SESSION 7A

DISEASE FORECASTING AND DIAGNOSTICS IN ARABLE CROPS

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RESEARCH AND DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DETECTION OF THE WHEAT PATHOGENS *SEPTORIA NODORUM* AND *SEPTORIA TRITICI*

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ABSTRACT

Diagnostics have been developed which utilize double-antibody sandwich direct-enzyme immunoassay technology for the presymptomatic detection, differentiation, and quantitation of the wheat pathogens *Septoria nodorum* and *Septoria tritici*. Initial studies were conducted in 1991 to evaluate the immunoassays' performance with field samples. Development program results indicated that the immunoassays can be utilized for specific and presymptomatic detection of *S. nodorum* and *S. tritici*, determination of the amount of infection present over the growing season, monitoring of disease progression from lower to higher foliar levels, and fungicide efficacy assessment. Correlations were also obtained between visual disease ratings and immunoassay results.

INTRODUCTION

The fungal disease Septoria is economically significant in nearly all wheat-growing regions of the world. Septoria is a disease complex, manifesting either as leaf blotch, caused by *Septoria nodorum* (*Leptosphaeria nodorum*) or *Septoria tritici* (*Mycosphaerella graminicola*) infection, or glume blotch, with *S. nodorum* traditionally attributed as the sole causative agent. Leaves initially become infected at the soil level, and inoculum is transported vertically to higher foliar levels by rain splash (Shaw & Royle, 1986; Shaw, 1967). If the infection is severe enough and wet weather conditions prevail, the flag leaf becomes infected and seed filling is impaired resulting in reduced crop yields.

Early visual diagnosis of initial infection on leaves is precluded by a latent period of 5-12 days for *S. nodorum* and 20 days for *S. tritici*, after which secondary spores are produced and symptoms are expressed. In addition, accuracy of diagnosis is compromised by other foliar pathogens which produce symptoms similar in appearance to or mask Septoria lesions. Because of the lack of early, accurate disease quantitation, traditional Septoria control has involved application of fungicides on a routine schedule based on cereal growth stages rather than disease progression. This Septoria control practice has varied greatly in effectiveness.

Accurate, objective quantitation of *S. nodorum* and *S. tritici* infection before visual disease appears would facilitate the implementation of fungicide application programs which are based on disease thresholds to determine optimal spray timings and rates. Immunoassay technology is frequently used in the medical field to diagnose and quantitate a plethora of infectious agents. Recently, this technology has been applied to agriculture and crop diseases, and commercial products have been introduced to the market (Miller *et al.*, 1988; MacDonald *et al.*, 1990; Smith *et al.*, 1990). Monoclonal antibody-based immunoassays in microplate well and membrane formats have been developed previously for the differentiation and confirmation of *S. nodorum* and *S. tritici* infection in wheat (Mittermeier *et al.*, 1990; Petersen *et al.*, 1990). This paper reports the development of polyclonal antibody-based immunoassays, which facilitate specific and presymptomatic detection and quantitation of S. *nodorum* and *S. tritici*.

MATERIALS AND METHODS

Immunoassay reagents and format

Rabbit immunogens consisted of culture supernatants or cell extracts. Polyclonal antisera were purified using protein A and immunoaffinity chromatography. The resulting purified IgG was used as an "antigen capture" reagent in the double-antibody sandwich direct-enzyme immunoassays configured in microtiter plate wells. IgG was conjugated with alkaline phosphatase and used as an "antigen detector" reagent.

Immunoassay standards were prepared from culture supernatants and extracts. The standards were calibrated and assigned antigen unit values per ml (AgU/ml) based on references, which were derived from wheat leaves that were heavily infected with *S. nodorum* or *S. tritici*. The optical density of standards was measured at 405-650 nm with a microplate reader to generate a standard curve.

Immunoassay specificity and validation studies

Reactivity of common foliar fungal pathogens and epiphytes with the *S. nodorum* and *S. tritici* immunoassay antibody reagents was examined using greenhouse-infected plant material and fungal culture material prepared as previously described. Culture and plant samples were assayed using the double-antibody sandwich direct-enzyme immunoassay format. Anti-*Septoria* sera were titered employing an ELISA format utilizing *S. nodorum* or *S. tritici* antigen-coated microtiter plate wells and goat anti-rabbit IgG conjugated to alkaline phosphatase as a detector reagent.

Wheat leaves (104) were collected from fields of the varieties Scipion, Nebraska, and Thesee, which had been infected with Septoria leaf blotch at a site in France. Each leaf was cut longitudinally into two pieces. One piece was homogenized with 2.5 ml Tris-buffered saline with 0.05% tween 20 (TBST) and assayed with the *S. nodorum* and *S. tritici* immunodiagnostics. The remaining piece was washed with tap water, placed on water agar, and incubated at 20°C for 24 h. After incubation, the leaves were examined using a binocular microscope (4X) for the presence of *S. nodorum* or *S. tritici* infection. Spores exuding from pycnidia were transferred onto a microscope slide and examined at 100X. Spores were differentiated based on morphology with *S. tritici* pycnidiospores being thin and thread-like and *S. nodorum* pycnidiospores characterized as smaller with rounded ends. Visual disease assessments were compared to immunoassay results.

Field studies and sample preparation

Twenty-five winter wheat main tillers were collected per replicate using an uniform, but random sampling pattern. Untreated checks and treatments consisted of four replications (100 m^2) each. A fungicide treatment consisting of a mixture of flusilazole and carbendazim was applied at growth stage 55 at rates of 200 g ai/ha and 100 g ai/ha respectively. Samples were collected at and between first node and after milky ripe at two sites in the United Kingdom. Visual disease assessments were performed noting foliar levels infected. Plant samples were prepared by homogenizing wheat leaves with 5 ml TBST per leaf in an electric food blender. Samples consisted of either individual leaves or 25 leaves bulked by foliar level, treatment, and collection date as listed above. Each sample was assayed with the *S. nodorum* and *S. tritici* immunodiagnostics.

RESULTS

Antibody reactivity

Although *S. nodorum* and *S. tritici* have been assigned to the same asexual taxonomic genus, they were found to be immunologically distinct. *S. nodorum* polyclonal antisera did not react with *S. tritici* antigen and vice versa (Fig. 1). The immunological characterization results were consistent with the classification of the fungi in two different sexual genuses, i.e. *Leptosphaeria* and *Mycosphaerella*. These results necessitated the development of two separate immunoassays in order to accurately diagnose the Septoria disease complex. All *S. nodorum* (20) and *S. tritici* (34) strains, which originated from France, Germany, the United Kingdom, New Zealand, and the U.S.A., produced antigen that was detected with the respective immunoassays. *S. avenae* (*Leptosphaeria avenaria* f. sp. *triticea*), a less common causative agent of Septoria leaf blotch and closely related to *S. nodorum* (*L. nodorum*), was found to be reactive with *S. nodorum* antibodies, but not *S. tritici* antisera.

Reactivity of antigens from common pathogens and epiphytes of wheat with *S. nodorum* and *S. tritici* antibodies was examined. Fungi tested included: *Alternaria solani*, *Aspergillus*, *Botrytis cinerea*, *Cephalosporium*, *Cladosporium herbarum*, *Didymella exitialis*, *Erysiphe graminus*, *Fusarium avenae*, *F. culmorum*, *F. equiseti*, *F. graminarium*, *F. moniliforme*, *F. nivale*, *F. oxysporum*, *F. roseum*, *F. semitectum*, *F. solani* (*cerealis*), *F. subglutinans*, *F. tricinctum*, *Gaemannomyces graminus*, *Helminthosporium sativum*, *Leptosphaeria microscopica*, *Penicillium*, *Pseudocercosporella herpotrichoides*, *Puccinia recondita*, *Rhizoctonia cerealis*, *R. solani*, *Rhizopus*, *Rhynchosporium secalis*, and *Sclerotinia minor*. Unpurified *S. nodorum* IgG was found to recognize *A. solani*, *H. sativum*, and *L. microscopica Karsten* antigens, while cross-reactivity was detected between *S. tritici* IgG and *F. avenae*, *F. oxysporum*, and *F. tricinctum* antigens. However, *Septoria* antibody reagents were rendered specific for *S. nodorum* and *S. tritici* via immunoaffinity purification of the polyclonal IgG. Uninfected wheat leaves did not react in the *Septoria* immunoassays.

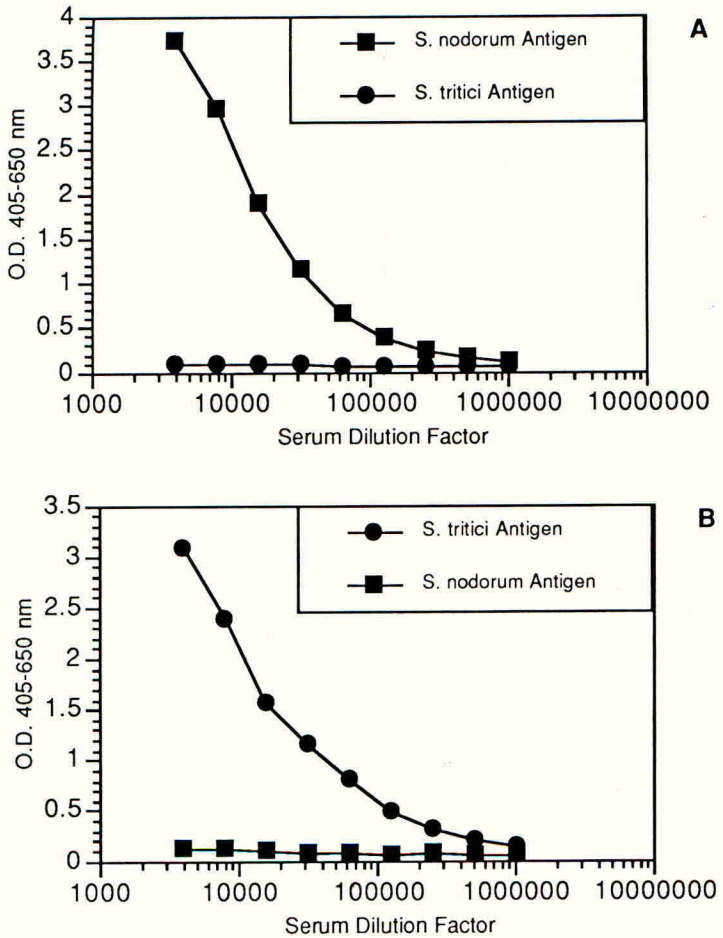

FIG. 1. (A) Anti-*S. nodorum* serum versus *S. nodorum* and *S. tritici* antigens. (B) Anti-*S. tritici* serum versus *S. tritici* and *S. nodorum* antigens.

Immunoassay standardization

The immunoassays have been designed to provide accurate quantification of the amount of *S. nodorum* or *S. tritici* infection present in wheat. Dilutions of calibrated *S. nodorum* and *S. tritici* antigen reagents are used to generate a standard curve for each immunoassay performed. The antigen concentrations of the diluted standards are expressed as AgU/ml. Standardization of the immunodiagnostics allows a comparison of assay results obtained from independent experiments. In contrast, a direct comparison of spectrophotometric measurements of wheat samples expressed in optical density units, which were obtained from independent experiments, are influenced by many uncontrolable factors including temperature fluctuations, operator precision, and minor differences in reagent activities. Figure 2 shows typical examples of standard curves for the *S. nodorum* and *S. tritici* immunoassays. Since both immunoassays have been calibrated to unique antigen references, AgU/ml values can not be compared directly between the two Septoria diagnostics (i.e. 100 AgU/ml in the *S. nodorum* immunoassay is not equivalent to 100 AgU/ml in the *S. tritici* immunoassay).

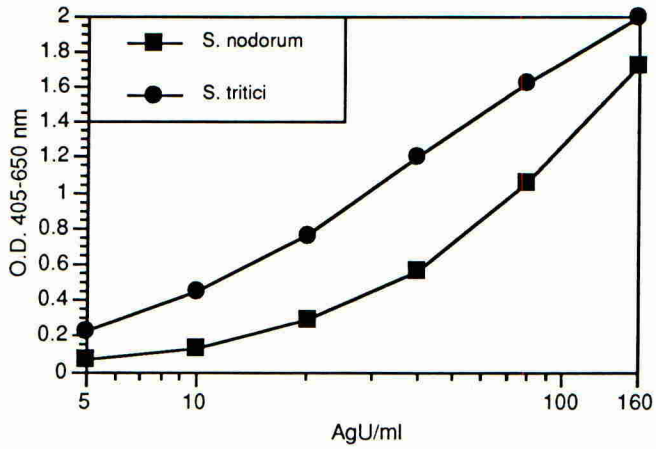

FIG. 2. Typical fungal antigen standard curves for the *S. nodorum* and *S. tritici* immunoassays.

Correlation of immunoassay results and isolation of *Septoria* from wheat leaves

A study was conducted to test the accuracy of the *S. nodorum* and *S. tritici* immunoassays. A total of 104 leaves were both assayed with the immunodiagnostics and microscopically examined for presence of *S. nodorum* and *S. tritici* infection. No false negative immunoassay results were obtained, i.e. symptom-bearing leaves with undetectable antigen. Some leaves (28% for *S. nodorum* and 5% for *S. tritici*) were found that lacked visual Septoria symptoms after incubation on water agar, but were immunoassay positive. Extremely low levels of *Septoria* infection in these leaves most likely prevented successful culturing and isolation of pycnidiospores. Antigen unit levels of asymptomatic leaves ranged from 5 AgU/ml to greater than 160 AgU/ml. These results suggest that the immunoassays are accurate and sensitive enough to detect presymptomatic infection of wheat leaves by *S. nodorum* and *S. tritici*. Figure 3 shows the relationship between isolation and visual symptom detection versus immunodiagnostic results.

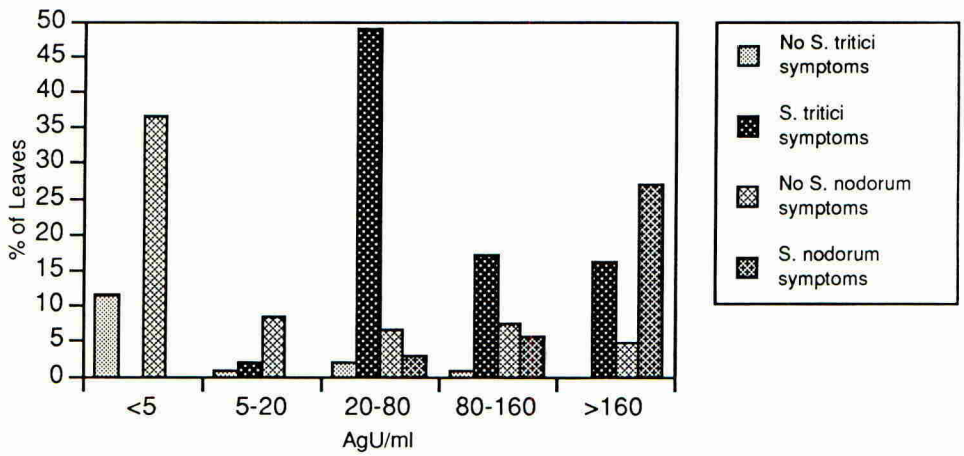

FIG. 3. Results obtained from 104 wheat leaves, which were tested with the *S. nodorum* and *S. tritici* immunoassays and visually diagnosed for infection.

Field studies

Septoria diagnosis

The *S. nodorum* and *S. tritici* immunoassays provided an objective and sensitive measurement of disease in the field. In general, quantitative results provided by the immunodiagnostics were found to be more accurate and precise than subjective visual disease assessment, particularly at the low (presymptomatic) and high (greater than 50% of the leaf area infected) points of the infection cycle. Data obtained from a field study conducted in the United Kingdom, included a total of 165 individual leaves which were assigned a visual rating of 0% Septoria infection. However, presymptomatic *S. nodorum* and/or *S. tritici* infection was immunologically detected in 73% of the leaf samples. A very strong correlation was obtained between visual disease scores and immunoassay results from the same field site as shown in Figure 4.

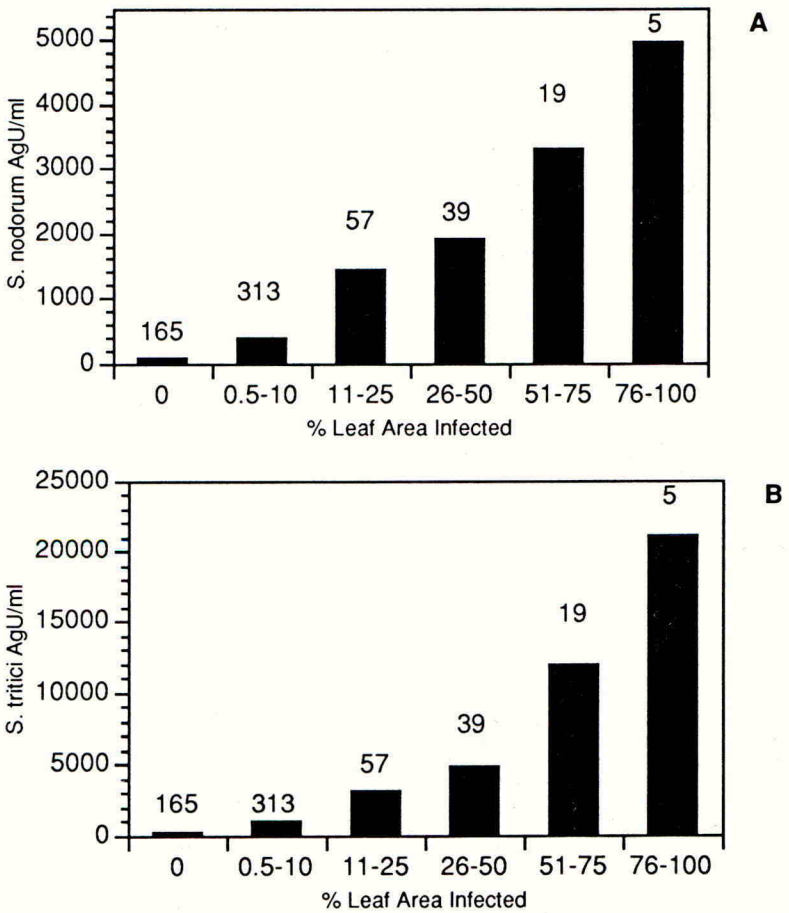

FIG. 4. (A) Visual disease scores, based on percent leaf area infected, compared to *S. nodorum* immunoassay results reported in AgU/ml. (B) Correlation between visual disease scores and *S. tritici* AgU/ml. Numbers above bars in (A) and (B) refer to number of leaves in each disease score classification.

Disease progression

The immunoassays proved to be very useful for quantitative monitoring of disease progression over the growing season and by foliar levels. *S. nodorum* and *S. tritici* antigen levels increased over time (Table 1). For example, between growth stages 51 and 73, *S. nodorum* infection on foliar level F-1 increased from 0 AgU/ml to 1397 AgU/ml. Likewise, *S. tritici* disease on F-1 increased from 0 AgU/ml to 5,313 AgU/ml at 50% head emergence to after milky ripe. Also, vertical spread of *Septoria* infection to higher foliar levels could be precisely quantitated with the use of the immunodiagnostics. Leaves closest to the soil level had the highest AgU/ml, and the antigen levels progressively decreased on upper foliage. These results agree with the current understanding of Septoria epidemiology.

Fungicide efficacy

The *S. nodorum* and *S. tritici* immunodiagnostics appear to be useful as tools for evaluating fungicide efficacy (Table 1). Although the amount of data is limited, the results obtained suggest that the fungicide application was successful in controlling the infection levels of *S. nodorum* and *S. tritici* on the flag leaf and the leaf below it. For *S. nodorum*, the flag leaves of the untreated and treated plots had respective infection levels of 426 AgU/ml and 235 AgU/ml. *S. tritici* disease in untreated flag leaves was 5,355 AgU/ml and decreased dramatically in fungicide-treated leaves to 962 AgU/ml.

Table 1. Septoria Disease Progression

Sampling Time	Foliar Level	Untreated Replicate Mean[a] *S. nodorum* AgU/ml	Flusilazole + Carbendazim[b] Replicate Mean *S. nodorum* AgU/ml	Untreated Replicate Mean *S. tritici* AgU/ml	Flusilazole + Carbendazim Replicate Mean *S. tritici* AgU/ml
First Node (31[c])	All[d]	0 (0)	0 (0)	28 (14)	32 (9)
50% Head Emergence (51)	F-1[e]	0 (0)	0 (0)	0 (0)	9 (6)
	F-2	3 (5)	13 (8)	22 (30)	21 (7)
	F-3	17 (6)	33 (7)	95 (14)	152 (91)
	F-4	46 (22)	99 (54)	279 (16)	686 (318)
After Milky Ripe (73)	F	426 (401)	235 (96)	5355 (3609)	962 (415)
	F-1	1397 (416)	699 (533)	5313 (3333)	2154 (1067)

[a]Mean of four replicates. Standard deviation is in parentheses.
[b]Applied at growth stage 55 at 200 g ai/ha for flusilazole and 100 g ai/ha for carbendazim.
[c]Cereal growth stage decimal code (Zadoks *et al.*, 1974).
[d]Sample consisted of all leaves from 25 main tillers.
[e]Foliar level designations are F for flag leaf(top leaf of main tiller), F-1 for leaf below flag leaf, etc.

DISCUSSION

The immunoassays described in this paper facilitate early, presymptomatic, quantitative detection of *S. nodorum* and *S. tritici*, which comprise the Septoria disease complex. The involvement of two major Septoria pathogens, which often infect wheat simultaneously, greatly complicates visual diagnosis and disease forecasting. In addition, the presence of pathogens that mask or look similar to Septoria symptoms may interfere with accurate visual disease assessment. Visual disease rating is also a subjective measurement, which may differ from person to person, while the immunoassays provide an objective measurement of fungal antigens enabling comparisons of disease pressure between different field sites and from year to year.

Extensive screening of major wheat fungal pathogens and epiphytes for cross-reactivity with the *S. nodorum* and *S. tritici* antibodies has been done to ensure specific detection and quantitation of Septoria. Antigen level increases, quantitated in AgU/ml, were shown to directly correlate with visual Septoria disease. Vertical disease progression was also successfully tracked by foliar level with the immunoassays. Quantitation of vertical transport of Septoria inoculum will be critical for establishing fungicide application guidelines, based on disease thresholds, for protection of the flag leaf.

The use of the Septoria immunodiagnostics will provide additional information for making fungicide application decisions. Immunoassay results, combined with disease prediction models, weather forecasts, and field history should provide the farmer with a more complete picture of Septoria disease pressure in the field. Thus, the farmer should be able to make a better informed, environmentally conscious decision on whether or not to apply a fungicide for control of Septoria.

Field study data suggested that the immunoassays may also be useful as tools for measuring fungicide efficacy. Quantitation of fungicide efficacy should allow future optimization of fungicide application timings, treatments, and rates for control of Septoria.

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FIELD EVALUATION OF AN IMMUNODIAGNOSTIC ASSAY FOR CEREAL EYESPOT

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ABSTRACT

Visual and immunodiagnostic methods were used to follow eyespot (*Pseudocercosporella herpotrichoides*) development in wheat crops at Long Ashton in 1991. Timing of inoculation, cultivar susceptibility and fungicides were used to attempt to generate plots with different eyespot levels. The immunoassay detected eyespot pre-symptomatically, and subsequently a good correlation was generally observed between visual symptoms and antigen levels. In the resistant cultivar, Rendezvous, a link between final yield and antigen levels was only evident at Growth Stage 85, and no threshold value was established to guide earlier fungicide treatments. Eyespot was more severe on the susceptible cultivar, Pastiche, and good correlations were apparent from GS 32 onwards between antigen levels and final disease severity, and these were always better than correlations based on visual symptoms.

INTRODUCTION

Effective use of fungicides depends on both accurate diagnosis and quantification in order to optimise spray timing. The advent of immunodiagnostic kits (Miller *et al.* 1988; Dewey, 1988; Petersen *et al.* 1990; Smith *et al.* 1990; Cagnieul *et al.* 1992) offers new approaches to detection and measurement of fungal diseases, which may be especially useful where visual diagnosis is difficult, or where pre-symptomatic detection is important. As with threshold criteria based on visual diagnosis, the value of serological tests is dependent on the correlations that can be established between antigenic measurements of disease levels and eventual yield loss. This requires field experiments to be carried out over a number of years during which a range of disease and environmental conditions will be encountered. Although the findings reported in this paper are based on just one year's experiment, it nevertheless describes the approach we are taking to establish an antigen threshold for guiding spray timing to control cereal eyespot (*Pseudocercosporella herpotrichoides* (Fron) Deighton) under UK conditions.

METHODS

Field experiment

A field experiment was carried out during 1990/91 on a site at Long Ashton Research Station where wheat had not been grown in at least the previous fifty years. Winter barley had, however, been grown in 1988-89, and oilseed rape in 1990. A randomised complete block design was used with three replicates and 12 m x 8 m plot sizes. A 1 m

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randomised complete block design was used with three replicates and 12 m x 8 m plot sizes. A 1 m strip around the edge of each plot was not sampled and served to isolate neighbouring plots. Cultivar differences, fungicide treatments, and inoculation timing were all used in an attempt to obtain different eyespot levels between plots. The two cultivars used were Pastiche (S) and Rendezvous (R) which differed in susceptibility to *P. herpotrichoides* (Anon. 1990). The two fungicides were prochloraz (Sportak-45) and flusilazole (Sanction). These were applied at Growth Stage (GS) 33 at recommended rates using a hand-held gas operated sprayer. Two inoculation times were used; one in November 1990, the other in February 1991. Inoculation was carried out according to the method described by Bruehl and Machtmes (1985). Five recently isolated *P. herpotrichoides* strains, all apparently rye pathotypes, were grown on autoclaved oat grains for six weeks at 10-16°C, dried and the different isolates mixed together before broadcasting onto plots at a rate of 400 ml infected grains per plot.

Sampling and visual assessments

Detailed assessments were carried out on five occasions; GS 25 (mid tillering); GS 32 (stem elongation); GS 37-39 (Flag leaf visible); GS 65 (Anthers) and GS 85 (Soft dough). Each time, 30 main shoots including their roots were randomly collected from each plot, avoiding a 1 m strip around the edge. On the first three occasions plants were rinsed with a minimum amount of tap water, whilst subsequent samples were simply shaken to remove soil. Stems were assessed visually for both occurrence and severity of eyespot generating an Eyespot Severity Index (ESI; Scott and Hollins, 1974) which ranged from zero to a maximum of 100.

Immunoassay

Assay procedures followed closely those given in the protocol notes supplied with DuPont diagnostic kits. These development kits differed from those now commercially available, since they incorporated a biotin-avidin step to enhance sensitivity (Smith *et al.* 1990). Each stem base, including leaf sheaths, was trimmed free of roots, cut into 40 mm pieces, frozen, and then either crushed in a polythene bag (GS 25), or macerated in a blender. Preliminary results showed that some additional antigen was released if extracts were allowed to stand before testing. To standardise the procedure extracts were prepared, and then allowed to stand overnight at 4°C before assay the following day.

RESULTS

Effect of cultivar on eyespot development

Eyespot symptoms were not observed on either cultivar before GS 32. Pre-symptomatic detection was possible using immunoassay, but infection and early development were no different in either cultivar. Only subsequently was development and symptom expression reduced on cv. Rendezvous compared to that on cv. Pastiche, with the result that differences were seen between cultivars from GS 37-39 onwards (Table 1). The drop in antigen levels observed at GS 37-39 may reflect a natural loss of infected leaf sheaths.

TABLE 1

Eyespot development on two wheat cultivars.
Results averaged over fungicide treatments and inoculum levels.

	Eyespot levels									
	GS 25		GS 32		GS 37-39		GS 65		GS 85	
	EAU*	ESI+	EAU	ESI	EAU	ESI	EAU	ESI	EAU	ESI
Rendezvous	1274	0	1738	3.4	578	7.1	1581	20.0	4882	40.7
Pastiche	1208	0	1338	8.7	772	18.4	4516	42.8	28,855	67.0
LSD 5%	474		180	3.1	132	3.2	1487	6.6	5434	9.0

* EAU Eyespot Antigen Units
+ ESI Eyespot Severity Index

Effect of fungicides on eyespot development

Assessments were made of sharp eyespot (*Rhizoctonia cerealis*) and Fusarium levels, but eyespot was clearly the major stem-base disease in this trial. Some foliar infection with *Septoria tritici* occurred later in the season. Both prochloraz and flusilazole achieved similar results, and within two weeks of spraying ESA and ESI measurements were always lower in treated than in untreated plots (Table 2). Control of eyespot was, however, only moderate, no doubt reflecting the high disease pressure, and that treatment should have occurred earlier. No interaction was seen between fungicides and either inoculation level or cultivar.

TABLE 2

Effect of fungicides on eyespot development.
Results averaged over cultivar and inoculum levels.

	Eyespot levels								Yield tonnes/ ha**
	GS 32*		GS 38		GS 65		GS 85		
	EAU	ESI	EAU	ESI	EAU	ESI	EAU	ESI	
No fungicide	1382	6.6	1040	18.9	4794	40.7	27,552	69.8	8.49
Prochloraz			495	11.0	1770	27.2	10,515	44.4	9.24
Flusilazole			491	9.4	2582	26.2	12,540	47.3	9.37
LSD 5%			132	3.2	1487	6.6	5,434	9.0	0.15

* Before fungicide application
** At 15% moisture content

Effect of inoculation level on eyespot development

Variation of inoculum timing and level was the most effective way used in this trial to influence eyespot levels. Inoculation increased eyespot severity and antigen levels from GS 32 onwards, especially on cv. Pastiche (Table 3). Eventually EAU levels reached 3-4 times those of uninoculated plots.

TABLE 3

Effect of inoculation timing on eyespot development.
Results averaged over cultivar and fungicide treatment.

	Eyespot levels									
	GS 25		GS 32		GS 37-39		GS 65		GS 85	
	EAU	ESI	EAU	ESI	EAU	ESI	EAU	ESI	EAU	ESI
No inoculation	1250	0	1450	2.1	474	9.2	1666	21.5	8,357	44.5
November 1990	1302	0	1800	10.0	893	16.1	4624	40.4	24,420	63.2
February 1991	1172	0	1546	6.1	614	12.9	3216	32.5	17,698	53.8
LSD 5%	474		180	3.1	132	3.2	1487	6.6	5434	9.0

DISCUSSION

Significant amounts of eyespot antigen were detected in both cultivars from GS 25 onwards, and before visual symptoms appeared. By GS 37-39 differences in both antigen and disease levels had developed because eyespot spread more slowly in cv. Rendezvous than in cv. Pastiche. It seems that initial infection is not reduced in cv. Rendezvous and using these two cultivars to generate different eyespot levels during early epidemic phases was not successful.

Prochloraz and flusilazole provided significant eyespot control on both cultivars, and generated differences between plots in antigen and disease levels. By delaying treatment until GS 33, when antigen levels at GS 32 were known, the opportunity to alter eyespot levels in the early phases of the epidemic was missed. Overall, results were similar to those reported by Bateman (1990) using just visual assessments to assess fungicide performance. Prochloraz and flusilazole performed similarly despite the fact that only rye pathotypes were used to inoculate plots, and in greenhouse trials prochloraz is more active against these pathotypes than is flusilazole (Cavelier *et al.*, 1987).

Inoculation timing produced differences in eyespot levels at GS 32 and later. This was especially so for the susceptible cultivar, Pastiche, although the extent of any differences was limited by the surprisingly high level of antigen (and later disease) in uninoculated plots, despite it being a first wheat crop. This suggests that dispersal may not be restricted to rainsplash and that other, perhaps wind borne, inoculum sources can play a significant part in eyespot epidemics.

TABLE 4

Correlation between eyespot antigen units and either visual disease assessments or yield. Percentage variance accounted for in a regression of eyespot antigen units and yield.

Growth stage	Correlation co-efficients		% variance accounted for	
	Rendezvous	Pastiche	Rendezvous	Pastiche
	Eyespot Antigen Units and Eyespot Severity Index			
32	0.3	0.61**	0	34.2
38	0.38	0.78**	11.3	58.7
65	0.54**	0.87**	25.8	78.0
85	0.78**	0.86**	59.1	80.5
	Eyespot Antigen Units and Yield			
32	0.06	0.04	0	0
37-39	0.19	0.63**	0	38.6
65	0.04	0.70**	0	46.2
85	0.61**	0.61**	36.5	29.6
	Yield and Eyespot Severity Index			
32	0.16	0.12	0	0
37-39	0.32	0.73**	6.9	46.7
65	0.02	0.50**	0	19.0
85	0.58**	0.51**	44.3	21.4

** Significant at 5% probability level.

Despite these limitations, some useful correlations were identified from the data available. There was an increasing correlation between Eyespot Severity Index and antigen units on both cultivars as the season progressed (Table 4). For Pastiche this was significant on all four sampling occasions, but only after GS 37-39 for Rendezvous. No correlation between yield and antigen units occurred at any growth stage for Rendezvous (Table 4), and it was not possible to determine an antigen threshold level at a growth stage where a fungicide would have a positive effect. Both flusilazole and prochloraz increased yields of cv. Rendezvous, but this may reflect control of Septoria and mildew rather than eyespot. Better correlations were obtained for cv. Pastiche (Table 4) where an association between yield and antigen level occurred from GS 37-39 onwards. The immunodiagnostic assay provided a more precise guide at this stage than visual symptoms to yield loss, with 350-700 antigen units per stem equating with a yield reduction of 0.75 tonnes per ha. This compared with a wider range at GS 37-39 of between 20-65% stem infection or an ESI from 5-30.

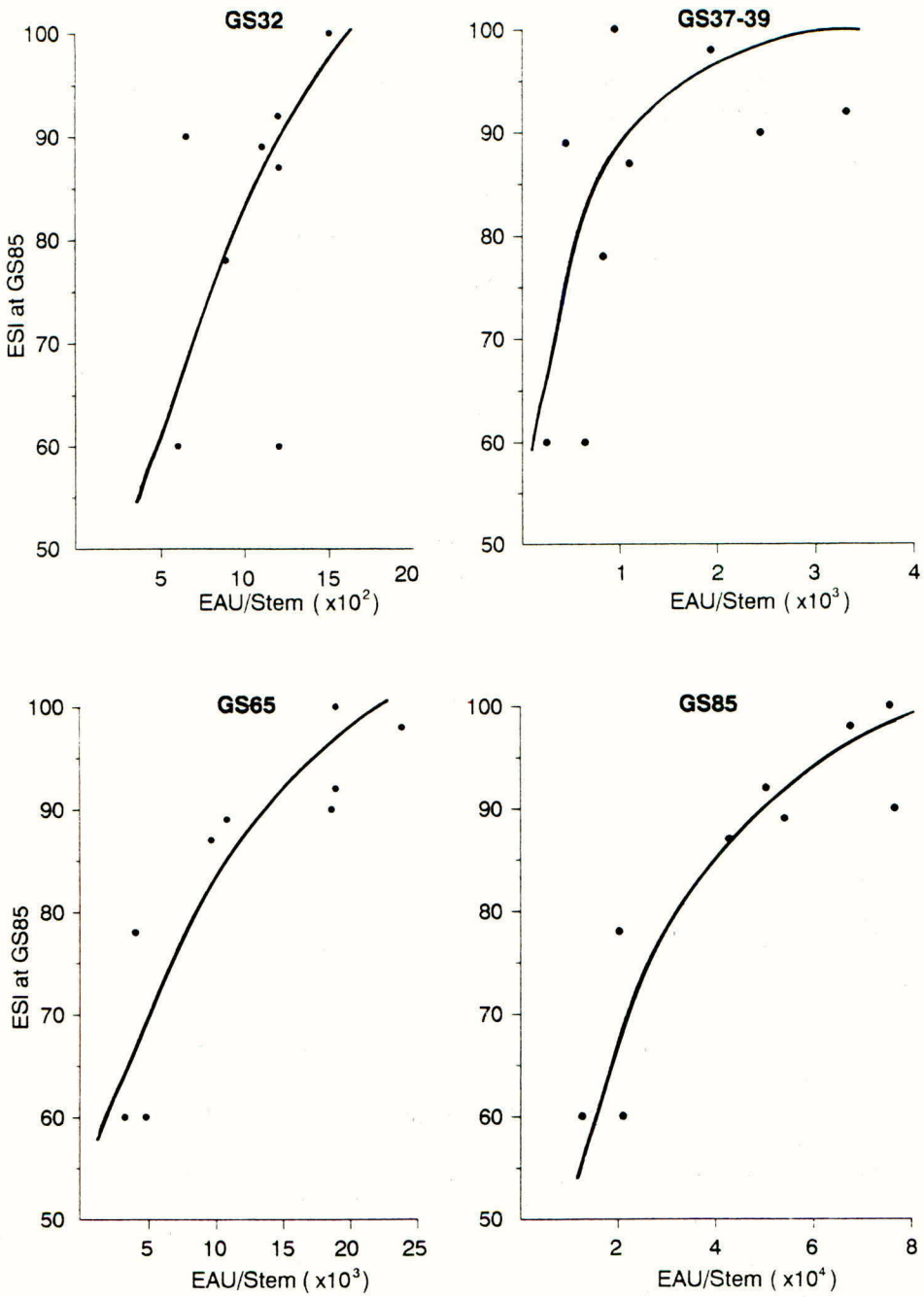

Figure Correlation between eyespot antigen units and eyespot severity index at four growth stages on the wheat cv. Pastiche. Values are for plots not treated with fungicide.

Thirty stems were routinely collected from each plot and used as a bulked sample. Immunoassays of 30 individual stems from the same plot indicated a standazrd error of 24% around the mean antigen level at a 95% confidence interval. Accuracy of thresholds could be increased at critical times by larger samples, but to double accuracy 120 stems would be needed which is, perhaps, impractical. From this one year's data it was not possible to identify a spray threshold at any growth stage, since infection levels as measured by EAUs, were high enough to justify treatment of both cultivars, even on uninoculated plots. More accurate spray thresholds would have been obtained if natural background antigen levels were lower, and fungicides applied at GS 25 or even earlier. Even so, whatever the threshold, standardised conditions for sample preparation and assay are also important.

Options based on disease assessment and meteorological data for improving spray timing have been critically reviewed by Fitt *et al.* (1988). In fact, the relationship between visual assessment of eyespot at GS 30-31 and final disease levels was poor. Serological measurements revealed a similar pattern of eyespot development to that established using traditional pathological methods, at least in a susceptible cultivar. Significant correlations were obtained during the period when fungicides may be used effectively (GS32 to 37-39), between EAUs and final disease incidence (Figure) or even yield, and these correlations were always better than those based on early visual assessments. Immunodiagnostics clearly offer the potential for better, more effective, eyespot control, and experiments of the type reported here should help to define that threshold more clearly for UK conditions.

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FUTURE PROSPECTS FOR THE INTRODUCTION OF DIAGNOSTICS IN AGRICULTURE

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ABSTRACT

In the Western European agricultural environment,new economic and ecological constraints will force farmers and their suppliers to reduce their costs and focus on quality aspects.
Immunoassay Diagnostic tools have clear potential to effectively help farmers in this challenge but have also some limitations.
A significant impact of Plant Disease and Residue Diagnostics is foreseen on farmer habits and on their suppliers in the near future. However, joint efforts will be required to make them successful and the chemical industry will actively participate in their introduction into practice.

INTRODUCTION

In modern agriculture, most farmers consider the use of crop protection chemicals absolutely necessary to avoid pest damage and get regularly high yields. Without crop protection products, the food supply would be significantly reduced and of lower quality. During recent decades, the chemical industry has invested a lot in Research and Development to provide farmers with highly effective active ingredients that can be used easily and safely by users.

Since crop protection chemical use began, a few non-target effects have occured: toxicity to wild life, residues in soil or at harvest, groundwater contamination,... which have raised some concerns in the public domain, such as: Are pesticides really safe for consumers? Do they respect our natural environment?. To answer these questions, many initiatives have appeared ranging from organic farming which rejects use of chemicals to Integrated Pest Management programmes that try to optimize their effective and safe use.

In the last two decades, a joint and intensive effort has been made by all partners of the agricultural community, including official registration agencies and the chemical industry, to answer these public concerns. Registration requirements have greatly increased to make sure that products can be applied safely by users with no risk for consumers and the environment. As a consequence,the costs to put a new active ingredient on the market have grown enormously.

Agricultural products must be used in their best way. New detection technologies such as Plant Disease or Residue Diagnostics can help to answer questions such as: Do I need to spray? At what time ? In my field conditions, am I sure that residues at harvest are below the established tolerances?

The objective is to analyse what are the new needs of the farmers and their suppliers in this changing European environment and to determine how Diagnostic tools can help satisfy them. The agrochemical industry is willing to participate in this general effort through innovation, but also must clearly understand how these new tools can affect the current use of products.

I-THE EUROPEAN AGRICULTURAL ENVIRONMENT

Following the introduction of a new Common Agricultural Policy, Western European agriculture has to face new ecological and economic challenges: higher environmental protection and lower prices of outputs. This will greatly stimulate a shift from an intensive agriculture aimed at providing the highest yields to a more qualitative, profit oriented agriculture. For example, farmers may tend to plant more breadmaking wheats instead of feed wheats that provide higher yields but that may not get an intervention price with the new CAP reform. This will have a direct impact on farmer objectives and therefore, on their suppliers.

1.1- Farmers are faced with a new challenge: how to maintain their profits when production prices are significantly reduced?

One obvious solution is to reduce costs in all possible ways, including the crop protection expenses which will be scrutinised more!

Herbicides and Insecticides can be sprayed according to the weed spectrum or insect presence in the field. Sprays are already limited to what is absolutely needed and their costs cannot be reduced further.

What about Fungicides which often are a major part of the expenses for crop protection?

Diseases are difficult to recognize and, usually, damage has already occured when they become easily visible! Unless reliable tools are provided that can detect the pathogens early enough, farmers will continue to rely upon their preventive fungicide sprays.

To be successful, Diagnostic tests have to help answering the following questions asked by farmers:

- **Do I need to spray my crop?** This implies that the overall complex of diseases damaging a crop is taken into account, not only a specific pathogen. In addition, the result from the test must quantify the infection level so that it helps to predict the risks of damage for the crop.
- **When should the treatment be made? What product and rate is the most adequate?** A very thorough field development programme has to be carried out to determine what timing, product and rate are best adapted to optimize activity at minimal costs. Treatment decisions must be made for each field according to its yield potential as well as disease development factors.

Other parameters must also be carefully integrated into the overall fungicide programme of a specific field:

- use of the most tolerant varieties to key diseases,
- apply the best seed coating technique and products to eliminate soil-borne pathogens,
- determine optimum sowing dates according to the most damageable disease infection periods,
- better timing of nitrogen inputs to avoid wash-off losses and increased sensitivity of plants to diseases.

Another way for farmers to maintain elevated profits is to acquire premium prices for high specific quality production. Contracts with food collectors and/or processors will develop to reach a quality level that perfectly meets customer requirements. To guarantee such quality labels, all cultural practices will be specified and residues at harvest checked. Cheap and easy-to-use detection tools will be of great help to achieve this quality goal.

1.2- The agricultural suppliers and advisors greatly depend on farm economics.

The European agrochemical market has been relatively stable in value over the last 3 years. However, margins have significantly decreased for several reasons:

- increasing registration costs,
- decreasing end-user prices mainly due to tougher competition at distributor (dealers, coops) level,
- more and more active ingredients coming out of patent,
- development of resistance.

To get an acceptable return from high investments, the manufacturers must keep their products profitable long after they have come out of patent. Providing a high quality service to their customers that brings a competitive edge and an added value is one way to achieve it. Diagnostics will be part of this service.

Distributors (dealers, wholesalers, coops) and crop specialists are also eager to maintain or improve their image for their customers: investing in a highly technical service that can help farmers to make the right decisions will become a key element of their success.

In the current European environment, farmers need help from all their suppliers to reduce costs while maintaining high quality production. What attributes should diagnostic tools have to get a real value for farmers?

II - KEY ATTRIBUTES FOR A DIAGNOSTIC TOOL

Plant Disease Diagnostics are tools to help farmers detect early infections of pathogens and evaluate the risks of crop damage. Residue Diagnostics tools can measure the level of residues in soil, water or plants during the growing season or at harvest. These Diagnostics must be easier to use and more cost effective than conventional detection methods or make possible new strategies.

Various technologies have been tried to achieve this goal. Immunoassays (based on the ELISA technique), first developed in the medical sector, have shown very promising results in the past few years in Agriculture. New technologies such as nucleic acid hybridization and amplification methods are under intensive development and may also have great potential.

2.1- Key attributes

A high quality Diagnostic test must be:

* Highly specific to the defined target:
  In the case of Plant diseases, it must:
  - either, detect all strains of a single fungal species and not react with other pathogens damaging the same plant (e.g. polyclonal and monoclonal antibodies)
  - or, detect one specific strain in a fungus population (e.g. nucleic acid probes and possibly monoclonal antibodies).

  Residue tests must detect traces of one specific molecule (or one chemical class of products) and not cross-react with any other molecule that can be found in soil, water or plant extracts.
  This requires high quality testing, in natural conditions of use, and comparison with the most sensitive conventional methods.

* Highly sensitive

A Plant Disease Diagnostic must be sensitive enough to detect a pathogen:
- either, pre-symptomatically, at an early stage of the initial infection and before the onset of symptoms, to avoid crop damages with a curative application of systemic fungicides. This strategy can more easily be applied for pathogens which have a long latent period and can be controlled curatively (e.g. cereal glume blotch).
- or, at early stages when visual symptoms are still scarce and the fungicide treatment cannot be delayed (e.g. cereal eyespot).

Residue Diagnostics must be sensitive enough to detect a molecule at its established Maximum Residue Limit (MRL) for a given crop or a specific consumer group.

* Allow quantitative measurements.

Presence or absence of a pathogen or a molecule may in some cases be good enough (for identification purposes). However, most pathogens are usually present in fields, and farmers need to know how much infection is present. Plant Disease and Residue Diagnostics must allow quantitative measures.
Good correlation with other existing scientific methods needs to be verified.

* Quick, easy to perform and less expensive than existing methods.

The visual examination of plant tissues is often the only quick way to detect plant pathogens. However, some pathogens are quite difficult to recognize and even highly skilled technicians may inaccurately diagnose when confronted with atypical symptoms.
Microscopic examinations are accurate but are laborious and require laboratory skills.
HPLC/GLC methods are currently the most accurate and sensitive ways to measure pesticide residues. However, they require expensive laboratory equipment and specifically skilled people.

Immunoassay Diagnostics can deliver rapid and accurate results at reduced costs and do not require specific skills to perform the analysis. A wide variety of test formats exists, such as:
- the microtiter plate providing highly precise, quantitative results in 2-3 hours, but requiring some lab equipment.
- user friendly kits (rapid membrane tests) that provide semi-quantitative results, but can be performed in 10-15 minutes, in the field, the back of a car or any other places, by everyone.

2.2- Some limitations

* Sampling and sample preparation

Whatever detection method is used, the most important factor in obtaining highly accurate results is the way the plant sample is collected in the field. The sample must accurately represent the infection level of the field as a whole. For each pathogen, a precise sampling procedure has to be defined according to its infection pattern in the field and the level of precision desired.
Often, a realistic compromise between high accuracy and practical field work has to be made.
Which part of the plant is kept in the final sample submitted to the test is also critical. Threshold levels are established under very precise sampling conditions: if they change, threshold levels must be checked again.

* Diagnostic tools give a clear picture of the infection or residue level at the time of sampling (the sample has to be processed soon after being collected or kept frozen). They are not in themselves predictive tools unless other influential parameters are considered such as the climatic conditions that occur after the test is made or the tolerance of the variety.
In the future, Diagnostics may have to be used in conjunction with mathematical models to achieve reliable damage predictions.

* A Diagnostic only detects the fungus/molecule it is made for: in practical field conditions, a complex of diseases damages each crop and many products are used to control them. To effectively help farmers or their technicians, a range of tests may be needed to get a good overall estimation of the risks and secure treatment decisions.
A long trip has yet to be done in this direction and will require sustained efforts from all partners in agriculture.

III - IMPACT OF DIAGNOSTIC TOOLS IN FUTURE AGRICULTURE

The market value for Diagnostics is still very limited. However, most market surveys predict a significant growth in the next decade, specially in the areas of Plant Disease and Residue detection. It is therefore important to try to understand what will be the impact of such Diagnostic tools on farmer habits and those of their suppliers.

3.1- At the farm level

A survey made in 1991 in France showed that 10-20 % of cereal farmers were ready to reconsider their preventive fungicide treatment strategy and would use Diagnostic kits to help decide their fungicide sprays. They would accept to pay for a full technical service given by their distributor or crop specialist, providing the cost did not exceed 10-20% of the treatment cost per hectare. Moreover, more than 80% of interviewed farmers showed a great interest to optimize their fungicide usage. Farmers will tend to reduce their off-season purchase of pesticides and buy the most adequate products at spraying time.

In some North-European countries, under the pressure of public opinion, a very significant reduction in pesticide amounts used each year, is planned. Farmers may soon need to justify their sprays, and field Diagnostics may then play an important role.

Some farmers are now looking for higher prices for their production through quality contracts that include very strict production programmes. The use of crop protection products are usually precisely defined and sprays made only when necessary. At harvest, acceptable levels of residues must be guaranteed: plant disease and residue diagnostics would help a lot.

3.2- at advisory level

Most important distributors (dealers, coops,...) and official or private advisors fully understand their need to enhance the quality of the service given to farmers and get in turn a higher loyalty. Therefore, many organizations are ready to re-orient their forces to better address technical issues, and not only sales issues. Diagnostics could be the tools they need to improve their technical image and build an effective and profitable technical service to farmers.

3.3- at manufacturer level.

In the highly competitive market environment of Western Europe, main manufacturers see as a key advantage to support their product line through a better service to their distibutors. They also recognize the continuing need to improve the agrochemical industry image in the public opinion and devote already large budgets to environment protection.

Diagnostics tools are one way of answering these needs.However, efforts will be focused to address the needs of the main European markets in cereals, grapes and some other important field crops. Most manufacturers have in house the necessary expertise to deliver high quality tools: good quality control and reliable supply are crucial points for their success.

There is still some fear of disturbing existing markets with the introduction of diagnostic tools. In the short term, the use of diagnostics will not significantly affect the markets because of their limited use. However, some shifts in product use will happen in the next 5 years to control only most damaging diseases. As farmers will tend to delay their purchases until the spraying time, prompt delivery to local distributors will become increasingly important. This is already happening in some European countries including the U.K.

CONCLUSION

In the new challenge that farmers are facing with the new Common Agricultural Policy and the public pressure for better Environment protection, the pesticide industry will actively participate to support its customers. As in the past, the main effort will be devoted to innovations. In that context, Plant Disease and Residue Diagnostics will be developed and introduced in key crops to optimize sprays. Such tools will play an increasing and significant role in the future to optimize crop protection usage. However, their success will largely depend on a close and open co-operation between all agricultural stakeholders working for the best benefit of farmers and consumers.

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EXPERIENCES WITH THE CEREAL DISEASE FORECAST SYSTEM EPIPRE IN SWITZERLAND AND PROSPECTS FOR THE USE OF DIAGNOSTICS TO MONITOR THE DISEASE STATE

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ABSTRACT

Decision support systems (DSS) for diseases in wheat growing have had little impact in Europe so far, apart from EPIPRE. Nevertheless DSS are valuable tools to reduce pesticide inputs. In Switzerland 30% of the fungicide applications and about 50% of the fungicide quantity could be saved using EPIPRE without losing a substantial amount of the grain yield. To promote the development and the use of DSS, more accurate diagnostic methods and forecast models could be useful. Immunodiagnostic assays for the detection of septorioses and eyespot are very promising ways to assess the current disease state in wheat fields. High correlations were found between the values of an eyespot immunoassay and visual assessment of wheat stems from a field trial with different varieties and treatments. Further developments of DSS and the use of immunoassays are discussed.

INTRODUCTION

In spite of demand from growers and intensive research on forecasting and risk assessment methods during the last three decades, the impact of decision support systems (DSS) for diseases seems to be rather low. An inquiry in seven countries of Western Europe in 1991 revealed that DSS for diseases in cereals are not used in three countries (Denmark, France and Italy). In the other four countries (Belgium, Germany, Netherlands and Switzerland) the use of DSS covers only 0.3 to 2% of the acreage of cereals (Bigler et al., 1992). Experiences with disease management systems will be discussed as well as prospects for such systems and for the utilization of new diagnostic methods to evalute the state of diseases in cereals .

Table 1. Number of wheat fields which participated in EPIPRE in the Netherlands (Daamen, 1991) and in Switzerland

Year	Number of fields NL	CH	Year	Number of fields NL	CH
1979	450	0	1986	862	486
1980	840	0	1987	586	684
1981	1155	24	1988	382	925
1982	1069	48	1989	306	1041
1983	1380	93	1990	50	1023
1984	1100	102	1991	-	939
1985	816	251	1992	-	612

NL: Netherlands; CH: Switzerland

EPIPRE is the only DSS in cereal growing that achieved international impact and was or is in operation in the Netherlands, Belgium, Sweden, Great Britain and Switzerland (Reinink, 1986). The numbers of fields that were registered with EPIPRE in the Netherlands and in Switzerland are summarized in Table 1.

CHARCTERISTICS OF EPIPRE

EPIPRE is a computer based DSS and makes spray recommendations for the following main diseases and pests of wheat:

- eyespot (Pseudocercosporella herpotrichoides)
- stripe/yellow rust (Puccinia striiformis)
- leaf/brown rust (Puccinia recondita)
- powdery mildew (Erysiphe graminis)
- glume blotch (Septoria nodorum)
- aphids (Sitobion avenae)

Basic data about the farmers wheat fields (e.g. cultivar, yield expectation, field exposition) are registered in the EPIPRE data base in early spring. In the season the farmer has to monitor the actual state of the diseases in his field. Using these data the EPIPRE program predicts the disease severity at the end of the prediction period and the corresponding yield losses (Zadoks, 1981; Daamen, 1991; Forrer, 1991).

Disease diagnosis and disease assessment

The farmer has to identify the pathogens himself. The diagnosis is based on the recognition of visible disease symptoms. For a correct diagnosis of the symptoms, farmers are trained in the field.

To determine the current disease state the farmer has to control 40 wheat tillers, collected on a diagonal line in the field. Then he has to count how many of the stems show symptoms of eyespot and - separately for all leaf diseases - how many of the 120 leaves show leaf symptoms. From growth stage 31 to 61 the participating farmer has to monitor his field three to six times depending on the disease situation.

IMPLEMENTATION AND SUPPORT OF EPIPRE

EPIPRE was implemented by our research station together with the Cantonal Plant Protection Services. Until 1984 (Table 1) EPIPRE was only accessible for farmers who agreed to compare EPIPRE with their conventional procedure and/or with untreated parts of their fields. Since 1985 EPIPRE has also been supported by the agricultural coooperatives. They financed a VIDEOTEX version and have administered this EPIPRE version since 1986/1987. By 1989 over 1000 fields were already managed under this system.

Validation of EPIPRE recommendations in field trials

The main variables used to evalute the recommendations of EPIPRE were the yield and the financial benefit. The results of the on-farm trials from 1981 to 1987 are summarized in Table 2. The results show that with

Table.2. Comparison of fungicide use and yield in farmers wheat field trials, 1981-1987 (Set A: only trials **with** treatments in EPIPRE; Set B: trials with or without treatments in EPIPRE).

Set (period)	Nb.of trials (N)	Mean nb.of treatments E	U	C	Grain yield EPIPRE t/ha	%	U %	C %	Cor.gross return* EPIPRE sFr/ha	%	U %	C %
A(81-87)	273	1.2	0.0	-	6.24	100	89	-	5700	100	94	-
B(82-87)	224	1.0	-	1.5	6.31	100	-	102	6220	100	-	100

*Corrected gross return = Gross return minus costs for product, machinery, labour and secondary costs (100.-sFr/ha and treatment)
E= EPIPRE U= Untreated C= Conventional

fungicide treatments according to EPIPRE there was 11% greater yield and 6% better financial return than without fungicide applications. Compared to the farmers procedure (conventional), there was a reduction of 30% in fungicide use. The EPIPRE yield was 2% lower, but the corrected gross return was equal in the two procedures.

Since 1984 we have compared EPIPRE with the following procedures: One fungicide application in growth stage (GS) 31/32 or GS 57, an application in GS 31/32 and GS 57 and no fungicide treatment (Table 3). The field trials had 4-5 replicates.

Table 3. Comparison of the yield and the benefit of different fungicide treatments with EPIPRE
(Mean values from 23 trials from 1984 to 1991 in Switzerland).

Treatment	Treatments Nb. of Ap.	AI	Grain yield t/ha	%	Du	Corrected gross return sFr/ha	%	Du
1 Untreated	0.0	0.0	5.38	100	d	5470	100	c
2 GS 31/32	1.0	1.6	5.72	106	c	5580	102	c
3 GS 57	1.0	2.0	6.08	113	b	5890	108	b
4 GS 31/32 & 57*	2.0	3.6	6.38	119	a	5970	109	ab
5 EPIPRE	1.3	1.8	6.38	119	a	6150	112	a

Ap.= applications AI = active ingredients Du = Duncan's multiple range test at P = 0.05 * = corresponding to conventional procedure in farms with relatively high intensity
(each trial was considered as one replicate)

In these trials the yields of EPIPRE and conventional were 19% above the untreated variant. With EPIPRE we obtained the same yield as with a standard two-spray programme using 30% fewer applications, 50% fewer active ingredients and about 50% less fungicide.

Information exchange with the extension service and the farmers

Since the introduction of EPIPRE in Switzerland in 1981 we have had intensive communcation with the extension service and farmers connected to the DSS. Since 1985, with the beginning of EPIPRE on VIDEOTEX, the raising number of participants and the reduction of the on-farm trials, we have lost more and more contact with farmers and the extension service. To get some idea how EPIPRE is working, the opinions and experiences of the participants were evaluated by survey (Table 4 & 5).

Most participants judged EPIPRE as a valuable way to learn how diseases can be monitored and to make treatments according to the disease situation. The main disadvantages of our DSS in farmers view are the time needed for monitoring (labour), uncertain forecasts and too high participation costs (Table 4). In 1987 when users of the older, post-card EPIPRE-system were surveyed, 45% of the farmers judged the time delay between disease monitoring and an answer from EPIPRE as too long. This point has lost importance since EPIPRE is managed by VIDEOTEX (Table 4).

Table 4. Advantages and disadvantages of the EPIPRE system in the view of the farmer. Results of a survey of 500 participants of the VIDEOTEX-EPIPRE system in 1989.

Advantage of EPIPRE	entries	Disadvantage of EPIPRE	entries
Education & knowledge	91%	No disadvantages	43%
Adjusted treatments	83%	Too labourious	25%
Saving of fungicides	45%	Uncertain forecasts	21%
Yield optimization	28%	Participation costs	20%
None	1%	Answers too late	8%

80% or more of the participants treated their fields as indicated by EPIPRE and a only two percent of the farmers who answered our inquiry did not follow the recommendations at all. 89 to 95% of them judged EPIPRE's indications as good. 18-24% stated that they would have applied more fungicides without EPIPRE (Table 5).

Table 5. Farmers attitude to the recommendations of EPIPRE. Results of inquiries from participants of VIDEOTEX-EPIPRE from 1989 to 1991.

Year/ Nb. of namings	How farmers followed the recommendations (%)			Farmers view of the quality of recommendations (%)			How farmers would use fungicides without EPIPRE(%)		
	wholely	partly	never	good	mediocre	bad	more	equal	less
1989/503	80	20	0	94	5	1	24	67	9
1990/430	87	13	0	95	4	1	24	70	6
1991/369	82	16	2	89	10	1	18	75	7

Yield and fungicide data from fields treated according to EPIPRE were evaluated and are summarized in table 6. In general the yield expectations of the farmers were fulfilled with exception of 1990. In this year wheat yields in Switzerland were generally low because of lodging problems promoted by a high mineralization of nitrogen in early spring. On average 1.0 fungicide and 0.1 insecticide treatments were enough to control the disease and pest situation.

Until 1985 each participant received an annual report. Since then our information input to participating farmers is low. Nevertheless we believe that a high and steady information exchange between scientists, extension service and the farmer is at least as important as good results from field trials to promote DSS in practice.

Table 6. Insecticide and fungicide use and expected and actual yield of VIDEOTEX participants following the recommendations of EPIPRE

Year	Nb..of fields total EPIPRE	evalu-ated	Mean Yield exp. kg/a	eff. kg/a	Insecticide(I) & Fungicide(F) use in EPIPRE fields Fields with I%	F%	Mean nb. of F-treatments
1988	781	521	-	64.5	2	82	1.1
1989	1010	503	63.8	68.2	9	74	1.0
1990	987	429	65.5	58.1	7	77	0.9
1991	911	369	65.8	64.2	1	77	1.0

exp. = yield expectation eff. = effective yield

IMPACT OF EPIPRE IN SWITZERLAND

Main benefits of EPIPRE

1. EPIPRE was crucial for a strong promotion of integrated pest management (IPM) in Switzerland. Right from the start in 1981 we could prove with our on-farm trials that IPM was not only an idea and that the principle of economic thresholds was useful not only in entomology but also in pathology.

2. EPIPRE has had in Switzerland a high educational value. 3'000 to 4'000 farmers have learned with EPIPRE how to monitor diseases in wheat. Since EPIPRE monitoring is part of plant protection training at agricultural schools and is used in the further education of farmers, it can be assumed, that at least 10'000 additional farmers took advantage from EPIPRE.

3. From the field trials (Table 2 & 3) we know that EPIPRE is a reliable way to reduce fungicide inputs in wheat production. From the economic point of view EPIPRE proved to be equal to the conventional procedure.

4. Based on the EPIPRE observations across the country a weekly disease bulletin is published. EPIPRE has proved to be also a very useful disease survey system, especially for cereal rusts.

Many other benefits resulted from the testing and application of the EPIPRE system. For example the economic evalution of EPIPRE trials served as a basis for subsidies for "low input" wheat. On the basis of EPIPRE we developed HORDEPROG, a DSS for barley diseases (Forrer & Amiet, 1989).

Utilization of EPIPRE and actual trends

From 1991 to 1992 EPIPRE lost 35 % of its participants (Table 1). From surveys and from discussions with farmers and plant protection officers we assume that the following reasons are crucial:

1. Since 1992 the Swiss government has subsidised low input wheat ("Extenso"-wheat), produced without use of fungicides, insecticides or growth regulators, by 800.-sFr./ha (320 £/ha). At the end of the 1991 season we asked the EPIPRE participants if they were interested to join the "Extenso" production. More then 30% of them stated that they would produce "Extenso"-wheat, if a subsidy of 800.-sFr./ha is guaranteed.

2. An other important reason for the loss of participants is probably our unsufficient stuardship of EPIPRE with too little feedback to the farmers.

3. The diagnosis of eyespot and of septoria diseases is difficult for farmers. If on-site support by skilled experts is not available the farmer soon gets uncertain and chooses the way seen as having the lowest risk.

4. EPIPRE has, at least at short term, no economic advantage over the conventional procedure. So why should a farmer choose the more laborious way if he makes the same profit without using EPIPRE?

5. Last, but not least, EPIPRE is already an "old" system. For the extension service and also for us it has lost interest mainly because we cannot learn much new from the programme. The accuracy of the forecasts should be improved by using more meterological data (Daamen, 1991) or other new systems should be tested to see if they lead to better decisions than EPIPRE e.g. the German Pro_Plant expert system (Frahm et al., 1991).

ROLE OF DETERMINATION OF THE CURRENT DISEASE STATE IN THE FIELD IN DECISION SUPPORT SYSTEMS LIKE EPIPRE

DSS for cereal disease and pest control decisions are primarily based on the situation in the field of a participant. The decisions depend on the composition of diseases and on the level of the different pathogens. Since an appropriate action is highly dependent on the disease state it should be determined as accurately as possible consistent with a minimal cost of time and resources .

Limits of visual assessment of diseases

Farmers often cannot distinguish the symptoms of eyespot (P.herpotrichoides) and sharp eyespot (Rhizoctonia cerealis), and we know that too many unnecessary eyespot treatments are applied. On the other hand

in mixed infections with fusaria eyespot symptoms are hardly recognizable and useful treatments may be missed.

For leaf blotch diseases similar problems of diagnosis in connection with mixed infections of different pathogens (e.g. Ascochyta sp.) exist. In addition, symptom expression can be influenced by different cultivars and the environment. The difficulty in diagnosis may lead to an overestimation of septorioses. Nevertheless, this may not lead to overtreatments, since the latency period of S. nodorum can be reduced by infections with other pathogens.

Possibilities to improve the quality of diagnosis.

For the diagnosis of eyespot and septoria diseases microscopic methods such as for example the "BAYER diagnosis system" (Vereet & Hoffmann, 1990) could be helpful. This method promises a good estimate of the actual amount of inoculum but its use seems to be too complicated and too labourious for many farmers. In addition the diagnostic instruments are very expensive.

Much more promising for applications in disease assessment seems to us the use of immunodiagnostic methods as ELISA (Enzyme-Linked Immunosorbent Assay). This method has proved to be a very specific and rapid diagnostic tool in the detection of plant viruses and is widely used. There are several examples of successful developments of immunoassays to identify and quantify fungal pathogens in plants (Mittermeier et. al., 1990).

The immunoassay for eyespot (P. herpotrichoides) proved to be highly specific and no cross reaction with other important cereal pathogens were observed. With the assay the pathogen can be detected before disease symptoms appear (Smith et al., 1990). Using an ELISA eyespot assay for treatment decisions, about 70% of the usual eyespot treatments could be saved in Germany (Unger et al., 1990).

For applications in wheat ELISA assays for S. nodorum and S. tritici are also promising (Mittermeier et al., 1990). Since these diseases can be detected at an early stage, there are good possibilities of disease control with low dosages of fungicides, if appropriate forecast models can be developed.

First experiences with immunoassays for the detection of fungal pathogens in Switzerland

In 1991 we had the opportunity to test the eyespot assay of Du Pont de Nemours (Smith et al., 1990). The assay was used for eyespot evaluations in an eyespot trial (12 procedures with 5 replicates) with the high and low susceptiblility cultivars Garmil and Rendezvous, a mixture of both cultivars and Garmil treated with fungicide.

To evaluate the disease state, bulk samples of 30 stem cuttings (5 cm) of untreated Garmil, Rendezvous and the mixture were assayed with the Du Pont test at GS 31, 32 and 37. On the same samples previously the percentage of the stems pieces with symptoms and the disease severity was rated on a scale from 0-5. The mean values averaged over the three growth stages are presented in Figure 1. The corrleation of the 9 (3 cultivars x 3 GS) values of the ELISA assay in antigen units (Smith et al., 1990) with the visual assessment of the incidence was 0.77 and with disease severity was 0.83. At GS 71 20 stem cuttings (10 cm) were assayed from all 60 plots.

For the visual assessment the length of symptoms covering at least 50% of the stem cicumference were measured. The results are presented in Figure 2. Each point in the graph represents the mean of the 5 replicates. Using these values a correlation coefficient of 0.96 between the ELISA assay and the visual assessment resulted.

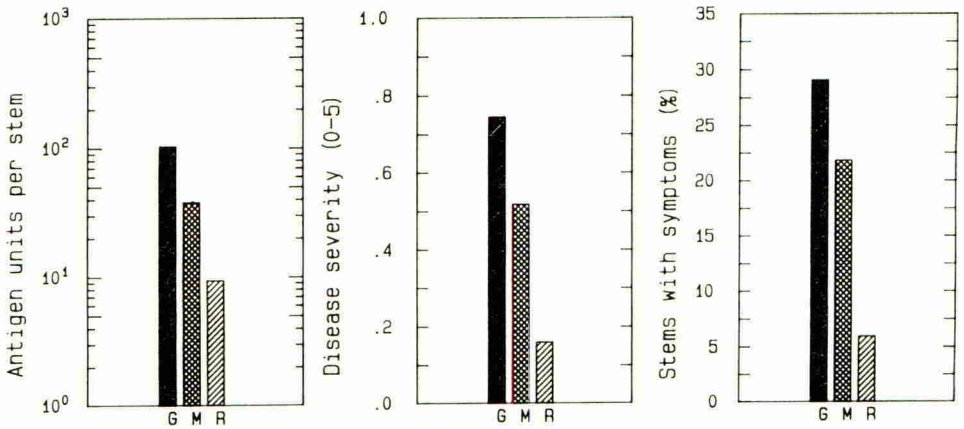

Figure 1. Comparison of P. herpotrichoides antigen levels from the two wheat cultivars Garmil(G), Rendezvous(R) and the mixture (M) of both with the results of two visual disease assessment methods (bars represent mean values from 3 evaluations in GS 31, 32 and 37; eyespot trial Zürich 1991)

Our results indicate that the ELISA eyespot assay is of high value

- to determine how much eyespot is present at spraying time,
- for the evaluation of fungicide efficacies
- and for the evaluation of the susceptibility of cultivars.

To establish the eyespot assay as a decision aid for eyespot treatments in farming practice we believe from our experience that the sampling procedure and the preparation of the stem cuttings should be refined and standardized (Hebeisen, 1991). Moreover we estimate that the accuracy of the decision can be improved if instead of one bulk sample, 3 subsamples (e.g. 3 samples with 15 stems) could be assayed.

CONCLUSIONS AND OUTLOOK

1. Experiences with the decision support system EPIPRE have proved that computer based DSS are highly valuable tools for the reduction of pesticide input in IPM strategies, and for the education of farmers.

2. To promote the use of DSS, the development and implementation of new, more accurate and easier to use DSS seems necessary. The

implementation and the use of DSS can only be successful if a high input of information and assistance to farmers is assured.

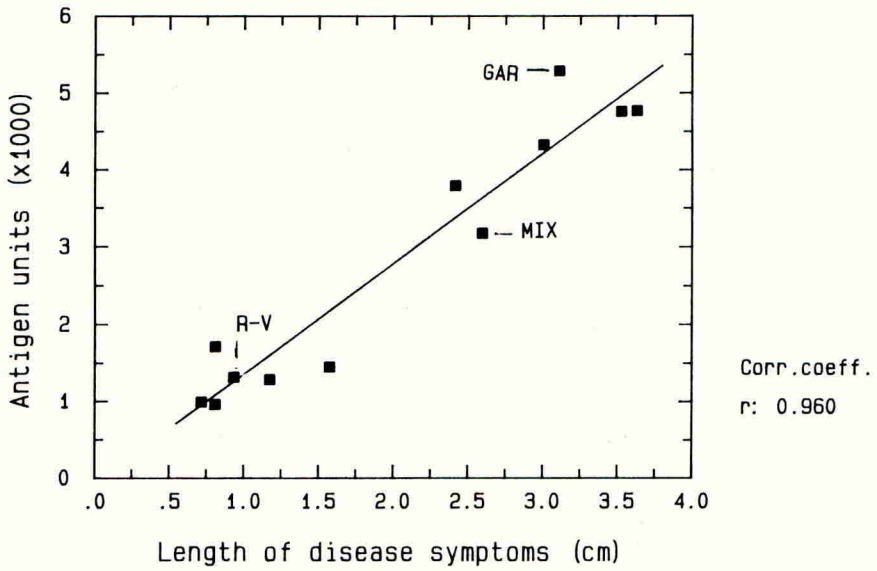

Figure 2. Relation between visible eyespot symptoms and eyespot antigen units in GS 71 (Fungicide trial Zuerich 1991, specified are the untreated variants with cv. Garmil(GAR), Rendezvous(R-V) & the mixture(MIX); each value is the mean of 5 replicates).

3. Since the determination of the current disease state in the field is crucial, more accurate diagnostic methods are needed. Our first experiences with immunodiagnostic assays for the detection and the assessment of pathogens are very promising. The accuracy of the laboratory eyespot assay with multiwell plates seems considerably better than those of the on-site assay.

4. If immunodiagnostic methods prove to be highly accurate and reproducible, the development of new, more accurate and international useful forecast methods will be made easier. Immunodiagnostics will also be a useful tool in the selection of disease resistent cultivars. They also could be used to assess diseases on seeds.

5. DSS allow a reasonable use of pesticides with a reduced danger to the environment. But since no economic benefit results for the farmers the use of DSS is limited. To promote the use of DSS political measures and/or the support of new developments could be useful.

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A PRACTITIONER'S VIEW OF DIAGNOSTICS AS AN AID TO DISEASE FORECASTING.

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INTRODUCTION

Central Science Laboratory (CSL)/ADAS disease surveys (Polley, 1990 & 1991) and CSL pesticide usage surveys (Davis *et al.*, 1990) testify to the widespread and, sometimes, intensive use of fungicides to control diseases of arable crops. Presumably all fungicide applications are made in response to a recognised disease threshold or a perceived risk of disease development or a preconceived plan for prophylactic treatment or a belief that one or more fungicides should be applied based on some other assessment of why or when.

There are systems available that attempt to support decision-making for fungicide use. Most have been integrated to some degree and provide a comprehensive approach to disease control in a single crop (eg winter wheat). These include the Managed Disease Control Schemes for major UK cereal crops (Anon., 1986), EPIPRE (Rijsdijk, 1983), and the Bayer system (Verreet & Hoffman, 1990). These combine one or more of the the criteria mentioned above in an attempt to provide a rational basis for deciding upon fungicide use.

However, none of these systems utilise disease forecasting to any significant extent. Disease forecasting in the UK is rarely used as a basis for deciding fungicide treatment. Stand-alone in-crop weather monitors are available that provide on-site recommendations for fungicide treatment against specific diseases. In addition the Meteorological Office calculates daily 'disease indices' for several diseases which are available to subscribers by FAX (Hough, pers. comm.). Clearly these two approaches to local disease forecasting are a step forward but the models upon which they are based require validation in the UK. Ideally such schemes should be part of systems that take account of previous fungicide application in assessing disease risk.

Given the quantity of work on disease epidemiology, the question arises as to why this work has never been used to formulate robust, practical disease forecasting schemes for successfully rationalising fungicide use. The answer is not a simple one but clearly validation is a complex problem demanding considerable resources and co-operation between various organisations. These are more likely to be seen as cost-effective if validation could be performed more readily and forecasting schemes implemented rapidly in UK agriculture. The cost-effectiveness of utilising forecasting schemes in practice can only be ascertained in a pilot exercise of appropriate scale.

WHY FORECAST?

During the last 15 years or so, much effort has been invested in comparing the disease control achieved by different fungicides, fungicide timings and programmes, fungicide/cultivar

interactions and application techniques. Only recently, has the understanding of fungicide activity taken a major step forward; results from the CSL/ADAS "PA51" experiment have revealed that the correct timing of virtually any cereal fungicide virtually complete control of any leaf disease of winter wheat (Hims & Cook, 1992). In addition it was possible to calculate the duration of the protectant and eradicant activity (in day °C) of recommended rates. Further work based on the "PA51" technique has indicated that the application of a fungicide mixture at rates of 0.25 - 0.75 of that recommended by manufacturers may achieve a similar degree of disease control, but only if applied during the apparent latent period (Paveley, pers. comm.).

Clearly an understanding of fungicide properties, in relation to either discrete infection events or some other means of determining a critical stage in disease development eg a threshold on a certain leaf layer, is a prerequisite to using fungicides more efficiently. But how may discrete infection events be identified with a degree of certainty that allows a chosen fungicide to be applied with absolute confidence? A thorough knowledge of disease epidemiology and a valid simulation model combined with recent, detailed meteorological data should indicate when an infection event occurred and hence a forecast of likely epidemic development. If we could quantify the amount of inoculum transported and/or identify successful infection events consistently and forecast the probability of epidemic development it should be possible to build a system that also takes account of fungicide activity ensuring that the minimum effective dose is always used. Thus forecasting could have a major role in maximising the efficiency of fungicide use. However, since nothing in biology is ever certain, forecasts are likely to remain imprecise. This lack of precision perhaps represents the niche that diagnostics could occupy (ie certain identification of successful infection events and the status of the pathogen during the latent period) as part of the discipline that attempts to increase the efficiency of fungicide use.

WHERE DO NOVEL DIAGNOSTICS FIT INTO DIAGNOSTIC PRACTICE?

Diagnosis can be accomplished readily only when characteristic symptoms or fungal structures are apparent. Correct disease diagnosis is important if control measures are to be used effectively. Despite the availability of broad-spectrum fungicides the use of appropriate doses may only succeed if diseases are diagnosed correctly. However the major problem is that of long incubation periods before symptoms appear to act as a guide on the need for control. For such diseases it would be an advantage in timing control measures if the disease could be diagnosed as early as possible during its' latent period. Clearly this is a role for diagnostic techniques. Agrochemical manufacturers and major research laboratories have realised the potential, both in terms of marketing their products and of providing an objective tool to assess the need for control measures.

It is unlikely that forecasting of every disease will benefit from the use of diagnostics. I have tried to sub-divide arable crop diseases on the basis of their biological/epidemiological features and rank them on the contribution that diagnostics may make in their forecasting.

Diseases in caegory 1 are those with long latent periods, initiated by meteorological events which vary in the degree to which they favour infection or those that are difficult to diagnose.

Diseases in category 2 are those caused by pathogens that may be dispersed over long

distances; the amount of primary inoculum may be a limiting factor but under favourable meteorological conditions the disease develops rapidly; early detection of inoculum and/or primary infection is important. However the real problem is that of scarcity of inoculum or rarity of infection (both sampling problems) not one of invisibility.

Diseases in category 3 may have short latent periods and develop from inoculum 'within' the crop ie they are primarily trash-borne and therefore there is no doubt about their presence, only their potential limited by meteorological events.

Category 4 diseases are those about which there is currently little epidemiological knowledge and whose significance and impact on crop loss is uncertain.

1. Septoria, eyespot, sharp eyespot, fusarium foot rot of cereals.

2. Rusts and mildew of cereals, potato blight, leaf rust and downy mildew of field beans.

3. Rhynchosporium, net blotch of barley, chocolate spot of field beans mycosphaerella leaf spot and flower/pod botrytis of peas, alternaria leaf and pod spot, light leaf spot, sclerotinia stem rot and phoma canker of oilseed rape.

4. Fusarium ear blight.

This list of diseases could provide a first attempt at prioritising development of diagnostics for research purposes if not for commerce. Organisations developing diagnostics presumably perceive some benefit from the development of diagnostic kits for septoria and eyespot in wheat since these are actively under development. Just what type of benefit is perceived may be commercially sensitive but may not be limited to one alone ie financial. However, in the future, the apparent need to apply a pesticide may have to be based on an objective test and not simply the perceived risk of or potential for a disease problem. Such a policy seems likely to be adopted in the Netherlands and will be the subject of legislation such that biological and integrated control must be used to reduce pesticide use (Anon., 1990)

FORECASTING AND DIAGNOSTICS - A SYNERGISTIC INTERACTION?

Forecasting should, on the basis of a robust model, provide an accurate prediction of disease development based on a synthesis of the dispersal, infection and sporulation processes. Since infection can only occur after inoculum has arrived in the crop diagnostics could confirm the arrival and accumulation of a given spore load and answer the question - is there sufficient inoculum present to provide a given, perhaps threshold, level of infection? Since spores may arrive over a considerable period of time perhaps some spore sampling device and immunoassay reaction vessel both monitored automatically could be devised. At the beginning such a system may be costly to initiate but complete automation may solve this problem. Such a system could prove to be valuable for sporadic diseases such as cereal rusts, potato blight, leaf rust and downy mildew of beans, alternaria of oilseed rape and fusarium ear blight where early detection of inoculum performed automatically is perhaps easier since it would rely on automated sampling rather than early detection of disease. Thereafter, having established the presence of inoculum, the forecasting element could take over the running of the system with

leaf sampling and testing at a suitable time interval to establish the success rate of infection from the known spore load. The cost and practicality of this approach would have to be carefully considered, but only a pilot exercise would be sufficient to judge its worth sensibly. However, the sporadic nature of inoculum dispersal may cause insoluble problems; sampling of airborne inoculum may be no more successful, as an early warning mechanism, than first detection of disease but simply easier to perform. Alternatively, assaying of leaf samples from trap plots of very susceptible cultivars in areas of high risk could replace the spore load assay for such sporadic diseases.

These two processes could, at a number of sites nationwide, act as a monitoring network for airborne diseases and as an early warning system for diseases that are dispersed over long distances. Clearly the success of such a system depends on the investment in resources eg a sampling tower, the sensitivity of the immunoassay and the monitoring equipment and manpower. It may be possible to utilise an existing sampling network to test its' feasibility.

Such a system could provide an overall estimate of inoculum. What happens thereafter depends on the degree to which meteorological events favour disease development. At this point the problem becomes more localised with local or regional patterns determining actual disease development. On a very local or individual farm basis, disease development potential as derived from indicator fields or specially sown tussocks of very susceptible varieties in "hot-spots" of traditional early disease development, could be used as a basis for an early warning scheme. This may be only a 'worst-case' estimate of the potential for local disease development rather than the more precise targeted use, admittedly for a much bigger area (region), referred to earlier. However, since this type of monitoring would be relevant to only a relatively small, 'local' area or even a single farm then this should provide the necessary sensitivity at minimal cost. For all diseases, early warning would then demand the use of a diagnostic immunoassay by farmers or advisers or consultants to test their own crops. Sampling procedures to reliably select sufficient leaf tissue likely to harbour the pathogen would need to be investigated. For trash-borne diseases, with the exception of eyespot perhaps, the need for diagnostic tests is not as high a priority. Inoculum is invariably present in the field crop and even low levels of disease may be apparent, usually from autumn-winter onwards, although some careful examination of crop plants may be needed to establish this fact. Certainly by early spring it should be possible to find evidence of most of the trash-borne diseases within crops at potential risk.

DISEASE DIAGNOSTICS - ARE THEY REQUIRED IN RESEARCH AND DEVELOPMENT?

Clearly, research and development on the forecasting of crop diseases would benefit from the application of diagnostics if only to increase the speed and accuracy with which infection and the current status of the pathogen could be confirmed. However, the cost and benefits of developing such diagnostic tools could be difficult to justify for some, if not many, arable crop diseases. High costs associated with the development of diagnostic kits might be justified if there was a compensating saving in resource ie manpower inputs into epidemiological studies.

Potato blight needs a more efficient forecasting method, ie summation of even the most marginal infection conditions, to produce an accurate indication of the number of infection

cycles completed. The problem with the current potato blight forecast criteria is that they are "all or nothing" with only a 'near miss' as a compromise for marginal infection conditions. They take no account of marginal conditions which may add a little, step-wise, to the totality of epidemic development. A diagnostic test would be a valuable aid to identifying the number of successful infections and the status of the fungus in the latent period under such marginal conditions. As already indicated the major problem with the sporadic disease, such as potato blight, is one of sampling in sufficient an extensive and/or intensive manner to ensure that the earliest of the primary infections/foci are detected. This problem may be overcome by the local use of "nurseries" monitoring the potential for local disease development using inoculated plants and small plots grown from infected tubers. The siting of these in areas of traditional early blight development plus the use of a diagnostic to detect the earliest successful artificial and natural infections would provide the early warning necessary for potato growers. Currently such a scheme may appear to be impractical. However a similar system is already operating in Switzerland (Gujer, 1991) and is meeting with considerable success. This system relies on extensive sampling rather than local intensive sampling and excellent co-operation and communication between growers, consultants and pathologists. There appears to be no good reason why a similar system should not operate in the UK.

The situation with regard to eyespot is less difficult; it is a localised, field by field disease. However, the problem of sampling and interpretation of results remains, especially since the current status of an eyespot epidemic must be assessed quantitatively. Since the current recommendation is for testing of a single sample per 10 ha. (McHale, pers. comm.), clearly a small number of plants/stems with a high antigen content could easily bias the test result if the remainder had a low antigen content. This may be corrected by careful sampling but this assumes users are willing to adopt such a technique. However, the antigen content of the outer leaf sheath bases may be irrelevant in the calculation of the threshold. Work in Germany (Wolf, pers. comm.) has shown that the pathogen must be present in the second to last innermost leaf sheath to have the potential of causing eyespot of sufficient severity to warrant fungicide treatment at the first node growth stage. In addition there is the complication of the different epidemiologies of the R and W pathotypes. Since the current diagnostic kit apparently does not distinguish between pathotypes (McHale, pers. comm.), the chance of it producing a spurious recommendation is considerable. Nevertheless, the use of an eyespot diagnostic may be an improvement on the situation currently in which there is no readily available test.

The threshold for phoma stem canker of winter oilseed rape is currently 25-50% of leaves with phoma lesions in the autumn, depending on cultivar susceptibility. These leaf lesions are characteristic and easily recognised. However, the leaf lesions are not the problem; rather it is the stem base lesion that results from infection following rain-splash dispersal of pycniospores from pycnidia in leaf lesions. Stem-base lesions are slow to develop and only become apparent at flowering or thereafter. Fungicide treatment must occur in mid-to-late autumn to prevent stem-base lesion development; moreover, the relationship between meteorological events, percentage leaf infection, stem base infection, cultivar susceptibility and lesion development needs defining far more precisely. In this respect a diagnostic test may be of considerable help.

Of all the diseases of arable crops, septoria must be the single most deserving of a diagnostic test. The relative uniformity of leaf infection likely to be present following a splash event of suitable intensity, ensures simpler interpretation of the results compared to those

obtained with most other diseases. In addition, the test could be used to calibrate "rainfall", "splash intensity" or "splash events" by the degree to which they favour upward dispersal of pycniospores, assuming that infection conditions were not limiting. Similarly the tests could calibrate infection conditions, the rate of incubation and length of the latent period and perhaps most important of all, they could provide a more accurate assessment under field conditions (where effective artificial inoculations may be difficult to operate) of protectant and curative fungicide activity. With respect to the reactivity of septoria diagnostics - do they detect mycelium (ie post-infection) or spores (pre-infection)? In order to confirm infection, the former is required; detection only of the latter could be misleading.

The case for the development of a diagnostic test for detecting mildew is not strong. Mildew tends to develop relatively slowly up to the flag leaf emergence stage and is therefore readily treated in good time before it is able to cause significant crop loss. Moreover, mildew is easily recognised and cultivar susceptibility is well known. For yellow rust the case for development of a diagnostic test is stronger, because of the problem of overwintering symptom-less infection. A diagnostic might establish the presence of the pathogen; thereafter a simulation model in relation to meteorological events could be used to forecast epidemic development. In addition the test could be used to help validate the forecasting scheme. A similar argument could be applied to the brown rust pathogen especially since early detection of inoculum or primary infection is important with respect to the rapidity of disease development and the opportunity to maximise fungicide efficiency. The problem of designing a suitable sampling technique remains but perhaps extensive trap plots/nurseries may be one solution.

Light leaf spot of oilseed rape may be similar to yellow rust of wheat; symptom-less infection of the growing point of plants during the winter period and the possibility of near systemic infection may be an important phase. The latter possibility has yet to be proven but results of fungicide spray timing experiments for light leaf spot control in Scotland appear to indicate that autumn infection may be an important phase of the disease (Wale *et al.*, 1990).

Downy mildew of field beans is almost certainly of insufficient importance to warrant the development of a diagnostic test at present. However, its status may change if cultivars with determinate growth dominate the market in the UK. Disease development in the growing point of such determinate plants may cause far more damage than in indeterminate plants and therefore a qualitative (presence or absence) test may be all that is required.

For those diseases where inoculum is unlikely to be limiting at the post-recognition stage, epidemic development is likely to be most damaging but almost wholly dependent upon meteorological events. Invariably there would be ample time for deciding upon the need for treatment. That is certainly so with rhynchosporium and net blotch of barley, alternaria leaf and pod spot of oilseed rape and flower/pod botrytis of peas. Thus there appears to be no need for a diagnostic test for these diseases. Sclerotinia stem rot of oilseed rape is slightly different in the sense that the critical phase of the disease appears particularly sensitive to three conditions ie (i) the presence of mature apothecia producing ascospores that coincide with (ii) wet weather of insufficient intensity to wash (iii) fallen petals from stems surfaces. The sensitivity of these three coincident factors appears to justify the development of a diagnostic test for ascospore infection of fallen petals. Such a diagnostic could replace the in-vitro testing of flower petals developed in western Canada to detect infection by *Sclerotinia sclerotiorum*

(Turkington *et al.*, 1991)

Mycosphaerella leaf spot of peas is almost entirely a seed-borne problem so that adequate attention to seed-hygiene and appropriate seed treatment should ensure freedom from the disease. However, if these precautions should fail then conventional visual monitoring would still give very adequate warning of a need to treat.

Fusarium ear blight is the most exceptional of all the diseases. Little detail is known of the epidemiology of this disease so in this respect a diagnostic test specific for each of the *Fusarium* spp. that is capable of causing disease in the ear should be of considerable help in understanding epidemic development and the influence of meteorological events. Such diagnostic tests, either specific or broad-spectrum, might be used extensively in commerce because of the reductions in quality and possibility of mycotoxin contamination caused by late ear disease.

The epidemiology and effect of two diseases, sharp eyespot and fusarium foot rot, for which there is currently no method of control is poorly understood. Diagnostic tests would certainly assist in investigating the epidemiology of these two diseases as part of the stem base disease complex. Quantitative tests would also assist in estimating of disease-crop loss relationships which would decide whether or not future control is likely to be economically worthwhile.

DISEASE DIAGNOSTICS - ARE THEY REQUIRED IN COMMERCIAL PRACTICE?

There appears to be a reasonable case for developing diagnostic tests for some of the diseases of arable crops for research purposes. However the level of commercial return from the necessary investment is uncertain due simply to the fact that the "market" ie the cropping area is relatively small when compared to cereals. Moreover, reductions in cropping areas under the CAP and possible restrictions on inputs eg nitrogen, could reduce still further the need for extensive and/or intensive disease control. However, development of a field based immunodiagnostic test for septoria seems to have a sound commercial basis perhaps in the UK alone. Wheat is the largest crop; 90-95% of wheat crops receive one or more fungicide applications, ostensibly for leaf disease control. Septoria is the most important leaf disease; the relative uniformity of leaf infection following a suitable splash event is likely to make sampling easy; the pathogen has a long latent period which offers the opportunity for tailoring fungicide timing and rate to the current status of the pathogen during the latent period.

Some of these arguments may be applied to eyespot although there are a number of problems including the sampling procedure, the degree of leaf sheath penetration and the differing epidemiologies of the R and W pathotypes. If these current problems could be resolved then there appears to be a commercial future for an eyespot immunodiagnostic test.

For the remainder of the arable crop diseases cost-effective commercial investments in diagnostic testing with an adequate return seems unlikely. However, the return required may not be directly related to technical need because the availability of such tests may increase the field credibility of commercial representatives, advisers and consultants.

CONCLUSION

Immunodiagnostic tests can make a valuable contribution to assisting research, hastening progress the understanding of pathogen epidemiology and its relationship with meteorological events, for most if not all arable crop diseases. Even if this cannot be translated into assisting decision support in commercial crop protection, then at the very least it should ensure that forecasting *per se* is considerably more reliable and therefore likely to be more acceptable and widely adopted. The acceptability and application of forecasting on the farm will depend on a clear demonstration of its' effectiveness. The latter depends to a large degree on resolving the problem of monitoring in sufficient detail at a practical level but with sufficient coverage and intensity to detect the events that really matter. The monitoring scheme adopted by Gujer in Switzerland seems to be an example worth emulating in the UK. We should at least try. Co-operation and collaboration plus good communication could resolve the disease monitoring problem that inevitably forms part of any disease forecasting scheme.The uncertainty factor inherent in any forecasting scheme may be impossible to remove but diagnostics could fill the niche created by this biological uncertainty and remove at least some, if not all of it.

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SESSION 7B

TRANSGENIC PLANTS FOR RESISTANCE TO PESTS AND DISEASES

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GENES FOR PROTECTING TRANSGENIC CROPS FROM CHEWING AND SAP-SUCKING INSECT PESTS

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ABSTRACT

Crops with genetically-engineered, inherent resistance to insect pests offer user-friendly, environment-friendly and consumer-friendly methods of crop protection for sustainable agriculture in the twenty-first century. *Bt.* toxin-expressing transgenics showed effective control in field trials, but the vulnerability of single factor resistance and the diversity of pests suggests *Bt.* alone is not enough. We are mimicking the multi-mechanistic protection systems which have evolved in plants themselves; using plant genes encoding insect control proteins with different modes of action. Combinations of these genes within transgenics giving multi-gene, multi-mechanistic resistance should increase both effectiveness and durability of insect control. Using plant-derived genes has extended control to sap-sucking, Homopteran pests. Thus, plant biotechnology is poised to offer a realistic alternative to chemical insecticides. Determination of optimum strategies for using transgenics within integrated pest management systems remains, but progress continues to be impressive.

World agricultural output is failing to meet the current world food requirements. The greater rate of increase in human population size compared with food production can only exacerbate this problem. A reduction in those crop losses which are attributable to insect pests would make a significant contribution to redressing this balance. Insects are not only responsible for massive losses of productivity directly as a result of their herbivoury, but also they serve as the vectors of many serious plant pathogens and the physical damage they cause to the plant may facilitate infection by various other soil or air-borne pathogenic organisms.

There is, however, growing concern that the conventional means employed in the recent past to protect crops from pests and realise high yields are becoming unsustainable; not least because of their potentially disastrous long-term ecological consequences. One such practice has been the over-use of synthetic chemical insecticides - to the extent that pesticide pollution has become almost a cliché in the catalogue of sins laid at the door of the agricultural industry. Without the use of these pesticides, however, crop losses to pests would reach catastrophic proportions. There is thus an urgent need to develop substitution technologies which would allow a much more limited use of synthetic pesticides yet provide protection for crops within a sustainable agricultural framework. It is in this area of crop protection that the recent advances in biotechnology, especially in genetic engineering, have been looked

to as offering the possibility of revolutionary new solutions.

Since the first reports of transgenic plants appeared in 1984 [Horsch *et al.*, 1984] there has been exceptionally rapid progress directed at using this new technology for the very real, practical ends of crop improvement. Protection of crops from their insect pests was quickly seized upon as a major goal of plant genetic engineering. Amongst the many reasons for this, not the least is the size of the insecticide market, estimated at *ca.* 7.6 billion dollar [1990, end-user value], which has attracted the attention of industrial research organisations.

Advances in molecular biology and plant tissue culture mean that it is now possible to produce insecticidal proteins and other crop protection agents within the plant itself for a wide range of crop species. Thus the protection is not applied to the general environment and is absolutely specific to [some of] those pests which actually attack the crop. Fine tuning of the genetic regulatory elements allows even more precise targetting - the genes can be switched on in defined organs [e.g. roots only] or at designated times [e.g. during insect attack]. Other advantages of genetically engineered insect resistance have been reviewed [e.g. Gatehouse *et al.*, 1992]. However, the source of suitable insect resistance genes remains a key question.

By far the greatest amount of research effort has been put into transgenic plants which express the crystal toxin genes produced by the insect pathogenic bacterium, *Bacillus thuringiensis* [*Bt*]. *Bt* has been in limited field use as a biological control agent for more than 25 years and modified genes encoding the *Bt* toxin provided the first examples of genetically engineering insect resistance in plants [Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987]. The effectiveness of transgenic plants expressing *Bt* toxin in resisting insect herbivoury, in those situations where it does work, is very impressive. Figure 1 illustrates the protection afforded by introduction of a plant-expression gene construct containing a truncated *Bt cry1A* toxin gene into tobacco plants when tested in laboratory whole plant bioassays against *Manduca sexta*, the tobacco hornworm. Monsanto Company have taken the lead in

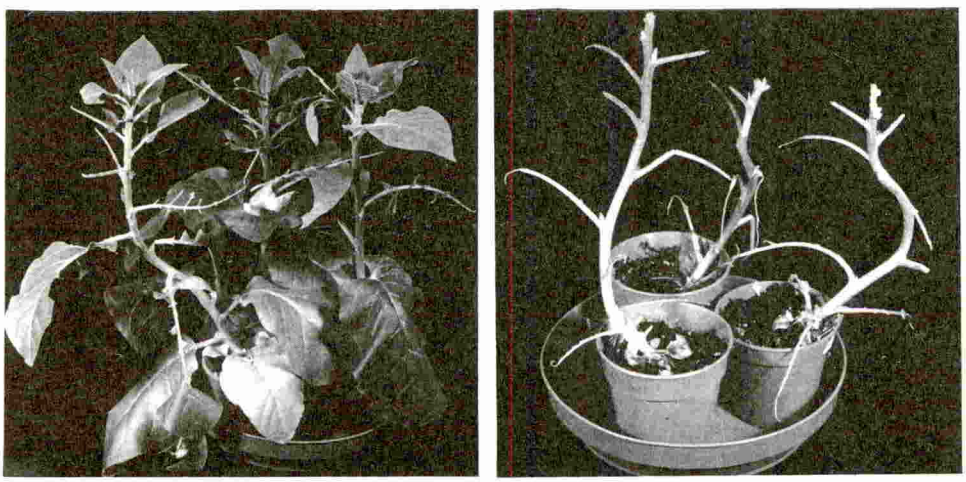

Figure 1. Transgenic [left] and control [right] tobacco plants [*N. tabacum var. 'Samsun'*] after 7d exposure to *M. sexta* larvae. The transgenics contain a truncated *Bt cry1A* gene driven from an enhanced CaMV 35S gene promoter.

development of *Bt*-expressing transgenic crops and have now demonstrated that such protection can be translated into commercially acceptable levels of field protection in such important crops as cotton and corn [against caterpillar pests] and potato [against Colorado potato beetle]. The development of *Bt*-expressing crops from the laboratory to the field has involved a massive research effort. In order to obtain adequate levels of expression in plants the *Bt*-encoding gene has to be substantially modified and has been effectively 'rebuilt' [Perlak *et al.*, 1990]. Commercialisation of the first of these genetically modified, insect resistant crops is expected within the present decade.

Despite the impressive progress which has been made with *Bt*-expressing crops, it does demonstrate some important problems which remain to be addressed. There is some concern that this further use of *Bt* toxin leads to dependence on a single factor resistance and that the potential for this to be broken down by the pests developing resistance to it is great. Numerous examples of resistance to *Bt* toxin are already known [see Anderson, 1992]. It has been shown that resistance to one type of *Bt* toxin does not determine resistance to others [Van Rie *et al.*, 1990], but reliance on the ability to introduce a new *Bt* toxin into crops before the previous one has broken down is an unsatisfactroy approach. This potential problem has, at least, been recognised before it becomes a reality, and efforts are being put into exploring the most effective strategies for utilizing transgenic crops to minimize the risks.

Another feature of Bt toxins is their inherent specificity. Toxin classes are known which are effective against certain Lepidopterans, against certain Coleopterans or against certain Dipterans. However, given the current pattern of chemical insecticide usage [Figure 2], it is clear that any replacement technology should be applicable to a broad range of insect pests.

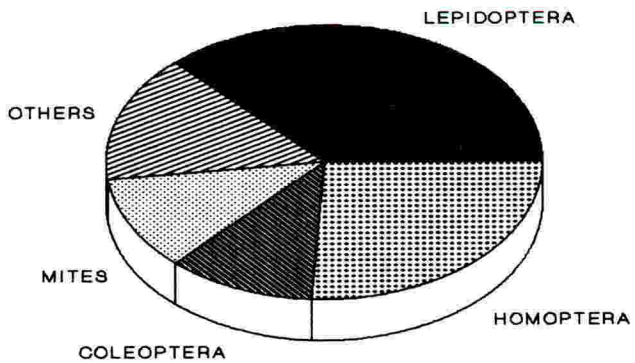

Figure 2. Insecticide usage by pest insect order; A.G.C. estimates [1990].

The technical strategy which we have adopted in the Durham University - Agricultural Genetics Company Insect Resistance Programme is to attempt to mimic the natural, multi-mechanistic protection systems which have developed in plants themselves over the course of their long co-evolution with insects - the plants' own solutions to the plants' problem. In nature, most plants are not eaten by the vast majority of insects. Each plant species has evolved a number of mechanisms to protect itself against insect attack and the combination of

these is generally sufficient to give durable resistance to most insect pests. Various factors in the artificial systems of agriculture have led to a breakdown of this balance [see Gatehouse *et al.*, 1992]. We have now isolated more than ten plant genes encoding different insect control proteins. Amongst these are representatives from more than six different classes of insect control proteins, each of which has a quite different mechanism of insecticidal activity. These genes, and selected combinations of them, are being tested in transgenic plants against a variety of insect pests.

The first example of this approach involved CpTI - the cowpea [*Vigna unguiculata*] trypsin inhibitor - the gene for which has been introduced into tobacco plants by *Agrobacterium*-mediated gene transfer [Hilder *et al.*, 1987]. Transgenic tobacco plants which express the cowpea protein [from the constitutive CaMV35S gene promoter] have been shown in bioassays to have enhanced resistance to a number of pest insects [Figure 3]. Insects on CpTI-expressing plants suffered a higher mortality, a reduced rate of development and consumed less of the plant. A commonly observed feature of plant defense mechanisms is their effectiveness against a wide range of potentially harmful organisms. CpTI has been shown in transgenic plants or in artificial diets to adversely affect the development and survival of number of different insects belonging to the orders Lepidoptera, Coleoptera and Orthoptera [see Gatehouse *et al.*, 1992].

Figure 3. CpTI-expressing [left] and control tobacco plants [*N. tabacum* var. 'Samsun'] after 7d exposure to *H. virescens* larvae.

Since these insect control proteins will be delivered to the insects as part of their food in transgenic crops, we have identified a number of candidate genes whose products are expected to adversely affect the insects' digestive system in different ways. The classes of compound include:

Serine protease inhibitors

- such as CpTI. Many insects, particularly the Lepidopterans, depend on serine proteases as their primary protein digestive enzymes. Inhibition of protein digestion leads to disruption of amino-acid metabolism.

Thiol protease inhibitors

- targetted at those insects, such as the major Coleopteran pests [e.g. *Diabrotica sps.* - the corn rootworms, and *Leptinotarsa decemlineata* - the Colorado potato beetle] which have acidic guts and rely on thiol rather than serine digestive proteases.

Alpha-amylase inhibitors

- disrupting carbohydrate metabolism by inhibiting the major enzyme responsible for carbohydrate digestion in the insects' gut.

Lectins

- sugar binding proteins which appear to interact with receptors in the gut wall of susceptible insects.

Ribosome Inactivating Proteins

- which have been shown in artificial diet studies to be more toxic to Coleopteran than to Lepidopteran insects [Gatehouse *et al.*, 1990].

Specific candidate genes for each of these classes have been identified and cloned, as cDNAs or by PCR amplification from genomic DNA, for introduction into transgenic plants, where they are being tested singly and in various combinations. Absence of toxicity to mammals has been a key consideration in our strategy for selecting candidate genes. Thus, for example, although CpTI will inhibit mammalian trypsin *in vitro*, rat feeding trials with the purified protein demonstrate it to be non-toxic *in vivo* [Pusztai *et al.*, 1992]. Such differential toxicity results from the entirely different organisation of the insect and mammalian guts; these differences can be exploited to select insecticidal proteins which are not harmful to the intended end-users of the crops [see Hilder *et al.*, 1992]. The selection of potentially useful insecticidal lectins provides another example of this strategy. Lectins are a heterogeneous class of proteins, specific members of which show a wide range of toxicity when fed to insects or to mammals. However, whilst wheatgerm agglutinnin [WGA], phytohaemagglutinnin [PHA] from *Phaseolus vulgaris* and the lectins from snowdrop, *Galanthus nivalis*, [GNA] and garden pea, *Pisum sativum* [PSA] are all toxic to bruchid beetles [*Callosobruchus maculatus*] when fed in artificial diets, the first two are also notorious anti-nutritional agents to man, whereas PSA and GNA are essentially harmless. Thus, the GNA gene has been selected for transfer into transgenic plants.

The use of these genes in combinations within transgenic plants to give multi-gene, multi-mechanistic resistance is expected to increase both the effectiveness of insect control and its durability, reducing the chances of resistant populations of insects developing. An initial demonstration of the principle of combining these genes has been made by cross breeding transgenic tobacco plants which have been individually transformed with the CpTI gene and the lectin gene from the garden pea [PSA]. In this case the genes had independent, additive effects in controlling *Heliothis* larvae [Figure 4] [Boulter *et al.*, 1990]. Protease inhibitors are likely to be particularly valuable components in such combinations since they should protect the other insect control proteins from premature hydrolysis in the insect gut.

Plant derived insect control proteins tend to be of relatively low absolute

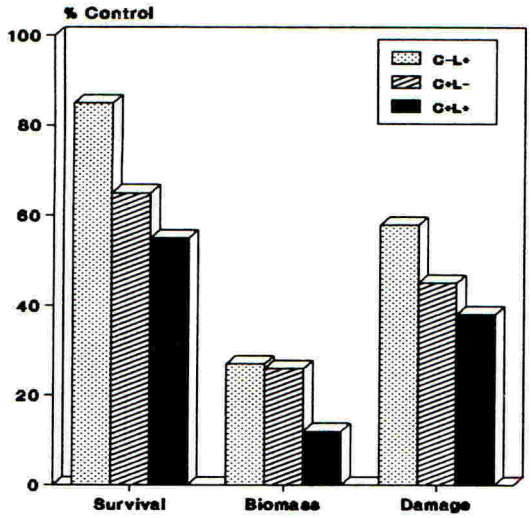

Figure 4. Effect of combining insect control protein genes in transgenic plants. Whole plant bioassay data from transgenic tobacco plants expressing pea lectin [C-L+], cowpea trypsin inhibitor [C+L-] or both [C+L+] foreign genes as % of null segregating controls, against *H. virescens* larvae.

toxicity compared to chemical pesticides or *Bt.* toxin. The question of whether expression of a foreign, plant derived protein in transgenic plants at the levels required for effective insect control incurs a yield penalty has been addressed using our population of CpTI-transformed tobacco plants under carefully controlled conditions in the growth room. Small, but significant differences were observed between transgenic and untransformed control plants in a number of morphological, vegetative and reproductive characteristics. These differences appear to result from the transformation process, not the expression of the foreign protein [Hilder & Gatehouse, 1991], since there is no correlation between the level of CpTI expression and the degree of divergence from the control [Figure 5]. This conclusion holds when the plants are subjected to environmental stresses such as water or nutrient deficiency.

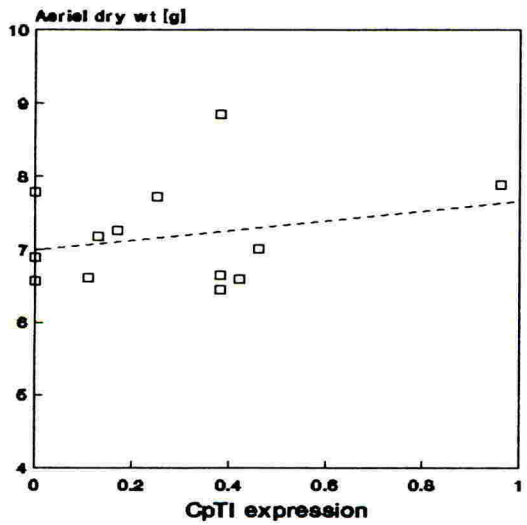

Figure 5. Effect of foreign gene expression on yield of transgenic tobacco. Mean aeriel dry weight [g] against CpTI expression level [% leaf total soluble protein] of 3 clonal replicate plants from 13 primary transformed lines.
$r = 0.2506$: $p[H_0:\rho=0] > 0.05$.

The pest insects which have been considered so far feed by chewing plant tissues; most of them belong to the orders Lepidoptera and Coleoptera. Those insects belonging to the order Homoptera - which have piercing and sucking mouthparts and feed upon plant sap - include many species which are serious pests of agricultural and horticultural crops and of ornamental plants. Crop damage caused by feeding of aphids, whitefies, planthoppers, leafhoppers etc. results in a number of ways. Extraction of sap deprives the plant of nutrients and water leading to loss of vigour and wilting. Phytotoxic substances present in the saliva of some species, and mechanical blockage of the phloem by feeding may result in distortion and necrosis of foliage [as in 'hopper-burn'] and in blindness or shrunken kernels in grain crops. Injury caused by insertion of the mouthparts leaves lesions through which plant pathogens may enter. Production of copious 'honeydew' may allow sooty moulds to develop or its stickiness may interfere with the harvesting of cereals and cotton. Some of the most serious damage caused by these pests is indirect, due to their role as vectors of plant viruses. Reduction of Homopteran insect populations would be particularly useful in limiting the spread of these and other viral diseases in crops.

Because of their specialised feeding behaviour and physiology the currently available transgenic plant technologies have so far not been successful in controlling them. Two major problems have had to be solved before this technology is applicable to sap-suckers:

i) the identification of effective gene products - *Bt* toxins with activity against Homopterans have not been described, and most characterised natural plant resistance mechanisms to them appear to be based on small, non-protein secondary metabolites

ii) the development of an effective means of delivery - expression is required in the phloem sap if the protein is to be ingested by the insects.

Using an artificial diet bioassay, whereby the insects feed on artificial sap through a parafilm membrane, we have now identified a number of plant proteins which are toxic or anti-metabolic to Homopterans [Table 1],[Powell *et al.*, 1992]. Amongst these proteins, it has been shown that the snowdrop lectin is effective against rice brown planthoppers [*Nilaparvata lugens*] and rice green leafhoppers [*Nephotettix nigropictus*] - both major sap sucking pests of rice [Figure 6] - and against peach potato aphids [*Myzus persicae*].

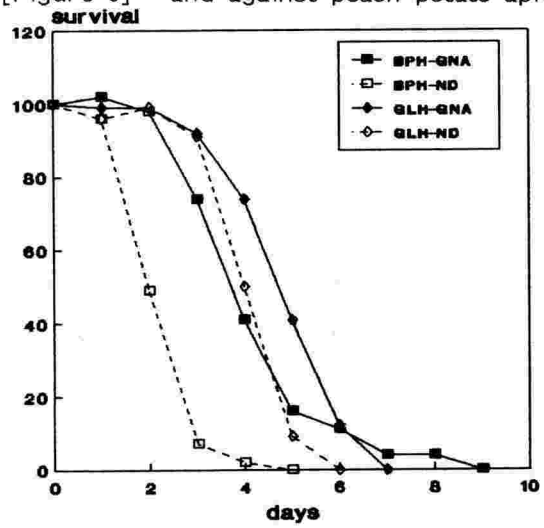

Figure 5. Artificial diet bioassay of GNA against sap-sucking pests the brown planthopper [BPH - squares] and green leafhopper [GLH - diamonds]. Filled symbols indicate survival relative to control diet in the presence of 0.1% [w/v] of the lectin, open symbols on nutrient free [ND] diet.

The promoter associated with the rice sucrose synthase 1 [RSs1] gene [Wang *et al.*, 1992] has been shown to direct phloem specific expression of *GUS* in transgenic plants. This promoter, and the RSs1 gene intron 1 have been linked to the gene encoding GNA and this construct is being introduced into transgenic rice and tobacco for testing *in planta* against the brown planthopper and peach potato aphids.

TABLE 1. Artificial diet bioassay of various proteins for anti-insect activity against *N. lugens*

PROTEIN[1]	CORRECTED[2] MORTALITY	G_{adj}[3]	$p_{[H0:CON=EXP]}$	
Inert proteins				
BSA	-138	12.554	<0.001	***
OVA	4	0.	>0.05	N.S
Lectins				
GNA	79	104.642	<0.001	***
PLA	-2	0.	>0.05	N.S
WGA	78	61.724	<0.001	***
LCA	22	4.336	<0.05	*
HGA	28	2.726	>0.05	N.S
JCA	-3	0.	>0.05	N.S
ConA	30	1.980	>0.05	N.S
PPL	-10	0.164	>0.05	N.S
Enzyme inhibitors				
CpTI	-13	0.160	>0.05	N.S
WAX	-4	0.	>0.05	N.S
Enzymes				
LPO	85	63.724	<0.001	***
PPO	12	0.330	>0.05	N.S

[1] Abreviations: OVA - ovalbumin; BSA - bovine serum albumin; GNA - snowdrop lectin; PLA - pea lectin A; WGA - wheatgerm agglutinin; LCA - lentil lectin; HGA - horse gram lectin; JCA - jacalin lectin; ConA - concanavalin A; PPL - potato lectin; CpTI - cowpea trypsin inhibitor; WAX - wheat α-amylase inhibitor; LPO - soyabean lipoxidase; PPO - polyphenol oxidase.

[2] Percent mortality, corrected according to Abbott [1925] on the day when all 'no diet' insects were dead. This formula produces a negative value if insect survival is enhanced in the presence of the test protein compared to controls.

[3] Survival frequencies were subjected to a G-test of independence. Yates' correction was applied to the data where appropriate [i.e. n<200].

Thus, plant biotechnology is well on the way to offering a realistic alternative to total dependence on chemical insecticides [or surrender to insect pests] in a wide variety of crop production systems. The technology is being transferred to 'orphan', third-world crops as well as the major crops of the developed world. Much careful work remains to be done to determine the optimum strategies for using transgenic crops as part of an integrated pest management system for sustainable agriculture, but the rate of progress over the last five years has been impressive.

ACKNOWLEDGEMENTS

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ENGINEERING FOR APPLE AND WALNUT RESISTANCE TO CODLING MOTH

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ABSTRACT

The production of transgenic plants expressing the insecticidal crystal protein(s) (ICP) of Bacillus thuringiensis (Bt) provide a useful approach toward a non-insecticide approach to combat insect pests. The ICPs of Bt have been used for many years and are highly toxic to many Lepidopteran insect pests. The main objective of our program is to achieve resistance to Cydia pomonella (codling moth, CM), the key pest in both apple and walnut. Purified insecticidal crystal protein fragments (ICPFs) of Bacillus thuringiensis encoded by crylA(c) and crylA(b) of this organism were shown to be lethal to codling moth. One of these genes, crylA(c), was used to transform walnut somatic embryos and apple leaf discs using Agrobacterium-mediated transformation. A binary vector (pWB139) was used to introduce this gene which was expressed as a protein fusion with the kanamycin resistance gene from bacteria. Transgenic walnut embryos representing individual transformation events were germinated to produce shoots that were maintained as micropropagated shoot clones in the laboratory or were grafted onto seedling rootstocks and introduced into the field. Transgenic apple plants were obtained through regeneration of apple shoots from leaf discs, these shoots were rooted to produce plants that have now been introduced into the field. DNA analysis (Southern blotting) was performed to confirm various segments of the introduced T-DNA and to document each transgenic line that was introduced into the field. Insect feeding trials were conducted using in vitro grown transgenic shoots or embryos corresponding to each transgenic line obtained with pWB139 and comparisons were made with both a control construct pWB149 (which does not contain the crylA(c) gene) or non-transformed lines. Field experiments are ongoing, however, micropropagated tissue have revealed very low levels of crylA(c) expression. To correct this problem we have introduced synthetic version of the crylA(c) gene in walnut. Results reveal strong levels of expression with high levels of C. pomonella mortality.

INTRODUCTION

The investigation and application of plant breeding in horticulture and forestry has made a significant impact on the domestication of a wide range of tree crop species, creating important industries and a demand for their products (fruit, nuts, wood, paper etc). The 21st century poses a new set of challenges, an increased demand for horticultural and forestry products that has to be met using novel production practices that sustain yield and quality while at the same time are less exploitive of the environment and the food chains vital to human health than in the past. The

technology and the potential to produce transgenic plants will have an important and powerful impact on some of the immediate problems such as disease and pests. However commercial success will depend upon their integration with the conventional technologies such as plant breeding, plant propagation, production physiology, integrated pest management and post harvest technology. The limiting step here will be "technology transfer".

The apple belongs to the genus Malus of the Rosaceae family. The genus Malus consists of at least 20 to 30 different species, most of the domestic cultivars being derived from M. pumila Mill. Today apple is the most widely grown deciduous fruit in temperate regions of the world with an annual production worth about $5.6 billion. The total production in the United States is worth in excess of $1 billion of which 70% is accounted for by just 4 cultivars ('Red' and 'Golden Delicious', 'McIntosh' and 'Rome Beauty'). California ranks 3rd in the nation for apple production. The commercial production of English or Persian walnuts (Juglans regia L.) on the other hand in the United States is almost entirely concentrated in California. Annual in-shell nut production is in excess of 200,000 metric tons with a farm value of over $240,000,000. Losses in walnut production in excess of $20 million occur each year due to pests, despite the extensive chemical control measures in operation. Codling moth is generally regarded as the number one insect pest for both species.

The chemical control of insect pests is a costly, time consuming and highly toxic process. Secondary insect outbreaks associated with chemical control are yet another impediment introduced by chemical insecticides. A good example of this is the secondary aphid infestation problems associated with the chemical control of Cydia pomonella (codling moth, CM) on walnut after 'Guthion' application. The passage of 'Proposition 65' and the development of the 'big green' initiative in California a few years ago represent growing public concern over environmental impact of chemical pesticides and food safety issues. There is a general feeling that future availability of many chemical pesticides is limited in view of the growing sensitivity to this issue. Research and regulatory costs are also escalating.

Genetic transformation, in theory at least, allows the introduction of any gene from any source, greatly enhancing the germplasm that can be used for a particular tree species. The major limitation apart from gene availability is the development of transformation/gene transfer technologies for the production of transgenic tree crops. We will focus on the following four areas in this review a) The nature of the techniques that have been used successfully to transfer foreign genes into apple and walnut, b) transfer and expression of a first generation construct of an ICP from Bt, c) current experiments involved with the introduction of first and second generation constructs containing a chemically synthesized gene encoding an ICP from Bt and d) management of transgenic tree crops and areas of future research.

TRANSFORMATION OF APPLE

Apple transformation was first reported by James *et al.,* 1989 using an Agrobacterium-mediated transformation of leaf discs of the apple cultivar 'Greensleeves' using the binary vector pBin6. This was possible because key tissue culture procedures had been successfully developed in apple including micropropagation, (Jones, 1976; Zimmermann, 1983) and adventitious bud formation (James, 1987). A detailed procedure for the transformation of apple has now been published for the transformation of the apple cultivar 'Greensleeves' which is an excellent experimental system for apple (James and Dandekar, 1991). The adventitious bud formation on leaf discs is a vital component of the transformation system to regenerate transgenic plants and, in the case of apple, it is highly variable. Factors that appear to affect regeneration frequencies include media (inorganic content), the presence of phytohormone (both type and amount), physiology of the explant source and cultivar of apple used. Regeneration has been reported in other varieties of apple and these include, 'McIntosh', 'Triple Red Delicious' (Fasolo et al., 1989), M26, M25, M9 and 'Greensleeves' (James, 1987; James et al., 1988).

Another problem is the low frequency of transformation observed in apple (James et al., 1989, James and Dandekar, 1991). Recent studies indicate that several factors could influence the

transformation frequency of apple including the type and physiology of explant tissue used, the strain of Agrobacterium and design of plasmid vectors (James et al., 1988; James and Dandekar, 1991; Dandekar et al., 1990). Agrobacterium strains containing pTiBo542 or pTiC58 and their derivatives thereof were found to be most virulent on apple (Dandekar et al., 1990). Virulence of the Agrobacterium strain could be enhanced either genetically by introducing additional copies of the virulence genes (Dandekar et al., 1990) or physiologically by growing the bacterium under conditions that induce the virulence genes (James et al., 1992). Many different transgenic apple plants have already been obtained and currently we have initiated (Spring 1992) the first field introduction of transgenic apple plants (permit # 91-218-03 of the U.S. Department of Agriculture's Animal Plant Health Inspection Service) after obtaining government regulatory approval.

TRANSFORMATION OF WALNUT

The transformation of walnut is also an Agrobacterium-mediated process, however, it has some unique features. First and foremost the target explant used in the transformation experiments is somatic embryos. Adventitious bud formation has not as yet been reported in walnut therefore procedures to regenerate transgenic plants by the leaf disc transformation system (Horsch et al., 1985) could not be used. However, walnut readily undergoes somatic embryogenesis when immature embryos are introduced into culture (Tulecke and McGranahan, 1985; Tulecke, 1987). These somatic embryos proliferate in culture producing numerous secondary embryos that appear to originate from single cells in the epidermal layer of the embryo (Tulecke and McGranahan, 1985; Polito et al., 1989). Selected somatic embryos can be germinated to produce plants. Unlike apple discussed above, various walnut tissues were found to be highly susceptible to infection with Agrobacterium (Dandekar et al., 1988) including embryos (McGranahan et al., 1988). High frequencies of transformation have been observed with single embryos capable of producing multiple independently transformed embryo lines (McGranahan et al., 1990). The procedure for selecting transformed embryo lines that represent independent transformation events and the regeneration of transgenic plants from these embryo lines has been published (Dandekar et al., 1989). After obtaining government regulatory approval, transgenic walnut trees were planted in the field in the spring of 1990 and now have begun to flower and produce nuts. The planted block includes trees that express marker genes like kanamycin phosphotransferase [APH(3')II] and β-glucuronidase (GUS) that have been previously reported (McGranahan et al., 1988, 1990). In addition many of the trees contain the cryIA(c) gene from Bt (Dandekar et al., 1992 a,b) and are described below.

TRANSFER AND EXPRESSION OF A FIRST GENERATION CONSTRUCT

Although different strains of the bacterium Bacillus thuringiensis make ICP's that are active against different groups of insects, the relationship between structure and function of the various ICPs is still being explored (Aronson et al. 1986; Höfte and Whiteley, 1989). At least 40 genes encoding ICP's effective against Lepidopteran, Dipteran and Coleopteran insects have been isolated and their DNA sequences compared (Höfte and Whiteley, 1989). We have tested purified preparations of the ICPs encoded by the cryIA(b) and cryIA(c) genes and found them to be very toxic to the target insect species codling moth (CM) Cydia pomonella (L.), a key walnut and apple pest (Vail et al., 1991). The cryIA(c) gene was introduced using the binary plasmid pWB139 into both walnut and apple and details of these experiments and the data will appear in separate publications (Dandekar et al., 1992a,b). The plasmid pWB139 was constructed by Dr. M. Barnes, Washington University School of Medicine, St. Louis, Missouri. This plasmid contains the cryIA(c) gene displaying the insecticidal activity from the HD-73 strain of Bacillus thuringiensis fused with the bacterial kanamycin resistance gene product APH(3')II. The fused gene sequences are transcriptionally regulated by the 35S promoter of the cauliflower mosaic virus (CaMV35S) and in addition are wound inducible. Wound inducibility is regulated by the presence of a fragment containing the sequences of exon 3 and part of the 3' region of the tomato protease inhibitor I gene. Therefore, successful expression of this chimeric fusion gene would give a product that should give resistance to the antibiotic kanamycin allowing selection of transformed plants as well as being toxic to insects by virtue of the presence of the cryIA(a) sequences. Experiments with the transgenic

plants in the field obtained as a result of transformation with this vector in both apple and walnut are in progress. However analysis of tissue of in vitro grown plants from both transgenic walnut (Dandekar et al., 1992a) and apple (Dandekar et al., 1992b) reveal low levels of expression.

INTRODUCTION AND EXPRESSION OF A SECOND GENERATION CONSTRUCT

Low levels of expression have been observed in plants expressing cryIA(b) or cryIA(c) in transgenic tobacco (Vaeck et al., 1987) and tomato (Fischoff et al., 1987) plants. However, in these transgenic plants although they expressed very low levels of detectable protein this was sufficient to protect the plants from damage caused by the feeding larvae of tobacco or tomato hornworm, and tobacco budworm, all known voracious pests (Fischoff et al., 1987; Vaeck et al., 1987). However, subsequent field trials revealed that a higher level of expression would be desirable for a commercial product (Delannay et al., 1989). Although these genes are expressed to extremely high levels in their native bacterial species (Bacillus thuringiensis), accumulating to concentrations of in excess of 50% of total cellular protein, the gene structure appears to be the most important factor limiting their expression in plants due to differences in codon usage pattern. RNA (transcript) instability has been reported and this may limit the amount of ICPs or ICPFs that accumulate in transgenic plants (Murray et al., 1991). The approach that has the most potential is the use of highly modified genes that have a codon usage bias more compatible with dicotyledonous plants (Perlak et al., 1990, 1991). In dicotyledonous plants, the use of synthetic gene sequences have worked very efficiently in cotton (Perlak et al., 1991) and in walnut (Dandekar et al., unpub.). We now have several embryo lines which give a high level of expression of the synthetic gene of cryIA(c) using the CaMV35S regulatory sequences (Dandekar et al., unpub). This synthetic cryIA(c) gene was supplied to us by Dr. Fischoff of Monsanto Co., St. Louis, Missouri.

FUTURE DIRECTIONS - RESISTANCE MANAGEMENT

Given their persistence and stability in the ecosystem it has been suggested that trees have considerable potential for the selection of resistance in insect populations (Raffa, 1989). This could be an important issue in a tree crop for the development of resistance to ICP's in target insects. The Bt encoded ICP's had a spotless 30 year commercial record and safe history of usefulness in the field. However, this is no longer true today with recent findings of resistance development (Ferré et al., 1991; Gibbons, 1991; Sims and Stone, 1991; Tabashnik et al., 1990; 1991). Reports have appeared on the development of resistance under conditions of extreme selection pressure with a stored grain products pest (McGaughey, 1985; McGaughey and Johnson, 1987 and McGaughey and Beeman, 1988), and deliberate selection conditions in the laboratory (Sims and Stone, 1991; Stone et al., 1989). These studies clearly indicate the need to study resistance mechanisms with the aim of developing management strategies to offset resistance development. There are many approaches that will slow down or limit the development of resistance (Roush, 1989). For instance, genetic regulation of the genes encoding ICPs in the transgenic plant could be used to target production of ICPs in selective tissues/organs or particular stages of development (Gould, 1988) or different structural combinations of the toxin protein could be tested to identify combinations which are more difficult to develop resistance to i.e., especially if they have different modes of action or do not share the same cellular target site in the insect. Recent studies on the mechanism of resistance indicates that it may be due to a decrease in the binding between the insect's midgut brush border membrane and the insecticidal protein (Van Rie et al., 1990; MacIntosh et al., 1991; Ferré et al., 1991). ICP-containing plants must be deployed carefully, so that insect populations are not placed under excessive selection for resistance. The use of integrated pest management strategies will be important.

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FIELD PERFORMANCE OF INSECT RESISTANCE TRANSGENIC CROP PLANTS: COTTON, POTATO AND CORN.

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(NO WRITTEN SUBMISSION)

THE FIELD RELEASE OF TRANSGENIC PLANTS

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ABSTRACT

The modification of plants by recombinant DNA methods presents new opportunities to introduce genes from a wide range of organisms. Following initial assessment of the expression and inheritance of transgenes in the laboratory and glasshouse, it is necessary to evaluate transgenic plants under field conditions. This allows a more comprehensive analysis of the transgenic plants under conditions closer to those used in agricultural practice. The initial experiments described were aimed at analyzing quantitative characteristics among 70 independently transformed potato plants and control plants. These showed that some constructs containing marker genes were associated with a mean decrease in plant height and tuber weight. There was also evidence of tissue culture induced variation (somaclonal variation) among the transgenic plants. With the aim of simplifying some of the procedures for handling transgenic plants outside containment, studies were carried out to determine the distance of pollen movement from potato and the likelihood of the transgenes being transferred to the solanaceous weed species *Solanum nigrum* and *S. dulcamara*. The amount of cross pollination between potato plants falls off sharply with increasing distance and no pollination was observed at a distance of 20m. There was no evidence that potato can cross pollinate with its two wild relatives. These data are being used to simplify the requirements for carrying out field experiments with transgenic potatoes.

INTRODUCTION

Using modern methods of genetic modification it is now possible to insert various types of novel genes into crop plants in the laboratory. The choice of genes available for crop improvement by traditional breeding methods is limited by sexual incompatibility. The use of recombinant DNA techniques to isolate and manipulate DNA, and transformation methods to introduce the modified DNA into plants (transgenic plants), makes it possible to transfer genes from a wide range of sources including from unrelated plants, bacteria, viruses and even animals.

Transgenic plants are first evaluated in the laboratory to confirm that the introduced gene (transgene) is being expressed as expected. The second phase of analysis is by growing the transgenic plants in a glasshouse to determine the temporal and spatial expression of the transgenes along with their stability of expression over sexual generations (or asexual generations in vegetatively propagated species). The

third phase of analysis needs to be carried out in a field environment close to that used in agricultural practice.

In early field releases of transgenic plants at the Cambridge laboratory from 1987 to 1989, our aim was to study the expression of the transgenes inserted, and the effect of the transformation process on the performance of the transgenic potatoes. During those early release experiments the procedures we were required to follow by the Advisory Committee on Genetic Modification (Planned Introduction Subcommittee), included: removing the flowers from all the plants in the trial site, removing any berries that might form, isolating the experiment from other potato crops by up to 600m and removing solanaceous weed species in the vicinity of the plot. Because the requirement to carry out these procedures made the field evaluation of transgenic plants cumbersome and not directly parallel to how potato plants are grown in agricultural practice, we decided to address some of the questions underlying the need for these elaborate procedures.

The procedures were necessary because of uncertainty about the extent of gene dispersal from potato by pollen. There were two primary questions:

1) is there the possibility that the transgenes might be transferred to related weed species of potato and become part of the gene-pool of those species and,

2) what distance can potato pollen travel and give successful cross pollination?

In other words, is it necessary to remove wild related solanaceous species from the vicinity of the transgenic potatoes to prevent transfer of the transgene by cross pollination; and, how far should transgenic potatoes be separated from ordinary crops of potato to prevent the transgenes being transferred by cross pollination? Even though in our experiments the potatoes carried marker genes considered to present no hazard if cross pollination did occur, genetic isolation during the initial evaluation of a novel transgenic plant is generally accepted as good practice.

The objective in this paper is to present the main conclusions from our experiments on the field performance of transgenic potatoes containing marker genes; and to summarise what we have learned about the extent of gene dispersal from transgenic potatoes, both to other potato plants and to related weed species.

THE TRANSGENIC PLANTS

Four gene constructs were inserted into potato by incubating tuber discs with *Agrobacterium tumefaciens* (Sheerman & Bevan, 1988). All four constructs contained the nopaline promoter sequences regulating the neomycin phosphotransferase gene (NPTII) which confers resistance to the antibiotic kanamycin, along with fragments (4 sizes) of the patatin promoter regulating the reporter gene beta-glucuronidase (GUS). Full details of the constructs are given by Jefferson et al. (1990). Seventy independently transformed potato plants were used in the field performance

experiment (Dale & McPartlan, 1992) and a subset of 8 transformants in the gene dispersal experiment. The dominant kanamycin resistance character was used to screen seedlings for evidence of cross pollination.

FIELD PERFORMANCE OF TRANSGENIC POTATO

The aim was to determine whether there was evidence of a) pleiotropic effects on quantitative plant characters from the introduced transgenes and b) somaclonal effects induced by the tissue culture procedure used during the transformation process.

The trial consisted of 156 lines which included: 70 transgenic lines, 45 lines regenerated from tuber discs but without the *Agrobacterium* transformation step, and 41 lines established from tuber node cuttings. The 156 lines were planted as tubers (grown in a field plot the previous year) in a randomised field plot with 3 replicates. The plants established from tuber nodal cuttings were the control plants, the plants regenerated from tuber discs gave a measure of somaclonal variation and the transgenic plants gave a measure of the effect of the transgenes on quantitative plant characters. The characters measured were, height of the tallest stem at plant maturity, total tuber weight and total tuber number. The following conclusions were drawn (see Dale & McPartlan, 1992 for full details).

1) There was a significant effect on plant growth associated with the four gene constructs (the characters were averaged over 10-30 independently transformed plant lines for each construct). In general the transgenic plants performed less well (lower mean height and tuber weight) than the node culture or tuber disc controls, but certain individual transformants performed as well as the controls.

2) There was a significant effect of somaclonal variation but lines could be selected that performed as well as the node culture controls.

3) Although the experiment was not designed specifically to test the effect of the kanamycin resistance gene on plant performance, the data suggested that there was no adverse effect of the gene on plant performance.

GENE DISPERSAL FROM TRANSGENIC POTATO

The experiment consisted of c750 transgenic potato plants, planted in a 20 x 20m central plot in standard sized ridges. Surrounding the central plot were a further eight plots, each with c 50 non-transgenic potato plants of the same variety (Desiree). Four of the plots were planted at 10m and four at 20m from the edge of the central plot. Four non-transgenic plots were also planted as an integral part of the transgenic central plot, with the non-transgenic plants at distances of 0-3m from the nearest transgenic plant. The non-transgenic potato plants acted as pollen recipient plants to measure the amount of cross pollination with the transgenic plants over the various

distances.

Plants of *Solanum nigrum* (black nightshade) and *S. dulcamara* (woody nightshade) were established by serial sowing in a glasshouse over several weeks and transplanted into the field just before the transgenic potatoes began to flower. Plants of the two solanaceous species were chosen for transplanting into the field so that they would flower at the same time as the transgenic potatoes.

Seeds were harvested from the potato plants and from *S. nigrum* and *S. dulcamara*, and screened for presence of the kanamycin resistance gene by germinating seeds on a culture medium containing kanamycin monosulphate (200mg/l) or by spraying the leaves of seedlings in a glasshouse with a solution of kanamycin at the same concentration. In a sample of plants, Southern blot analysis was used to confirm that the kanamycin resistant seedlings contained the resistance gene and the susceptible seedlings did not. Transgenic seedlings known to contain the kanamycin resistance gene were tested for potato and for *S. nigrum* to confirm the efficiency of the selection procedure. The number of kanamycin resistant seedlings, as a proportion of the total number harvested, was used as a measure of the extent of cross pollination. The conclusions from these studies were as follows.

1) None of the *S. nigrum* seedlings (sample size c3000) nor the *S. dulcamara* seedlings (sample size c1000) were resistant to kanamycin. There was therefore no evidence of cross pollination between potato and the two common solanaceous weed species.

2) The frequency of cross pollination between transgenic potato and non-transgenic potato decreased sharply with increasing distance (Table 1) and no hybrids were detected at 20m.

TABLE 1. Percentage of kanamycin resistant hybrids formed between transgenic potato carrying the kanamycin resistance marker gene and non-transgenic potato plants growing at various distances away in a field plot. Seeds were harvested from the non-transgenic plants and screened on kanamycin at a concentration of 200mg/l.

Distance (m)	% hybrids	Number of seedlings screened
0	24	> 1000
0-3	2	> 3000
10	0.017	>11000
20	0	>12000

Additional data

During the course of these experiments two other sources of evidence became available on the sexual compatibility of potato with related weed species and on the

distance of pollen transfer between potato plants.

A research group in the Netherlands (W. Stiekema, personal communication) carried out over 2000 manual pollinations between potato and *S. nigrum* and over 500 between potato and *S. dulcamara*. No berries were formed from pollinations with *S. dulcamara* and seedless berries were formed from hybridizations with *S. nigrum*. Approximately 24,000 immature embryos were rescued from hybridizations with *S. nigrum* and two male sterile hybrids were produced. The two hybrids were obtained by culturing very immature embryos at 10-23 days after pollination and no seeds were produced when berries were allowed to mature naturally (see also Dale et al. 1992).

An experiment in New Zealand has also provided evidence of the distance potato pollen travels (Tynan et al. 1990). In their studies there was no evidence of cross pollination between the transgenic and non-transgenic potatoes at distances of greater than 4.5m.

The isolation procedures used in 1992.

The data on the extent of gene dispersal through pollen has contributed to a simplification of procedures for experiments with transgenic plants in potato. There is no longer a requirement to remove flower buds or berries from transgenic potatoes and no requirement to remove solanaceous weeds from the trial site. The isolation distance between transgenic and non-transgenic plants has (at least in some instances) been reduced to 5m with a distance of 15m specified for monitoring plants around the plot for 3 years following the trial, to identify and remove any seedlings that may contain the transgenes (Dale et al., 1992).

CONCLUSIONS

The prospects of introducing a much wider range of genes than has hitherto been possible by traditional breeding, will give opportunities to modify crops in many novel ways. As with all significant developments in biology, new opportunities bring new responsibilities. In the case of genetically modified plants it is necessary to ensure that transgenic varieties are safe to use by the consumer and do not present a risk to the environment. There are currently several levels of international discussion on the development of regulations to govern the release of transgenic plants. Because it is now possible to introduce genes into plants that are outside the experience of conventional plant breeding, some form of regulation is necessary, however, the formulation of regulations presents some important challenges. In formulating these regulations the objective must be to ensure that they are:

1) workable and "user-friendly",

2) based on criteria that command the respect of scientists, environmentalists and the general public,

3) amenable to the principle of case by case assessment, streamlining and

eventually the de-regulation of certain transgenes or constructs,

4) allow the use of transgenic plants for crossing in traditional breeding programmes with no, or with minimal, regulation,

5) internationally equitable in the amount of information required, and the time and cost of processing a proposal for the release of transgenic plants, both for small scale experiments and for commercialisation of transgenic varieties.

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FOREIGN PHYTOALEXIN EXPRESSION IN PLANTS RESULTS IN INCREASED DISEASE RESISTANCE

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ABSTRACT:

Stilbene phytoalexins with fungicidal potential are synthesized in several unrelated plants, e. g. in peanuts (Arachis hypogaea), in grapevines (Vitis vinifera) and pine trees (Pinus sylvestris). With the objective of increasing the resistance of plants to pathogens and to study defense-related genes in transgenic plants we have isolated grapevine (Vitis vinifera) stilbene synthase genes and transferred them to tobacco (Nicotiana tabacum). These tobacco plants accumulate trans-resveratrol (3,4',5 Trihydroxystilbene) upon fungal infection at the infection sites. Transgenic tobacco plants grown in the greenhouse under controlled conditions showed a significant increase in resistance to the test pathogen Botrytis cinerea. Preliminary data indicate a positive correlation of resveratrol synthesis and resistance to B. cinerea in tobacco.

INTRODUCTION

Upon pathogen attack a number of defense-related genes are activated in plants and highly co-ordinated molecular events are induced in the vicinity of the infection sites. Several different defense mechanisms of plants, which can be either constitutive or induced, have been analysed at the molecular level. Due to such defense mechanisms plants can be resistant to pathogens (for review Bell, 1981 and Sequeira, 1983). Among these reactions the microscopically detectable ones like hypersensitive cell death, formation of small local lesions, the formation of cell wall bound phenolics, callose formation and the subsequent accumulation of phytoalexins are most obvious. Phytoalexins are fungitoxic molecules supposed to play an important role in the early defense mechanisms of plants in incompatible host pathogen relationships or in the defense of non-obligate biotrophic pathogens (Ebel, 1986; Scheel et al., 1986; Jahnen and Hahlbrock, 1988).

It may now be possible to engineer the resistance of plants towards fungal pathogens by the introduction of genes coding for the synthesis of phytoalexins. For the engineering of plants to produce foreign phytoalexins, it is necessary that only a limited number of genes has to be transferred. This is the case with hydroxystilbenes whose formation requires, besides the

normal pathways of plant metabolism, stilbene synthase only. As an example of practical potential the formation of stilbene-type phytoalexins (Gorham, 1980) in plants has been discussed (Hain et al., 1990). Stilbenes are present in several unrelated plants either as constitutive components of the woody parts or their synthesis can be induced by stress, UV-light, fungal elicitors, pathogenic fungi or wounding (Ingham, 1976; Schöppner and Kindl, 1979; Vornam et al., 1988).

Peanut and grapevine stilbene synthase cDNA and gene sequences have been isolated (Schröder et al., 1988; Melchior and Kindl, 1990). Furthermore, full length genomic clones have been isolated (Hain et al., 1992) offering the possibility to study the formation of stilbenes in heterologous plant systems and to evaluate the biological effects of the additional stilbene formation on disease resistance in transgenic plants.

Since there is evidence that the synthesis of stilbenes contributes to the resistance of grapevine to B. cinerea (Stein and Blaich, 1985; Langcake and Pryce, 1977; Langcake and McCarthy, 1979; Dercks and Creasy, 1989), a potential use of stilbene synthase genes in agriculture can be implicated.

We have studied the expression of stilbene synthase genes in heterologous plant systems and evaluated the effect of stilbene formation on disease resistance in these plants. B. cinerea being a perthotrophic pathogen with a broad host range but with no economic importance in tobacco cultivation has been chosen as a test pathogen because of the positive correlation of resveratrol content of grapevine varieties and their resistance to greymould (Stein and Blaich, 1985; Dercks and Creasy, 1989).

MATERIALS AND METHODS

Plant Material and Transformation Procedures

Nicotiana tabacum cv. Petit Havana SR1 and Nicotina tabacum var. Samsun containing stilbene synthase genes with their natural promotors have been generated either by direct gene transfer or Agrobacterium-mediated transformation (Hain et al., 1985). Transgenes contain either one or two full-size stilbene synthase gene from Vitis vinifera cv. Optima (Hain et al., 1992).

Northern Blot Analysis

RNA was isolated and purified as previously described (Schröder et al., 1988) and separated on a denaturing 1,5% agarose formaldehyde gel and transferred to nitrocellulose filters according to Maniatis et al., (1982). A stilbene synthase cDNA fragment isolated from grapevine (Melchior and Kindl 1990) as probe was used and labelled by nick translation (Amersham Buchler) to a specific activity > 4 x 10^8cpm/µg DNA. Hybridization was for 24 h at 42°C and washes at 50 °C in 0.1XSSC and 0.1% SDS. X-Ray films were exposed for 24 h at -70°C using intensifying screens.

In situ RNA Hybridizations

Tissue fixation, paraffin embedding, sectioning and hybridization

To prepare paraffin sections tobacco leaflets were prefixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 2 h on ice according to Schmelzer et al., (1989). The fixed tissue was subsequently dehydrated in a series of aqueous ethanol solutions (Schmelzer et al., 1989). The tissue was then infiltrated with tertiary

butanol and paraplast as described by Schmelzer et al., (1989) and was incubated for further 2 days at 60°C. After cooling and hardening of the paraplast, small paraffin blocks containing leaflets were mounted to a holder, trimmed and sectioned at 7 µm thickness with a standard microtome (Reichert Jung). Sections were transferred to water droplets on microscopic slides coated with poly-L-lysine (Angerer et al., 1988). After relaxation of the sections, the water was removed and the slides were exposed for 2h to 40°C. Subsequent deparaffinization of the sections was performed in xylol/ethanol and re-hydrated in aqueous ethanol according to Schmelzer et al., 1989). Sections were then treated with pronase and post-fixed (Somssich et al., 1988). Sections were then dehydrated in 30%, 60%, 80%, 95% and 100% of ethanol for 5 min each.

Dehydrated sections were hybridized with ^{35}S labelled sense (control) and antisense RNA transcripts obtained from grapevine stilbene synthase cDNAs (Melchior and Kindl, 1990). Conditions for the in situ hybridization and detection of double stranded RNA by microautoradiography were the same as described by Somssich et al., (1988). For in vitro transcription the cDNAs were subcloned into the vector pBluescript II SK+ (Statagene, La Jolla, CA). The ^{35}S-labelled transcripts were synthesized from an isolated BssHII fragment containing the cDNA flanked by T3 and T7 promoters as described by the supplier. The transcripts were subsequently hydrolysed in alkali to give fragments of about 50-150 nucleotide in length (Somssich et al., 1988). Control hybridizations with sense transcripts gave in all case only low background signals statistically distributed over the slide surface. Photomicrographs were taken under a Zeiss IM 35 microscope equipped with darkfield and epifluorescence as described by Schmelzer et al., (1989).

Inoculation of tobacco plants with Botrytis cinerea

For the in situ RNA hybridization experiments shoot tips of sterile SR1 tobacco shoot cultures were dipped in B. cinerea spore suspensions ($5x10^5$ spores/ml) and placed on solidified LS Medium (Linsmaier and Skoog 1965).

For biological testing and for northern blot analysis of stilbene synthase gene expression in leaves transgenic SR1 tobacco plants and wild-type plants as controls were multiplied by in vitro shoot culture and then transferred to the greenhouse. Plants were grown in the greenhouse at 22-25°C and 80% rel. humidity and inoculated 4-6 weeks after transfer having 4-5 fully expanded leaves. Furthermore experiments were performed with F1 generations of Nicotiana tabacum var. Samsun (E1.3.1) and wild-type Samsun controls. Transgenic tobacco plants and SR1 controls were incubated in tents and inoculated with spore suspensions of B. cinerea (10^5 spores/ml). After incubation for 4-7 days at 19°C and 100% relative humidity, infection density was evaluated on six leaves numbered from bottom to the top.

Experiments for the demonstration of a correlation of resveratrol formation in transgenic SR1 tobacco were performed with homozygous F3 generation plants containing stilbene synthase gene 1 (Vst1).

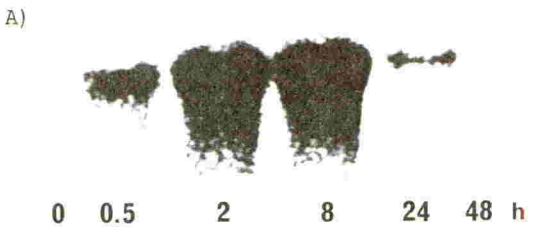
Analysis of resveratrol formation in transgenic tobacco

Resveratrol production was analysed using the ELISA test with antisera raised against synthetic resveratrol (Hain et al., 1990). Plant material was homogenized in liquid nitrogen and extracted with 5ml methanol/g plant material for 1h in the dark on a rotary shaker and centrifuged at 3500 rpm at 4°C. The supernatant was used in the ELISA assay for the analysis of resveratrol.

RESULTS

Expression of stilbene synthase genes in heterologous plants

In order to study the expression of newly introduced disease-related genes (Hain et al., 1990, 1992) coding for the synthesis of the phytoalexin resveratrol in heterologous plant systems we isolated stilbene synthase genes from grapevines known to express at a high level (Hain et al., 1992). A genomic clone containing two full size stilbene synthase genes was transferred to tobacco, tomato, oilseed rape and potato by Agrobacterium-mediated transformation. Upon induction by fungal attack or elicitor treatments stilbene synthase mRNA accumulates in these plants. Detailed analysis of heterologous stilbene synthase gene expression was performed with tobacco. In elicitor treated tobacco cell cultures as well as in infected tobacco leaves a pathogen-inducible, transient stilbene synthase gene expression has been demonstrated (Fig. 1a,b, Hain et al., 1992). Furthermore, in situ RNA hybridization experiments have demonstrated that the stilbene synthase genes are expressed to a high level at the vicinity of the fungal infection site (Fig. 2, Hain et al., 1992). 8 h post inoculation cells around the infection site accumulate stilbene synthase mRNA (about 75 µm in diameter).



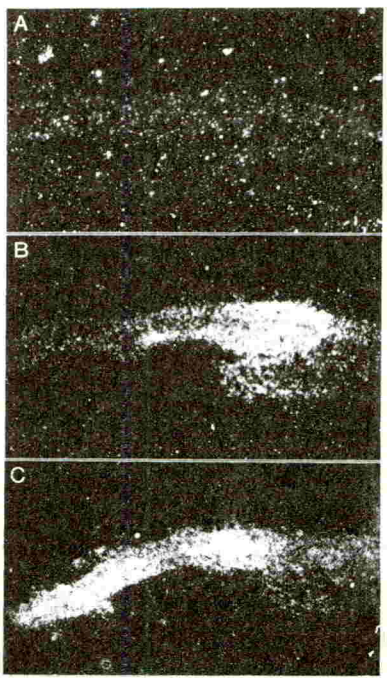


Fig. 1: Left: Northern blot showing inducible and transient expression of grapevine stilbene synthase genes in cell suspension cultures (elicitor-treated, **A**) and tobacco leaves inoculated with B. cinerea (**B**).

Fig. 2: Right: Darkfield micrograph of transgenic tobacco leaf cross sections after in situ RNA hybridizations with grapevine stilbene synthase sense (**A**, control) and antisense (**B**) transcripts. Leaflets had been exposed to B. cinerea for 8 h. When leaflets were analysed 24 h p.i. areas of elevated stilbene synthase mRNA accumulation were larger (**C**).

Enzymatic activity of stilbene synthase and stilbene formation in tobacco

Stilbene synthase activity was detectable in crude extracts of transgenic tobacco cell suspension cultures after induction with PMG elicitor or Botrytis cinerea (Fig. 3). Enzymatic activity reached a maximum 12 h after induction and decayed.

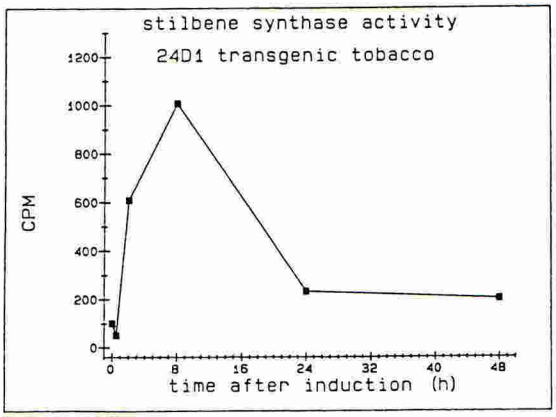


Fig. 3: Kinetics of enzymatic activity in crude extracts of transgenic tobacco 24D1 suspension cultures induced with crude elicitor preparations of Phytophthora megasperma f.sp. glycinea

Influence of stilbene synthase genes on resistance of tobacco to B. cinerea

Transgenic tobacco plants grown in the greenhouse under controlled conditions showed a higher resistance to the test pathogen B. cinerea compared to wild-type SR1 or Samsun control plants (Table. 1). Summing up all experiments the effects were reproducibly observed in transgenic line 23D17 and E1.3.1. Only in one of six experiments 23D17 plants were diseased, e.g. like the wildtype tobacco control and F1 plants of E1.3 showed in all of three experiments enhanced resistance. The reduction in disease incidence mediated by stilbene synthase gene expression was in both tobacco varieties at the same level with an average of about 49% reduction (23D17) and 41% reduction (E1.3.1). Transgenic tobacco line 15D15 (Hain et al., 1990) containing a peanut stilbene synthase gene producing only low amounts of resveratrol served as transgenic control. 15D15 showed no significant increased resistance to B. cinerea.

Hypersensitive reactions of transgenic tobacco plants were rarely observed, the exception beeing transgenic Samsun tobacco plants (E1.3.1). Some plants produced only necroses but no lesions, whereas other plants being less infected than controls showed compatible interaction but fewer lesions. B. cinerea was able to sporulate in these lesions after a certain incubation period under high humidity conditions. At the necrotic infection sites fungal development had stopped completely. In general in transgenic plants more often necrotic lesions occured together with "normal" lesions than only hypersensitive type of necroses.

The effectiveness of the stilbene synthase genes in enhancing resistance to other pathogens than Botrytis is not yet proven. Alternaria longipes e.g., being an important leaf pathogen of tobacco in the field, was not influenced in its development in first greenhouse trials.

Three individual experiments (Table. 2) illustrate the stable effects in line 23D17 and E1.3.1 to B. cinerea. For a perthotrophic leaf pathogen like Botrytis disease incidence is correlated with leaf age. Older leaves of wildtype tobacco plants are more susceptible than younger leaves. Also in transgenic tobacco plants disease incidence was dependent on the leaf age (Table 2). Compared to wild-type SR1 tobacco plants there is a tendency to a higher level of resistance on younger leaves in transgenic tobacco plants. The highest reduction in disease incidence on transgenic SR1 tobacco plants was on the youngest leaves (No.5 and 6).

Tab. 1: Influence of stilbene synthase genes on resistance of tobacco to B. cinera: Summary of biological testing in the greenhouse

Clone	number of trials with difference in disease incidence[1] compared to controls (a, wildtype SR1 or b, Samsun) >25% increased	+/-25% no diff.	>25% reduction	% average disease reduction	No. of trials	No. of plants
a)						
15D15	2	3	1	-11,8[2] (+/-19,7)[3]	6	86
23D17	0	1	5	49,0 (+/-10,2)	6	77
24D1	1	3	2	12,3 (+/-16,6)	6	78
b)						
E1.3.1	0	0	3	41,4 (+/-7,2)	3	97

[1] disease incidence = average of percent diseased leaf area on leaves No. 1-6
[2] increase in disease incidence
[3] SE of the mean in brackets

Tab. 2: Disease incidence on leaves of wildtype* and transgenic tobacco with B. cinerea in dependence of leaf age

clone	% diseased leaf area on leaves 1-6 old ----------> young leaves 1	2	3	4	5	6	% average diseased leaf area	% disease reduction	No. of plants
Trial A									
SR1*	15,0	16,0	16,5	11,5	8,6	2,8	11,7	-	15
15D15	13,8	11,2	9,7	6,8	6,1	2,2	8,3	29	14
23D17	6,4	8,6	8,8	2,9	1,7	0,9	4,9	58,1	13
24D1	10,8	8,1	3,7	3,6	3,0	0,8	5,0	57,3	9
Trial B									
SR1*	11,3	13,6	9,5	13,9	3,8	0,8	8,7	-	21
15D15	16,6	16,8	9,7	5,1	2,6	0,4	8,5	2,3	21
23D17	3,7	4,0	1,1	0,7	0,4	0,0	1,6	81,6	14
24D1	7,3	9,9	8,8	8,3	6,6	0,8	6,7	23,0	14
Trial C									
Samsun*	11,4	15,2	19,7	18,8	9,0	1,8	12,6	-	32
E1.3.1	4,2	5,0	7,1	9,0	8,7	2,9	6,1	51,6	32

Correlation of resveratrol production and susceptibility of transgenic tobacco to Botrytis cinerea infection

Transgenic tobacco plants homozygous for stilbene synthase gene 1 (Vst1, Hain et al., 1992) and respective controls were inoculated with B. cinerea and after 24, 48, 72 and 96 h half of the leaves were cut off, analysed for resveratrol content and in the remaining half of the leaves (leaves 2-7, from bottom to top) the disease incidence was evaluated after 120 h. The amounts of resveratrol determined in the extracts of the leaves were plotted versus percent disease incidence evaluated after 120 h (Fig.4). Obviously, a basal level of resveratrol has to be synthesized to reduce disease incidence in transgenic tobacco. Furthermore, these data indicate a correlation of resveratrol concentration in leaves and reduction of disease incidence in tobacco (Fig. 4).

We have compared the kinetics of resveratrol accumulation in leaves with high and low disease incidence (Fig. 5) and found that leaves accumulating high levels of resveratrol 48 h after inoculation were more resistant to B. cinerea infection than controls or transgenic leaves which did not accumulate high levels at that time.

In summary, these results indicate that a certain basal level of resveratrol is necessary combined with a high level of resveratrol 48h after inoculation for an increased resistance of tobacco leaves to B. cinerea.

Fig. 4: Relation of % disease incidence versus amounts of resveratrol. Extracts from 48 leaves were analysed and corrected

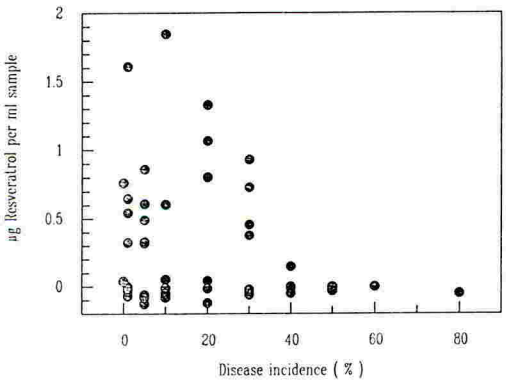
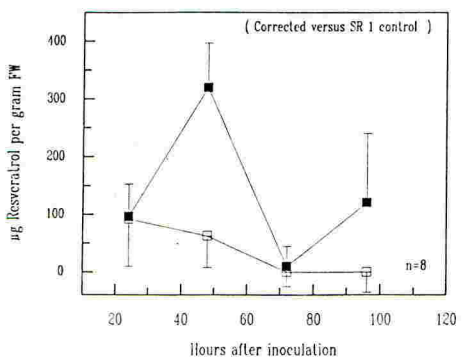


Fig. 5: Kinetics of resveratrol accumulation in leaves. Comparison of leaves with high and low disease incidence.



Low disease incidence = 0 - 15 % leaf area infected mean : 11 %
High disease incidence = > 16 % leaf area infected mean: 28 %
SR 1 control mean: 23 % n=6

DISCUSSION

The presented work reports the finding that expression, in tobacco, of a heterologous gene encoding an enzyme that produces a phytoalexin is sufficient to confer enhanced resistance to a fungal pathogen that is sensitive to the phytoalexin (Hain et al., 1992).

In previous work we have demonstrated that a foreign phytoalexin can be synthesized in a novel plant (Hain et al., 1990). However, the expression of the particular peanut gene was not sufficient to produce levels of the phytoalexin resveratrol affecting the resistance of the transgenic tobacco 15D15.

Stilbene synthase genes from grapevine supposed to be expressed to a high level were isolated and identified by direct gene transfer and demonstration of foreign stilbene synthase activity in transgenic tobacco. These stilbene synthase genes were expressed at a high level and were rapidly activated in heterologous tobacco cells and plants. In cell cultures of tobacco transient expression of stilbene synthase genes resulted in highest accumulation of stilbene synthase mRNA between 2 and 12 h post induction with elicitor. Parallel to mRNA accumulation enzymatic activity can be measured in crude extracts of these cultures. Also in leaves of transgenic SR1 tobacco plants, inoculated with B. cinerea, transient expression of stilbene synthase genes occurred with a maximum accumulation of specific RNA between 24 and 48 h. Furthermore, in situ hybridization experiments have demonstrated high and localized expression in the vicinity of fungal infection sites. The pathogen-induced, transient and localized expression pattern of stilbene synthase genes in both grapevine and the heterologous tobacco indicates conserved signal perception and transduction mechanisms for the activation of the defense-related genes (Hain et al., 1992). We assume the signal chain to be present in more or less all plants since we have also found pathogen-induced expression of stilbene synthase genes in transgenic tomato, potato and oilseed rape. The results of the expression studies in tobacco strongly supports the model character of stilbene synthase genes for defense-related genes. These genes are activated upon request (infection) leading to a localized transient accumulation of an antimicrobial metabolite, which under certain conditions and local concentrations of the phytoalexin can prevent infection of the host plant. In the case of resveratrol a proposed effect on disease resistance of a plant seems to depend on a rapid pathogen-inducible expression of the stilbene synthase gene used and the amount of resveratrol provided at the infection site.

The amounts of resveratrol formed in tobacco containing a peanut gene have been analysed previously by HPLC and antisera raised against synthetic resveratrol (Hain et al., 1990). In these tobacco cultures (15D15, Hain et al., 1990) only 30-50 ng/g fresh plant material were detected after induction with elicitor. The level of resveratrol in regenerated plants of 15D15 was not analysed but was obviously not sufficient to enhance the resistance of this transgenic line (see Tables 1 and 2). In transgenic SR1 plants containing stilbene synthase genes from grapevine up to 300 µg resveratrol/g fresh weight were detected. The comparison of transgenic leaves with high or low disease incidence evaluated after 120 h with the accumulation of resveratrol within the leaves during the incubation period indicates that a high concentration of resveratrol 48 h after inoculation is needed for enhancing disease resistance. We do not know yet the actual concentration of resveratrol at the infection sites. However, from our in situ RNA hybridization experiments the area of stilbene synthase mRNA accumulation has been estimated to be about 75µm in diameter around the infection sites 8 h post inoculation. This opens the possibility of estimating the local concentration of resveratrol.

We have tested four transgenic tobacco lines for enhanced disease resistance to B. cinerea in trials in the greenhouse. Only in one experiment with transgenic tobacco (Samsun) hypersensitive response (necroses) has been observed on the transgenic line E1.3.1 typical also for the response in grapevine/ Plasmopara viticola interaction (Dercks and Creasy, 1989). In this interaction the necrotic reaction of plant tissue after inoculation was correlated with phytoalexin accumulation rather than reproduction of P. viticola (Dercks and Creasy 1989).

However, two transgenic lines (23D17 and E1.3.1) were more resistant to B. cinerea than the wild-type controls. Interestingly transgenic line 24D1 was not reproducibly more resistant than control tobacco as was line 23D17. Analysis of the kinetics of stilbene synthase mRNA accumulation in leaves of both lines (24D1 and 23D17) revealed that line 23D17 more rapidly accumulated high amounts of specific mRNA. In 23D17 shortly after inoculation stilbene synthase mRNA was detectable reaching a maximum between 6 and 24 h (data not shown). In line 24D1 there was no stilbene synthase mRNA detectable shortly after inoculation and maximum accumulation between 24 and 48h post inoculation. The reason for the difference in the speed of stilbene synthase gene activation in these lines is not known, but might be due to position effects resulting from different locations of the transferred DNA in the genome of tobacco.

Nevertheless, these results in combination with our findings that tobacco leaves accumulating high amounts of resveratrol shortly after inoculation (Fig. 5) are more resistant to B. cinerea strongly support that a resveratrol based increased resistance depends on rapid synthesis of high amounts of the phytoalexin.

Phytoalexins have previously been shown to be vitally important in certain pathogen resistance interactions; especially, virulence of Nectria hematococca on pea depends on its ability to degrade the host phytoalexin (Tegtmeier and VanEtten 1982). Furthermore, an avirulent Cochliobolus heterostrophus transformed with a gene encoding the phytoalexin degrading enzyme of Nectria became virulent on pea (Schäfer et al., 1989). Thus, our work confirms and extends the findings that phytoalexins can determine resistance by moving a phytoalexin from one species to another rather than moving detoxification genes from one pathogen to another.

Finally for practical application and use of the stilbene synthase genes in molecular plant breeding studies have to prove the resistance mechanism to be active also in other host/pathogen interactions.

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