DEPARTMENT for ENVIRONMENT, FOOD and RURAL AFFAIRS

CSG 15

Research and Development

Final Project Report

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Project title	Chrysanthemum White Rust: Estimation of prevalence of strains of Puccinia horiana on chrysanthemum tolerant to strobilurin and triazole fungicides and the development of a protocol for identifying tolerant strains.
DEFRA project code	**PHO 182**
Contractor organisation and location	Horticultural Development Council Bradbourne House, East Malling, Kent, ME19 6DZ
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Executive summary (maximum 2 sides A4)

For the past twenty years, Chrysanthemum White Rust (CWR) caused by the fungus *Puccinia horiana,* has been effectively controlled by triazole fungicides, e.g. propiconazole (as Tilt), and more recently in conjunction with strolilurin fungicides e.g. azoxystrobin (as Amistar). However in 1999, some growers reported a loss of control, which the Central Science Laboratory (CSL) confirmed was due to the development of fungicide tolerance (resistance). The following year, strains of *P. horiana* tolerant to both propiconazole (as Tilt or it's replacement Bumper) and azoxystrobin (as Amistar) were identified by CSL. This project aimed to determine the prevalence of fungicide resistance to both strobilurin and triazole fungicides and to investigate more rapid methods for identifying tolerant isolates.

Currently, tests determining fungicide activity are performed in a 5 week *in planta* test on artificially infected plants because the fungus cannot be cultured outside host tissue. A more rapid test is highly desirable so that growers can identify if they have tolerant isolates on site in order to select the most appropriate control measures. Recent research has identified genes responsible for strobilurin and triazole tolerance in other fungi. This project aimed to investigate whether strobilurin and triazole tolerance in *P. horiana* is controlled by the

same genes responsible for tolerance in these other fungi and to investigate in a step-wise approach whether these gene markers could be used in development of a rapid diagnostic service screening for tolerance.

A questionnaire was devised to determine the prevalence of fungicide tolerance in England and Wales and to source material for the study. Growers experiencing poor disease control were asked to send CWR samples to CSL for *in planta* testing. In addition, ad hoc samples were received from the Plant Health and Seeds Inspectorate (PHSI) and the Agriculture, Development and Advisory Service (ADAS). CSL received 41 samples in total. Of these, 8 (from 5 sites) were typed *in planta* as tolerant of propiconazole (Bumper) only, 3 (from 3 sites) were tolerant of azoxystrobin (Amistar) only, 4 (from 3 sites) were tolerant of both propiconazole and azoxystrobin and 6 (from 4 sites) where sensitive to both fungicides. The remaining 20 samples (from 10 sites) could not be multiplied on receptor plants or were multiplied but there were too few pustules for fungicide testing.

To investigate whether the gene for strobilurin tolerance in CWR was the same gene as that linked to strobilurin tolerance in certain other fungi – such as wheat powdery mildew, DNA from tolerant and sensitive CWR pustules was extracted using a commercial kit, together with DNA from *Blumeria graminis* (wheat powdery mildew - the main fungal species in published work (Fraaije *et al*, 2000)). The PCR (polymerase chain reaction) primer set CBF1/CBR3 was used to amplify the region of the cytochrome b gene with the G143A mutation, which confers tolerance of strobilurin in *B. graminis*. For *B. graminis*, the correct sized amplicon was successfully produced for both sensitive and tolerant isolates, though not on all occasions. A possible explanation for this may be that only partial annealing of the primers was occurring. For CWR, DNA from sensitive pustules was amplified sporadically and DNA from tolerant pustules could not be amplified. A possible explanation for this may be that tolerant pustules may contain chemicals that inhibit the PCR reaction. Due to difficulties of reproducibility with the CBF1/CBR3 primer set, a second set of cytochrome b primers STBF and STBR (Conyers, CSL, unpublished) were used to amplify the region of the cytochrome b gene containing the G143A mutation. These primers always amplified DNA from the *B. graminis* samples. There was no amplification however using DNA from azoxystrobin tolerant CWR pustules, though amplification was achieved on one occasion with one sensitive CWR isolate. However, this could not be repeated and sequence data could not prove that the amplicon was that of the cytochrome b region. In conclusion, although the technology could be reproduced on isolates of *B. graminis*, the technology could not be extended for use on CWR.

For investigating whether the gene for triazole tolerance was the same in CWR as that published for other fungi e.g. *Uncinular necator* (grapevine powdery mildew) DNA from CWR pustules was tested with the primer pair C14/C14R, designed to amplify the P-450_{14DM} gene with the tolerant mutation. Neither sensitive nor tolerant P. *horiana* DNA was amplified. The failure of the primer pair C14/C14R to amplify *P. horiana* could be explained by the specificity of the primers to *U. necator.* These findings were substantiated through communication with Dr. C Delye, the author of the original work on *U. necator* who confirmed that these primers were designed to be species specific to *U. nectator* and therefore would not be guaranteed to amplify DNA from *P. horiana*. The published work on *U. necator* was reproduced at CSL, but, as with the strobilurin work, could not be transferred to *P. horiana*. In conclusion, it was not possible to determine if the genes governing strobilurin and triazole tolerance in other fungi were responsible for tolerance of these fungicides by *P. horiana*. The published strobilurin and triazole work was reproduced at CSL on the published species (ascomycetes), but, the technology could not be transferred to *P. horiana* (basidiomycete). A comprehensive literature review revealed that this was the first time that tolerance studies for strobilurin and triazole fungicides had been attempted on a basidiomycete.

Scientific report (maximum 20 sides A4)

INTRODUCTION

For two decades, propiconazole (as Tilt/Bumper) has controlled CWR caused by *P. horiana* on *Dendranthemum morifolium* in the UK (Dickens, 1991). It has been known for a long time that tolerance to an unrelated group of fungicides (oxycarboxin and benodanil) was present in continental Europe (Dirske *et al*., 1982). However, from March 1999, reports began to accumulate of growers in the Midlands failing to control the disease with propiconazole on various cultivars. In September 1999, samples of cv. White Fresco with suspected tolerance to triazole fungicides from a Manchester nursery were examined at CSL. The tests showed unequivocally that this isolate was tolerant to five times the maximum permitted concentration of propiconazole. It was also tolerant to the same high concentration of another triazole, myclobutanil (as Systhane). However, the strobilurin fungicide azoxystrobin (as Amistar), shown to be effective on outdoor chrysanthemums (O'Neill & Pye, 1997), was still effective against this particular isolate.

In August 2000, growers in Cambridgeshire and West Sussex failed to control CWR with both propiconazole and azoxystrobin on cultivars including White Fresco, White Reagan and Dark Rosy Reagan. The presence of a strain of CWR tolerant to both triazoles and strobilurins (doubly tolerant) was suspected and tests confirmed that isolates from these sites were tolerant to both fungicides. Moreover, in laboratory experiments they were tolerant to protectant as well as to curative sprays of these fungicides. All the while, an isolate collected from Wales, where no fungicide sprays had been used, remained sensitive to both chemicals applied either as curative or protectant sprays in laboratory experiments (Cook, 2001).

The current testing process for CWR fungicide tolerance testing is very time-consuming and laborious because the rust is an obligate parasite and cannot be cultured outside living host tissue. Therefore, an *in planta* test is currently used, but, this requires at least 5 weeks to complete. A more rapid test is highly desirable to enable growers to quickly identify if they have tolerant isolates and to allow them to select an appropriate control strategy. Recent research on strobilurin tolerance in other fungi (e.g. Ascomycetes - *Alternaria, Blumeria, Sphaerotheca* and *Mycosphaerella* and the Oomycetes - *Plasmopara* and *Pseudoperonospora)* has shown that it is controlled by a single gene (Fraaije *et al*., 2000, Heaney *et al*., 2000). It was thought likely that the same gene may be responsible for strobilurin tolerance in CWR. Research on triazole tolerance in powdery mildews (as a sterol demethylation-inhibiting fungicide or DMI) has indicated that this involves a gene in another part of the genome (Delye *et al*., 1997). This project aimed to investigate whether this technology could be transferred from these other fungi and applied to CWR to develop a rapid diagnostic test for fungicide tolerance to strobilurins and triazoles.

AIMS, OBJECTIVES AND MILESTONES

Aims

To estimate prevalence of CWR tolerant strains to strobilurin and/or triazole fungicides and to develop a protocol for their identification.

Specific Objectives

1. To assess the extent of fungicide tolerance in England and Wales and to determine sources of test isolates by way of a questionnaire to growers.
2. To investigate whether strobilurin tolerance in CWR is caused by the same gene as in certain other fungi, enabling the development of a molecular diagnostic protocol.
3. To investigate whether triazole tolerance in CWR is caused by the same gene as in other fungi, enabling the development of a molecular diagnostic protocol.
4. Identify if CWR fungicide tolerance has the same genetic basis as described for other fungi and use this to develop diagnostic tests for tolerant strains.

Primary Milestones

01 Obtain, maintain and test *P. horiana* strains from growers *in planta* for fungicide tolerance.

02 Determine if the published strobilurin tolerance gene is also responsible for tolerance in CWR.

03 Determine if the published triazole tolerance gene is also responsible for tolerance in CWR.

04 Review objectives and produce testing protocol for grower material if objectives 2 and 3 are successful.

Secondary Milestone

S 1.1 Produce grower questionnaire.

S 1.2 Send questionnaires to identified growers.

S 1.3 Analyse questionnaire and identify growers from which *P. horiana* strains can be obtained.

S 1.4 Obtain CWR strains from growers.

S 1.5 Test the obtained grower strains *in planta* for fungicide tolerance.

S 2.1 Maintain CWR isolates.

S 2.2 Develop method to extract DNA from CWR pustules.

S 2.3 Analyse stored DNA for the published strobilurin tolerance gene.

S 2.4 Produce assay to detect the strobilurin tolerance gene.

S 2.5 Use assay on the *P. horiana* strains from 1.4.

S 3.1 Assess a protocol to detect triazole tolerance in *P. horiana* strains.

S 4.1 Review objectives 2 and 3 and if successful, develop a rapid service for typing tolerance.

OBJECTIVE 1: To assess the extent of fungicide tolerance in England and Wales and to identify potential sources of test isolates by way of a questionnaire to growers.

Grower questionnaire

A questionnaire, produced by the Horticulture Development Council (HDC), Fargro Ltd, Southern Glasshouse Produce (SGP) and CSL asking for reports of CWR incidence, details of fungicide spray regime, environmental conditions and efficacy of control strategies was circulated to 221 HDC chrysanthemum growers in England and Wales (S1.1 and S1.2). Thirty-three survey forms (15 %) were returned. Twenty-two growers, identified by the questionnaire as experiencing problems with controlling CWR with fungicides, were asked to send samples to CSL for *in planta* testing (S1.3). Of these, 12 growers sent 24 samples to CSL (S1.4). A further 17 samples from eight growers were received from the PHSI and ADAS following routine nursery visits. In total, CSL received 41 samples of CWR. All samples were allocated a code (Appendix 1). Further information on growers who were surveyed and samples that were sent to CSL can be found in Appendix 2.

Fungicide tolerance testing in planta

Method

Three infected leaves from each submitted sample were stored at -80°C for molecular analysis (S2.1, Appendix 1; i). The remaining leaves were used to bulk up infected material for fungicide sensitivity tests. This was achieved following a method adapted from Dickens, 1991 which involved suspending infected leaves, pustule side downwards, above ten healthy receptor plants (c.v. Sunny Margaret) in a humid chamber in a glasshouse maintained at 18°C and natural light. After 3 days, these plants were removed from the chamber and placed on the glasshouse bench. Healthy, untreated control plants were placed amongst the test plants to monitor potential cross contamination (sentinel plants). Plants were watered from beneath as required. When the first symptoms of CWR appeared on the plants which had previously been in the infection chamber (i.e. 7-10 days after exposure to *P. horiana*), three infected leaves were removed and stored at -80°C as source material for molecular studies (Appendix 1; ii). These plants were then suspended in a humid chamber above three plants, which had been sprayed to run-off using a hand held sprayer with one of the following treatments:

- Water
- Propiconazole (Bumper) one application at 100ppm
- Propiconazole (Bumper) one application at 500ppm
- Azoxystrobin (Amistar) one application at 100ppm
- Azoxystrobin (Amistar) one application at 500ppm

For azoxystrobin spray concentrations were equal to and five times the manufacturers recommended application rate respectively. Propiconazole was sprayed at a higher concentration (2.5 and 8 times) than recommended by the manufacturer (after methodology of Cook, 2001).

Following exposure to *P. horiana* for 3 days, the sprayed plants were removed from the humid chamber, placed on the glasshouse bench and tagged above the topmost fully expanded leaves. Sentinel plants were again inserted amongst the sprayed plants to monitor any possible cross-contamination. Following 14 and 21 days after exposure to CWR infected plants, fully expanded leaves below the tag on each sprayed plant were visually assessed for CWR pustules. Pustules on fungicide treated leaves were scored as fungicide tolerant and a sample of these infected leaves was stored at -80°C for subsequent molecular analysis (Appendix 1; iii) (S1.5).

Grower questionnaire

Grower incidence reports:

Twenty-four of the 33 growers who returned questionnaires reported CWR infection on their sites over the periods surveyed (Table 1). These outbreaks were catagorised into 4 groups based on the % area of each site infected (Table 2). The growers responses revealed the following:

- CWR infection was observed throughout the year.
- Three high level outbreaks (>50%) were reported during the period Jan- Aug 00 (Sites G, I and M).
- During the autumn period Sept - Dec 00 more outbreaks were reported with an increase in the total area affected, but only one outbreak (Site C) affected >50% of production area.
- Fewer outbreaks occurred during the period Jan - July 01 with diminishing % areas affected on each site (except for A and L).
- For Aug 01 onwards - even though there were outbreaks on 12 sites - the total production area affected was much reduced (8,206m^2 v. 27,073m^2 the previous autumn) and the infection areas on 11 sites were 20% or below. Only Site L had an increase in the overall area affected (>50%).

Whilst it is difficult to draw detailed conclusions from this data set the main trends indicate that more severe and extensive CWR outbreaks occurred during 2000 especially in autumn 00 than in 2001. The majority of growers appeared to achieve reasonable control of CWR, especially those which were able to bring severe infections under control (Sites C, G, I and M) although others (A, B, K and L) appeared to have persistent problems.

Table 1 Period when CWR infection occurred on 24 of the 33 survey sites and production area (m^2) affected by CWR – (% of total production area for the affected site).

	Survey period			
Grower	Jan-Aug 00	Sept-Dec 00	Jan-July 01	Aug 01-onwards
A	5300 (20)	5300 (20)	7950 (20)	N/D
B	1800 (20)	3600 (40)	1800 (20)	1800 (20)
C	-	11200 (100)	-	900 (15)
D	-	710 (5)	-	710 (5)
E	-	-	2112 (30)	-
F	-	800 (13)	-	1100 (18)
G	3600 (64)	-	-	-
H	-	-	-	1600 (10)
I	2800 (70)	-	-	-
K	-	1920 (40)	240 (5)	960 (20)
L	260 (20)	130 (10)	520 (40)	780 (60)
M	7500 (75)	340 (2)	-	170 (1)
N	-	87 (30)	29 (10)	6 (2)
O	-	-	30 (10)	-
P	-	-	120 (10)	-
Q	-	1300 (16)	-	-
R	-	16 (2)	-	-
S	1240 (10)	620 (5)	-	-
T	-	1050 (15)	-	-
U	515 (18)	N/D	-	-
Cc	-	-	-	N/D
Pp	225 (1.5)	-	-	-
1	-	-	-	180 (11)
3	415 (2)	-	-	-
Number of infected sites	10	14	8	12
Total area affected (m^2).	23,655	27,073	12,801	8,206
Mean area affected (%)	30	23	18	16

On three of the infected sites (A, U and Cc) for three periods no infection data was given (N/D). No production figures were available for two of the sites on which no CWR was observed, whilst for the remaining 31 sites the total chrysanthemum production area covered by the survey was 238,894m^2.

Table 2. Degrees of site infection relative to survey periods.

	Number of sites infected with CWR out of the 33 sites surveyed			
% area of sites affected	Jan – Aug 00.	Sept - Dec 00	Jan - July 01	Aug 01 – onwards
1-10%	3	5	4	4
11-20%	4	4	2	5
21-50%	0	3	2	0
> 50%	3	1	0	1

N.B. For grower details see Table 1 and Appendix 2.

Of the 24 sites where CWR was observed, five growers suspected poor environmental conditions were responsible for failure to control CWR, four believed fungicide tolerance was the reason for poor control and eight suspected that both factors were involved (Appendix 2). While five growers did not suspect either fungicide tolerance or poor environmental control and two growers gave insufficient information for analysis.

Fungicide tolerance testing in planta

Results

Twenty-one samples were successfully typed *in planta* (Table 3). Eight isolates were typed as tolerant to propiconazole and of these, five were tolerant of propiconazole at 500 ppm (Appendix 1). Three of these isolates were typed *in planta* as tolerant of azoxystrobin and all three were tolerant of azoxystrobin at 500 ppm. Four isolates were tolerant of both fungicides: one of these was tolerant of azoxystrobin at 500 ppm; two were tolerant of propiconazole at 500ppm and six isolates were sensitive to both fungicides. Of the remaining 20 samples, six isolates were used to successfully infect receptor plants, but there were too few pustules to complete fungicide tests and 14 isolates could not be transferred. No infection was recorded on the sentinel plants in any of the trials indicating that no cross-contamination occurred.

Table 3. Results of *in planta* tests showing the number and percentage of samples typed as tolerant to one or both fungicides.

Result of *in planta* typing	Number and (%) of samples	Number of infected sites
Tolerant of Bumper only	8 (38)	4
Tolerant of Amistar only	3 (14)	3
Tolerant of Amistar and Bumper	4 (19)	3
Sensitive to Amistar and Bumper	6 (29)	4

N.B. For sample details see Appendix 1.

Interpretation of grower survey reports in light of in planta test results

a) Tolerant of propiconazole:

Growers reported nine sites with suspected tolerance of propiconazole (A, B, D, E, H, O, S, T and U; Appendix 2) but of the samples typed *in planta* only four sites displayed tolerance of propiconazole (Dd, E, F and J; Appendix 1). Site E had suspected tolerance to propiconazole and had just started to use azoxystrobin. Their control strategy during CWR outbreak was alternative sprays of triazole fungicides - propiconazole and myclobutanil (Systhane) at 7/8 day interval. Site F (3 samples) did not suspect any problems but had used an extensive propiconazole spray programme (up to 4 successive applications of propiconazole at 5-7 days interval) before switching to myclobutanil and azoxystrobin. Dd (2 samples) and J (2 samples J1 and J2) were

obtained from PHSI/ADAS and background information was not collected from these sites. (A, B, D, H and T could not be typed. O, S and U did not send samples).

b) Tolerant of azoxystrobin:

Growers reported two sites where tolerance of azoxystrobin was suspected (A and B) but unfortunately these could not be typed *in planta*. However three samples were typed *in planta* as tolerant of azoxystrobin (Aa2, Cc and Pp). Cultural and chemical information from sites Cc and Pp indicated that neither grower suspected a problem with this chemical and Cc attributed control problems to poor environmental conditions, which were conducive to disease development. Aa2 was obtained from PHSI/ADAS and background information was not collected from this site. On examination of Cc's spray regime it was noted that azoxystrobin (alone) was used routinely following an outbreak when no disease was visible. Azoxystrobin and propiconazole had been used to control the outbreak. However Pp did not report use of azoxystrobin (iprodione/Rovral and benomyl/Benlate had been used) – so the typing for tolerance to azoxystrobin is confusing but may relate to origin of isolate or previous spray history.

c) Tolerant of both propiconazole and azoxystrobin:

None of the grower survey samples were typed as tolerant of both propiconazole and azoxystrobin but three samples from PHSI/ADAS were classified in this category, Oo, W(2) and Y. Unfortunately no cultural or chemical history was obtained to aid interpretation.

d) Others:

Samples from Sites A and B, where tolerance of both propiconazole and azoxystrobin was suspected, and three sites where tolerance to propiconazole was suspected could not be typed *in planta,* either because there was insufficient original sample to infect receptor plants, or where infection did develop on receptor plants there were too few pustules to complete fungicide tests. All of the growers had applied fungicide treatments a few days prior to samples being sent into CSL and it is possible that these had killed the CWR pustules. Secondly, infective basidiospores may have been released in transit to CSL and therefore pustules were no longer capable of infecting receptor plants. A further three sites where tolerance to propiconazole was suspected were asked to provide samples for testing but, as indicated earlier, these were not received by CSL. The remaining samples classified as sensitive (Aa, J3, Qq and V) were received from PHSI/ADAS so no cultural/chemical interpretation is possible.

Discussion and Conclusions

The number of samples that could not be 'typed' *in planta* was high (49 %; Appendix 1). The failure of many isolates to infect receptor plants may have been due to insufficient fresh pustule material on the submitted

sample, efficacy of the last fungicide application prior to the sample being despatched to CSL, or premature sporidial release in transit. An experiment was completed on a single sample in the latter stages of the project to assess the viability of CWR pustules sent to CSL. This involved suspending an infected leaf over an open Petri dish containing tap water agar in a humid chamber for 24 hours. The plate was assessed for the presence of germinating basidiospores. In this case there was none and it was concluded that the pustules on this sample were no longer viable since no spores had been released under ideal conditions. A second sample which was not used in the project was assessed as above and germinating basidiospores were seen under microscopic observation. In the light of this experiment, it is recommended that CWR samples should be tested for pustule viability alongside or before the standard *in-planta* infection tests. Also, growers should be requested to send at least 20–30 infected leaves with fresh infection (white pustules).

For the samples which could be typed the *in planta* testing showed fungicide tolerance of CWR to fungicides to be high (15 out of 21 samples (71%) from 10 out of 13 sites) with tolerance to propiconazole being more prevalent than that of strobilurin (Figure 1). This supports the grower's view expressed in the survey that tolerance of fungicides, in particular propiconazole, is a problem for CWR control in crops.

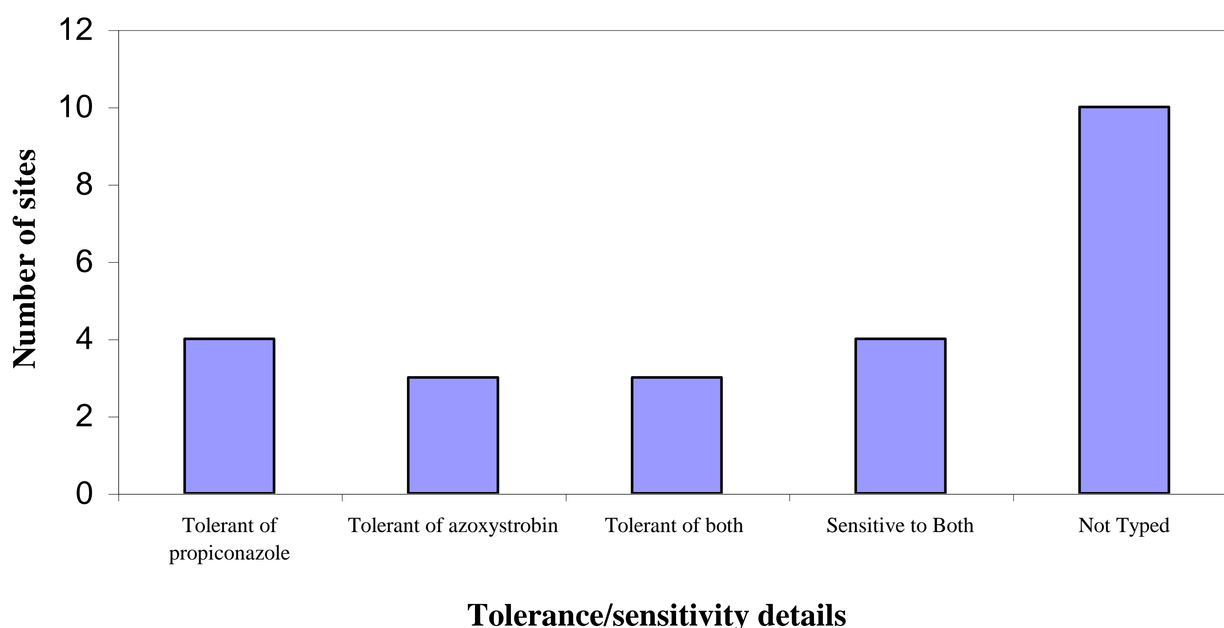

Figure 1. Number of sites typed *in planta* as tolerant of and sensitive to propiconazole and azoxystrobin.

The number of PHSI/ADAS samples that were typed (15 out of 21; 71%) was much greater than those from survey growers (6 out of 21; 29%). Of the PHSI samples, the isolates from site J should be noted (Appendix 1). In January 2002 two samples from this site were typed as being tolerant of propiconazole only (J1 and J2), then in June, two further samples were tested. This time one sample was multiplied and typed as sensitive to both fungicides (J3) while the other could not be multiplied on receptor plants (J4). Unfortunately no spray information is available for this site but it appears that in the six months between sampling either tolerance to propiconazole has been lost on this site or a new strain of CWR has been introduced to this site which is susceptible to propiconazole. Samples Aa1 and Aa2 should also be noted, these were also obtained by the PHSI from the same site, on the same day. Sample Aa1 was sensitive to both propiconazole and azoxystrobin while sample Aa2 was shown to be tolerant of azoxystrobin, demonstrating that two CWR strains can be present on the same site at the same time.

In conclusion, objective 01 was achieved; *P. horiana* strains from growers were obtained and a proportion were successfully tested *in planta* for fungicide tolerance (Figure 1).

GENERAL METHODOLOGY FOR MOLECULAR STUDIES (OBJECTIVES 2 AND 3)

Development of method to extract DNA from CWR pustules.

Method

Initially, DNA was extracted using a NucleoSpin Plant Kit, (ABgene), but the method proved inconsistent and did not always recover DNA for PCR testing. This procedure was compared with two other DNA extraction methods: a second commercial kit, DNeasy Plant Mini Kit (Qiagen) and a CTAB (cetyltrimethyl ammonium bromide) extraction method. The presence of DNA in extracted samples was confirmed either by running neat DNA on an agarose gel, or by performing PCR reactions on the samples using the universal primers ITS1 and ITS4 (White *et al.,* 1990). This primer set amplifies the internal transcribed spacer regions (ITS) and the conserved 5.8S region of the nuclear ribosomal gene. The DNeasy kit was selected for further work as it extracted DNA from both sensitive and tolerant pustules and formed brighter and more compact bands on gels than the CTAB extraction method. DNA was extracted from pustule material removed from infected receptor plants from twelve isolates. Fungal material from pustules removed from receptor plants was used since this *in planta* stage yielded the most test material. DNA was eluted into 100µl TE buffer which was aliquoted into smaller volumes (10µl) and stored at -80°C. Ten fold dilutions were prepared in molecular grade water for each sample for subsequent DNA amplification by PCR. This reduced freeze-thawing effects, thereby reducing

lyses and degradation of the DNA. Neat DNA was amplified using the universal primers ITS1 and ITS4 and visualised on an agarose gel. All primers used are described in Appendix 3.

Results

Twenty-two out of 24 samples produced PCR bands. In one sample, three bands were produced and DNA from each of these bands was sequenced and compared with sequences on the National Centre for Biotechnology Information (NCBI) nucleotide database for identification. The heaviest band (700 bp) was identified as *Dendranthemum morifolium* DNA, (cultivated chrysanthemum) and the lightest (550 bp) as *Cladosporium oxysporum,* a well documented hyperparasite of CWR. The third band (650 bp) was suspected to be *P. horiana,* although this is difficult to confirm as there are only sequences for unrelated *Puccinia* species published. Of the remainder nineteen produced an amplicon of 700 bp, two produced two PCR bands (550 bp and 700 bp).

Discussion and Conclusions

Fungal material from leaves removed from donor plants were used since the receptor *in planta* stage yielded the most test material. Original isolates sent from growers and samples from the fungicide tests did not have enough pustule material to undertake DNA extraction. Indeed, it had been noted during the *in planta* studies that the number and size of pustules decreased from the receptor stage to the fungicide treatment stage which may reflect the presence and action of the fungicide. For the propiconazole sprayed plants in particular, often, there would be three or four very tiny pustules. Experiments confirmed that 10-20 large pustules were needed to produce enough DNA to be visualised on an agarose gel (20 ng). Although, microscopic examination of pustules from infected leaves showed it to be predominantly *P. horiana* in origin, sequencing of PCR products produced using ITS1 and ITS4 showed that DNA from other organisms were preferentially amplified. This could be due to PCR inhibitors binding to the *P. horiana* DNA or it requiring different PCR primers for its amplification.

Objective 2. To investigate whether strobilurin tolerance in CWR is caused by the same gene as in other fungi, enabling the development of a molecular diagnostic protocol

Analysis of stored DNA for the published strobilurin tolerance gene

Method

For PCR detection of the strobilurin tolerance mutation G143A in the cytochrome b gene, general cytochrome b PCR primers CBF1 and CBR3 (Fraaije *et al*., 2000) were tested with DNA from the following CWR isolates:

V2 (sensitive to azoxystrobin and propiconazole); Pp (tolerant of azoxystrobin only) and Oo (tolerant of propiconazole and azoxystrobin). Two *Blumeria graminis f. sp. tritici* (wheat powdery mildew) isolates, one strobilurin sensitive, the other strobilurin tolerant, and DNA from other fungal species of unknown fungicide tolerance were run as experimental controls.

Two pair's of internal primers, 143C/R5 and 143G/R4 were also assessed which have been shown to preferentially detect either isolates of *B. graminis* with the strobilurin tolerance gene or isolates without the mutation (*Fraaije et al*., 2000).

Results

Strobilurin sensitive *P. horiana* isolates were amplified occasionally with the cytochrome b primer set but amplification of strobilurin tolerant *P. horiana* isolates was not achieved. Detection of the *B. graminis* controls and the other fungal species was sporadic. The internal primer set 143C/R5 however always successfully detected the *B. graminis* isolate with the mutation conferring tolerance, whilst 143G/R4 only amplified the sensitive *B. graminis* isolate *P. horiana* DNA was not detected by either primer set.

Discussion and Conclusions

Although CBRF1/CBR3 primers were designed to be a general fungal primer set, it was not possible to amplify the region of the cytochrome b gene of interest with *P. horiana* DNA. Despite repeated efforts to optimise and develop the PCR reaction including working with other scientists at CSL and Dr. Fraaije (the original author of the work) amplification of strobilurin tolerant *P. horiana* isolates was not possible. As it was not possible to confirm if the tolerance gene in *B. graminis* is responsible for tolerance in *P. horiana* by way of the published universal cytochrome b primers CBF1/CBR3, further efforts to obtain the information by using a second set of cytochrome b flanking primers known to amplify the area containing the gene responsible for strobilurin tolerance were taken. Christine Conyers (CSL) designed this primer set, STBF and STBR from sequences of *B. graminis*. Initially, these primers showed great promise as two sensitive CWR isolates were amplified together with the *B. graminis* controls. In subsequent experiments, however, *P. horiana* DNA was not amplified, although *B. graminis* was successfully amplified. A possible explanation for this may be that only partial annealing of the primers was occurring allowing sporadic amplification of *P. horiana* DNA, or the DNA extracted was of too poor a quality for reproducible PCR amplification. From the amplified bands, CWR sensitive isolates were sequenced. The sequence data was found to contain numerous base pair mismatches indicative of non-optimal PCR conditions, making sequence interpretation difficult. If the sequence data had been complete, it had been our intention to design internal primers specific to *P. horiana*. However since this

was not possible, two sets of forward and reverse degenerate primers inside STBF and STBR at points of partial sequence were designed (CWRCBF1/CWRCBR1 and CWRCBF2/CWRCBR2) to amplify a readable section of the cytochrome b sequence. These primer sets were run with STBF and STBR in different combinations. However, neither *P. horiana* DNA nor *B. graminis* DNA were amplified. The most likely reason that *B. graminis* was not amplified was that the new primers were complementary to *B. graminis* DNA, while, optimal PCR conditions for amplification of *P. horiana* could not be achieved. Extensive optimisation experiments on a gradient thermocycler using the two sets of in-house primers were undertaken to amplify the portion of the cytochrome b gene of interest. Unfortunately, since amplification and sequencing of CWR isolates was not achieved it was not possible to determine if the G143A mutation was present. One possible explanation for this may be that *P. horiana* is a basidiomycete. All the published strobilurin work has been completed on ascomycetes and oomycetes thus basidiomycete research may require the use of other primers to amplify the area of interest with the cytochrome b gene. Secondly, it is possible that the *P. horiana* DNA in the extracted DNA samples was not amplifiable and contained PCR inhibitors. The internal primers worked well replicating the results on *B. graminis* (Fraaije *et al*., 2000).

In conclusion, milestone S 2.2 was completed. Some stored DNA was analysed for the published strobilurin resistance gene. However, milestones S 2.3, 2.4 and 2.5 could not be achieved, as it was not possible to determine if the published tolerance gene in *B. graminis* is also responsible for strobilurin tolerance in *P. horiana*. Experiments in the published strobilurin literature were repeated at CSL for *B. graminis,* however, the strobilurin tolerance technology could not be transferred to *P. horiana*. Hence the overall aim of objective 2 could not be achieved. This study did however, demonstrate that information from published sources on genes for strobilurin tolerance and associated molecular methodology did not transfer readily to CWR. Hence, in order to develop a rapid diagnostic test for growers experiencing CWR fungicide tolerance problems on their nurseries more strategic research is needed. Initial research should concentrate on examining ways of extracting DNA from basidiospores released by *P. horiana.* This would ensure that pure *P. horiana* DNA is extracted and that no DNA from other fungal species that live saprophytically on CWR pustules or host DNA is assessed. Also, if it was possible to add basidiospores directly to the PCR reaction, the speed of the diagnostic test would be increased, enabling growers to select appropriate control measures within 48hrs of dispatch of the original CWR sample. Research on *P. horiana* would then facilitate better analysis of genes that have been shown in other fungi to contain mutations conferring fungicide resistance e.g. cytochrome B gene.

OBJECTIVE 3: To investigate whether triazole tolerance in CWR is caused by the same gene as in other fungi, enabling the development of a molecular diagnostic protocol.

Methods

Flanking primers, C14/C14R (*Delye et al., 1997*), which amplify the P-450_{14DM} gene which contains the mutation thought to govern triazole tolerance, were screened against four *U. necator* isolates (grapevine powdery mildew). The *U. necator* isolates were provided by Dr. MF Corio-Costet of the Institut National de la Recherche Agronomique, an author of the original work. Two of the isolates were tolerant strains with the MUT136 mutation and two were sensitive. Additionally, nine *Rhyncosporium secalis* isolates of known varying sensitivities to the triazoles, epoxiconazole and flusilazole (Dr. L.Cooke, Queens University Belfast) and three CWR isolates, V2 (sensitive to propiconazole and azoxystrobin), J2 (tolerant of propiconazole only) and Oo (tolerant of propiconazole and azoxystrobin) were tested with the primer set. Internal primers MUT1 and U14DM (Delye *et al,* 1997) which were designed specifically to amplify P-450_{14DM} sequences exhibiting the A-T mutation at codon 136 were also screened against the four *U. necator* isolates, *P. horiana* and *B. graminis*.

Results

C14/C14R amplified all four *U. necator* isolates but not DNA from the other fungi. The internal primers MUT1 and U14DM amplified as expected both triazole tolerant *U. necator* samples, whilst neither of the sensitive *U. necator* isolates or DNA from the other fungi were amplified. The failure of the primers to amplify *P. horiana* DNA was discussed with C. Delye (an original author of the work) who confirmed that this result was not unexpected since his primers were designed to *U. necator* and could not be guaranteed to amplify DNA from other fungal species. In conclusion, it was not possible to confirm that the same gene conferring tolerance in *U. necator* is causing tolerance in *P. horiana* (03). This was most likely due to the specificity of the published primer set to *U. necator*. The experiments outlined in the published work were reproduced successfully at CSL, however the method was not transferable to *P. horiana*. A search was undertaken to find other triazole primer sets, but, none were identified.

OBJECTIVE 4: To review the project and if tolerant genes are identified to develop diagnostic test to identify tolerant strains

Unfortunately, as the published techniques for detection of strobilurin and triazole fungicide tolerance genes in other specific fungi could not be used for *P. horiana*, it was not possible to develop diagnostic tests to identify

tolerant strains. Further, more strategic research is needed to develop a rapid test for identifying *P. horiana* strains tolerant to strobilurin and triazole fungicides. Research should centre on extracting pure *P. horiana* DNA either from basidiospores or from CWR pustules. The former would be more appropriate, since it would guarantee that pure *P. horiana* DNA was extracted as DNA extracted from pustule material may contain small amounts of host and saprophyte DNA. Secondly, pure *P. horiana* DNA would make it possible to sequence genes that contain mutations conferring strobilurin and triazole resistance and design primers specific to *P. horiana.*

OVERALL CONCLUSIONS

- A questionnaire was sent to growers to estimate the prevalence of CWR in England and Wales. Of the questionnaires sent out by the HDC, 33 (15%) were completed and returned.
- Forty-one CWR samples were obtained from growers: 8 (4 sites) were characterised *in planta* as tolerant of propiconazole (Bumper) only; 3 (3 sites) were tolerant of azoxystrobin (Amistar) only; 4 (3 sites) were tolerant of both propiconazole and azoxystrobin; and 6 (4 sites) were sensitive to both fungicides. Twenty samples could not be multiplied on receptor plants for fungicide screening.
- The ability to multiply up inoculum for *in planta* testing was dependent on the quality of samples received. e.g. samples recently sprayed with fungicide or old looking (grey / black) could not be multiplied up on receptor plants. Therefore sample procedure has been reviewed and it is recommended that growers submit at least 15-20 leaves each containing several healthy, white, viable pustules for testing. This approach was validated on later samples submitted by the PHSI and ADAS.
- It is also recommended that *in planta* fungicide testing should be supplemented by an initial *in vitro* test for basidiospore germination from the pustules received. This will provide basic, quick information on the potential for CWR infection, which will enable additional samples to be obtained quickly if the original samples have poor levels of germination.
- It was not possible to determine if the gene responsible for strobilurin tolerance in other fungi was the same governing tolerance in *P. horiana* since the published primer set did not amplify *P. horiana* DNA routinely, probably because only partial annealing of the primers was occurring. Although the published work for this gene was reproduced consistently on *B. graminis,* the technology could not be transferred to *P. horiana*. A second set of cytochrome b primers were used to amplify the potential area containing the gene responsible for strobilurin tolerance in *P. horiana.* However despite comprehensive PCR optimisation experiments conditions could not be identified for the amplification of *P. horiana* DNA.
- It was not possible to determine if the gene responsible for triazole resistance in other fungi was the same governing tolerance in *P. horiana* using primers designed to *U. nectator.* The published work was reproduced on *U. necator* but the technology could not be transferred to *P. horiana*.
- More strategic research is needed to develop a rapid molecular test for identifying *P. horiana* strains tolerant to strobilurin and triazole fungicides.
- Further research should concentrate on extracting pure DNA directly from basidiospores of this obligate pathogen. This would enable unequivocal sequencing of genes of interest, e.g the cytochrome b gene, the P-450_{14DM} or other genes, which may be responsible for fungicide tolerance. This research would then allow isolates, such as those typed in this project, to be compared on a molecular level.

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Appendix 1. Source of CWR pustules and tolerance/sensitivity details of samples frozen at -80°C for molecular analysis (isolates coded relative to origin).

ISOLATE DETAILS	**SOURCE OF PUSTULES**		
	(i) Pustules removed from original sample	**(ii) Pustules removed from receptor plants**	**(iii) Pustules removed from plants sprayed with fungicides**
Tolerant of propiconazole (Bumper) only.	Dd1+	Dd1	Dd1*
	Dd2+	Dd2	Dd2
	E1	E1	-
	F1	F1	-
	F2	F2	F2*
	-	F3	F3*
	J1+	J1	J1*
	J2+	J2	J2*
Tolerant of azoxystrobin (Amistar) only.	-	-	Cc**
	-	-	Aa2**+
	Pp	Pp	Pp**
Tolerant to both propiconazole (Bumper) and azoxystrobin (Amistar) at 100ppm (a.i).	Oo+	Oo	Oo**
	W1+	W1	W1
	W2+	W2	W2*
	Y+	Y	Y*
Sensitive to both propiconazole (Bumper) and azoxystrobin (Amistar) at 100ppm (a.i).	Aa1+	Aa1	Aa1
	J3+	J3	J3
	Qq+	Qq	Qq
	V2 +	V2	V2
	V3+	V3	V3
	V4+	V4	V4
Isolates that could not be transferred to receptor plants.	A1		
	A2		
	B1		
	B2		
	B5		
	B7		
	C		
	D		
	H		
	J4+		
	K		
	T1		
	T2		
	V1+		
Isolates that were transferred to receptor plants but produced too few pustules for fungicide testing.	-	A3	
	-	A4	
	-	B3	
	-	B4	
	-	B6	
	I	I	
Total	**33**	**25**	**19**

Letters without + are samples collected from survey growers. Number after grower letter indicates multiple samples

* Isolates tolerant of propiconazole (Bumper) at 500ppm (a.i.)

** Isolates tolerant of azoxystrobin (Amistar) at 500ppm (a.i.)

\+ PHSI/ADAS samples. Number after grower letter indicates multiple samples

- Samples that were not stored at -80°C

Appendix 2

Spray regime and environmental control details of Chrysanthemum growers in survey

Group	CSL Ref	Environmental Controls			Spray Regime	Suspected reason for poor control	
		Heating	Ventilation	Irrigation		Environment	Tolerance
SURVEY ISOLATES TYPED *IN PLANTA* AS TOLERANT OF PROPICONAZOLE (BUMPER)	E	X	X	X	During CWR outbreak	√	Tilt
	F (3)	√	√	√	During CWR outbreak	X	X
SURVEY ISOLATES TYPED *IN PLANTA* AS TOLERANT OF AZOXYSTROBIN (AMISTAR)	Cc	√	√	√	Seasonal During CWR outbreak	√	X
	Pp	√	√	√	Year round	X	X
SURVEY SITES WITH CWR THAT SENT ISOLATES TO CSL BUT COULD NOT BE TYPED *IN PLANTA*	A (4)	√	√	X	Year round	√	Bravo, Amistar, Tilt
	B (7)	√	√	X	Year round Seasonal	√	Amistar, Tilt
	C	√	√	√	Year round Seasonal	-	-
	D	√	√	X	Seasonal	√	Tilt
	H	√	√	X	Year round Seasonal	X	Tilt
	I	√	√	√	During CWR outbreak	X	-
	K	X	√	X	Seasonal	√	X
	T (2)	√	√	√	Year round During CWR outbreak	X	Tilt
SURVEY SITES WITH CWR THAT WERE ASKED FOR SAMPLES BUT FROM WHOM CSL RECEIVED NO SAMPLE	G	√	√	X	During CWR outbreak	√	Tilt
	L	√	√	X	Year round	√	Plantvax, Bravo
	M	√	√	X	Year round	√	X
	N	X	X	X	Seasonal During CWR outbreak	X	X
	O	X	X	X	Year round	X	Tilt
	P	X	X	X	Seasonal	√	X
	Q	√	√	X	During CWR outbreak	X	X
	R	X	X	X	Seasonal During CWR outbreak	√	X
	S	√	√	√	During CWR outbreak	√	Tilt
	U	X	X	X	Seasonal During CWR outbreak	X	Radar
SURVEY SITES WITH CWR BUT NOT ASKED FOR SAMPLE	1	X	X	X	Year round During CWR outbreak	X	X
	3	√	√	√	During CWR outbreak	√	Bravo 500, Systhane
SURVEY SITES WITH NO CWR	4	X	X	X	-	-	-
	5	X	√	X	-	-	-
	6	X	X	X	-	-	-
	7	X	X	X	-	-	-
	8	X	X	X	Year round	-	-
	9	√	√	√	-	-	-
	10	√	√	√	-	-	-
	11	X	X	X	-	-	-
	12	X	X	X	Year round	X	X

Numbers in brackets equal the number of samples sent from the site. √ = yes, X = no. Tilt (as Bumper) (a.i. = propiconazole–triazole fungicide), Amistar (a.i. = azoxystrobin–strobilurin fungicide), Bravo & Bravo 500 (a.i.= chlorothalonil–chlorophenyl fungicide), Plantvax (a.i. = oxycarboxin–carboxamide fungicide), Systhane (a.i. = myclobutanil–conazole fungicide), Radar (a.i.= propiconazole). PHSI/ADAS samples have not been included in this table for *in planta* typing details refer to Appendix 1 (tolerant of propiconazole, sites Dd and J; tolerant of azoxystrobin, site Aa; tolerant to both Oo, W and Y; sensitive to both Aa, J, Qq and V). - = no information available.

Appendix 3. Primer sequences and combinations for PCR reactions.

Primer Name	Primer Sequence (5' to 3')
Flanking Primers (strobilurin)	
CBF1	TAT TAT GAG AGA TGT AAA TAA TGG
CBR3	CCT AAT AAT TTA TTA GGT ATA GAT CTT
Internal Primers (strobilurin)	CAG ATG AGC CAC TGG GG
143GF	TAA TAT TGC ATA GAA GGG CAG
R4	CAG ATG AGC CAC TGG GC
143CF	ACT CCG GTA CAA TAG CAG CC
R5	
	CGG ATC ATA TAG AGC ACC AAG AAC
STBF	GGA TTA CCA GAT CCT GCA CTA TCG
STBR	
	CAT TAG TAT GAA CAA TWG GTG
CWRCBF1	CAT TAA TGC TAA **R**GC AGC TAA
CWRCBR1	CAT TAG TTT GAA CWA T**W**G GTG
CWRCBF2	CAC TAT CGT GTA **R**TG CTA TTA A
CWRCBR2	
Flanking Primers (triazole)	TAA GGT AGT ATT GAG GCG GG
C14	TTC TAA CCC TAA CAC CTG CC
C14R	
Internal Primers (triazole)	ATG TAC ATT GCT GAC ATT TTG TCG G
U14DM	AAT TTG GAC AAT CAA
MUT1	
Universal Primers	TCC GTA GGT GAA CCT GCG G
ITS1	TCC TCC GCT TAT TGA TAT GC
ITS4	

Nucleotide code used in primer sequences. C= cytosine, A= alanine, G=guanine, T= thymine. Letters in bold are degenerate bases **R=** A+g; **W**= A+T

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