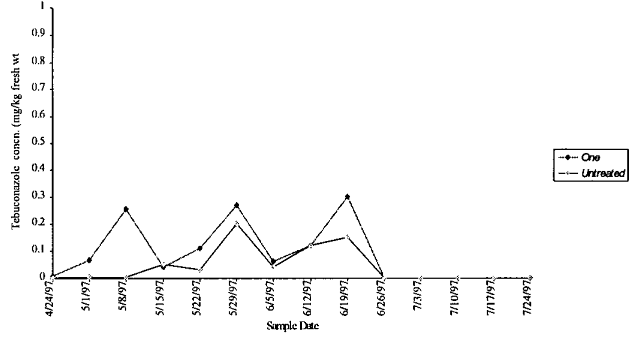
APPENDIX SIX
Experiment Two - SAC Edinburgh 1997

S. tritici infection - Leaf F2 - Trial 2 -1997
4 weeks pre-leaf emergence sprays



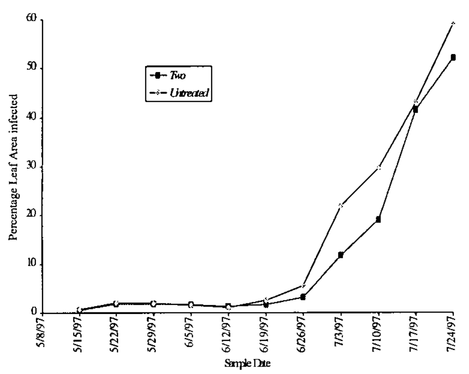
Spray Date 23/4/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
4 weeks pre-leaf emergence spray



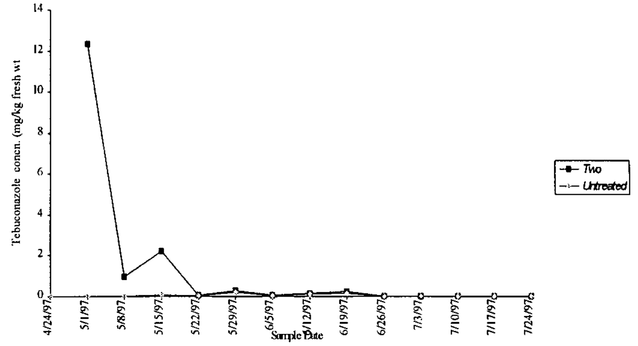
Spray Date 23/4/97

S. tritici infection - Leaf F2 - Trial 2 -1997
3 week pre-leaf emergence spray



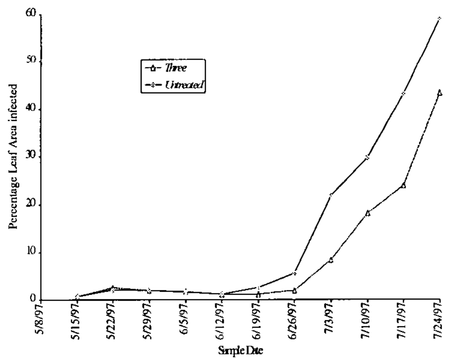
Spray Date 30/4/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
3 weeks pre-leaf emergence spray



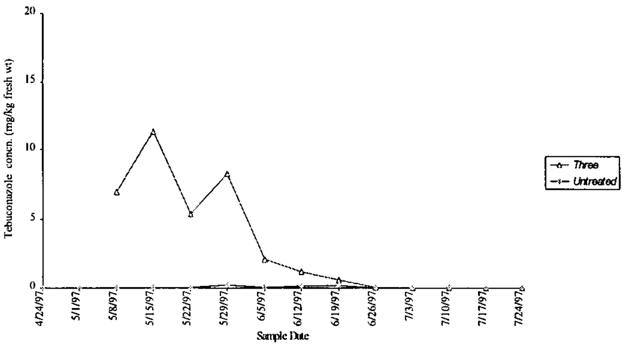
Spray Date 30/4/97

S. tritici infection - Leaf F2 - Trial 2 -1997
2 weeks pre-leaf emergence spray



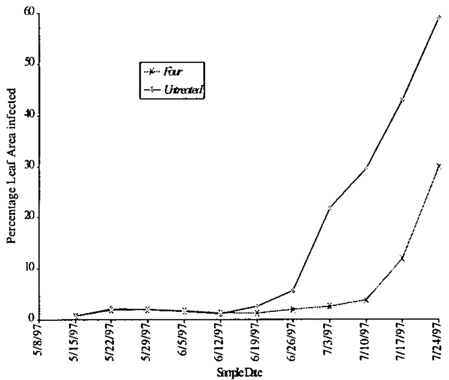
Spray Date 7/5/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
2 weeks pre-leaf emergence spray



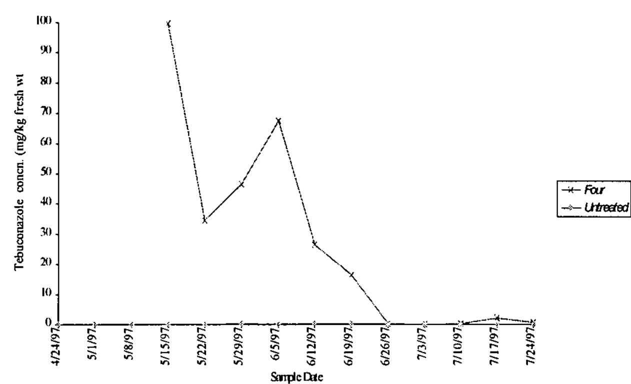
Spray Date 7/5/97

S. tritici infection - Leaf F2 - Trial 2 -1997
1 week pre-leaf emergence spray

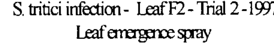


Spray Date 14/5/97

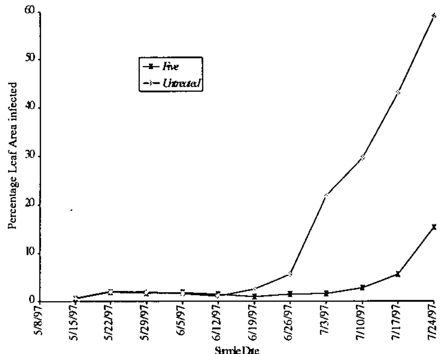
Tebuconazole Residue- Leaf F2 - Trial 2 -1997
1 week pre-leaf emergence spray



Spray Date 14/5/97

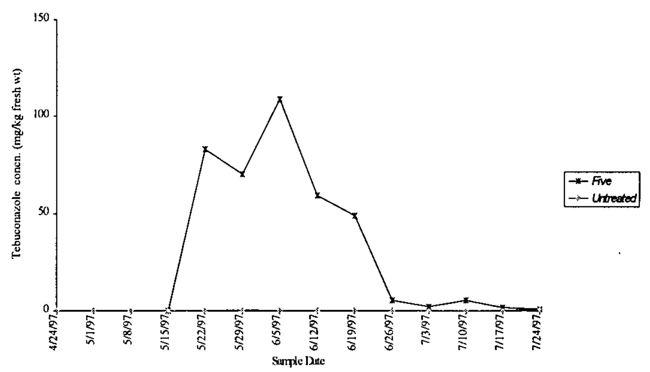


S. tritici infection - Leaf F2 - Trial 2 -1997
Leaf emergence spray



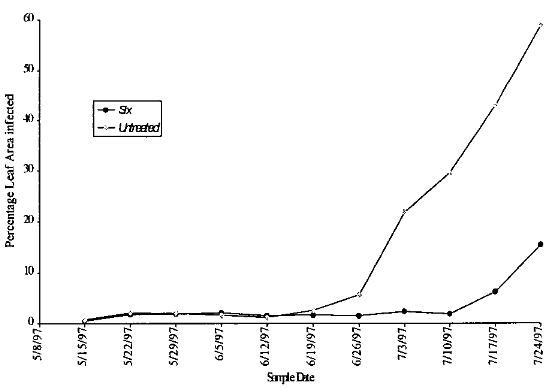
Spray Date 22/5/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
Sprayed at leaf emergence



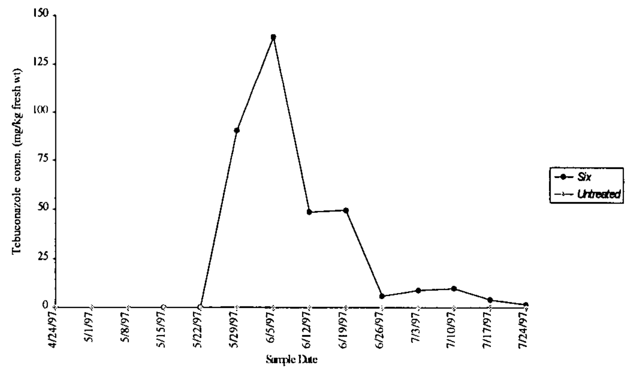
Spray Date 22/5/97

S. tritici infection - Leaf F2 - Trial 2 -1997
1 week post-leaf emergence spray



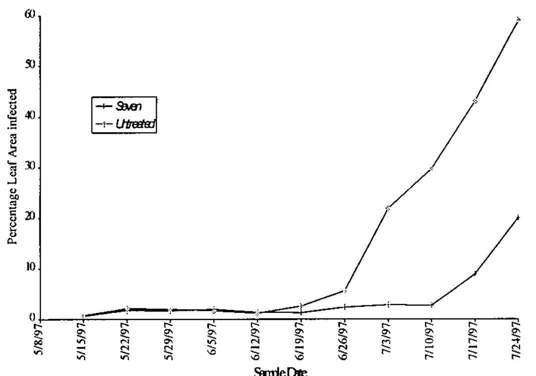
Spray Date 28/5/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
1 week post -leaf emergence spray



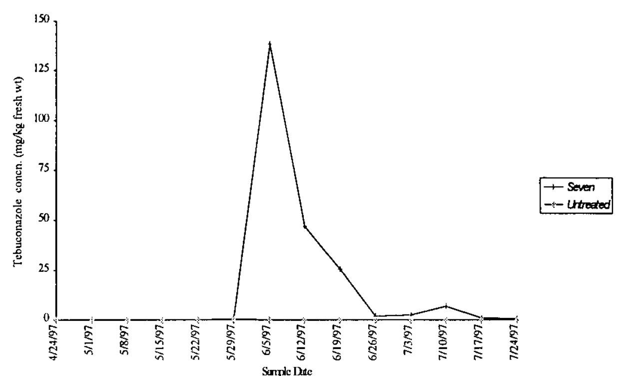
Spray Date 28/5/97

S. tritici infection - Leaf F2 - Trial 2 -1997
2 weeks post-leaf emergence spray



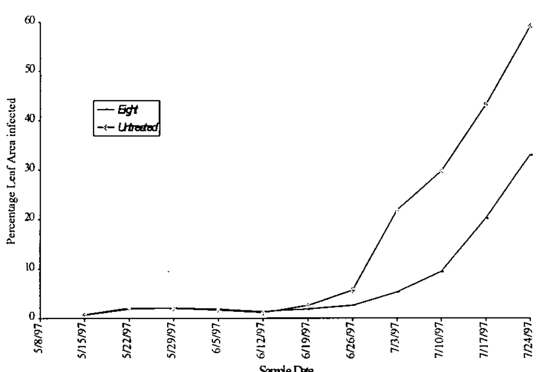
Spray Date 4/6/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
2 weeks post-leaf emergence spray



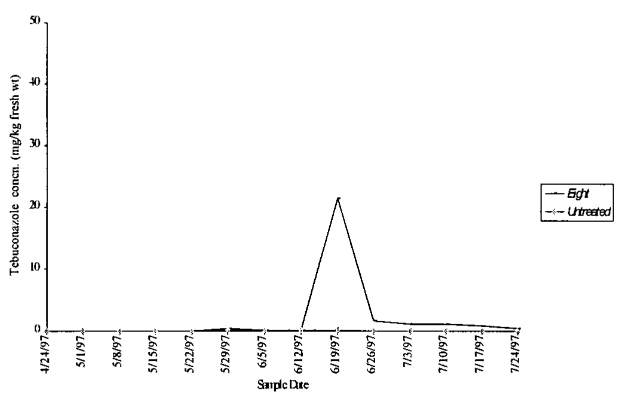
Spray Date 4/6/97

S. tritici infection - Leaf F2 - Trial 2 -1997
3 weeks post-leaf emergence spray

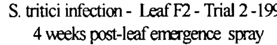


Spray Date 16/6/97

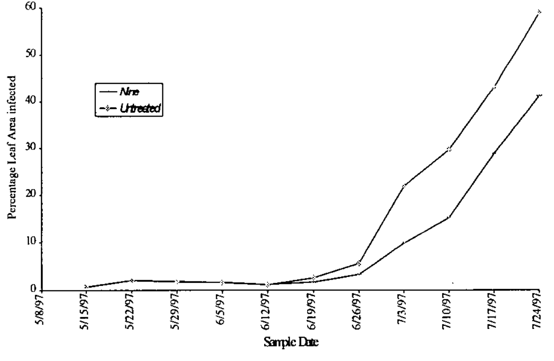
Tebuconazole Residue- Leaf F2 - Trial 2 -1997
3 weeks post-leaf emergence spray



Spray Date 16/6/97

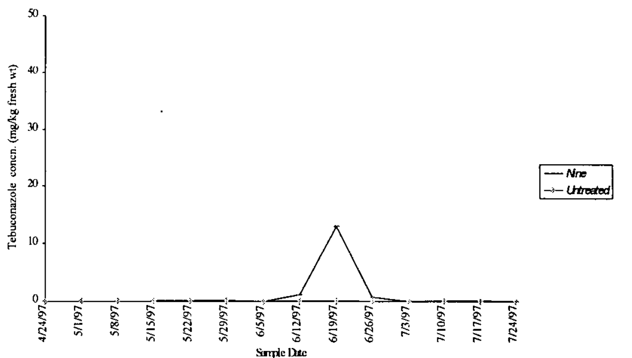


S. tritici infection - Leaf F2 - Trial 2 -1997
4 weeks post-leaf emergence spray



Spray Date 18/6/97

Tebuconazole Residue- Leaf F2 - Trial 2 -1997
4 weeks post-leaf emergence spray



Spray Date 18/6/97