PHENYLAMIDE RESISTANCE IN POTATO LATE BLIGHT (*Phytophthora infestans*) IN THE UNITED KINGDOM IN 1993

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ABSTRACT

A modified floating leaf disc technique was used to test isolates of *P.infestans* collected from primary sources of infection and commercial field crops of potatoes for sensitivity to phenylamide containing fungicides. The survey in 1993 detected resistance in 76.6% of all sites and 66.2% of all isolates tested. High levels of resistance were detected in primary infection sources (82% of sites and 72.8% of isolates tested). The implications of the results and interpretation of the data are discussed.

INTRODUCTION

Since the mid-1980's, ADAS has tested bulk-isolates of potato late blight (*Phytophthora infestans*) in England and Wales for sensitivity to phenylamide fungicides. Samples collected from blight infected field crops, private gardens and experimental plots were tested using a modified floating leaf disc method. Since the mid-1980's there has been an apparent increase in the level of phenylamide resistance from 31.7% of samples tested in 1986 to 81% in 1988 and 1989. A decline in resistance was recorded thereafter and in 1992, 46.3% of samples contained phenylamide resistant *P. infestans*. There is no published information on the phenylamide resistance status of primary sources of potato blight in England and Wales early in the season before fungicides have been applied and allowed to exert selection pressure.

The data presented here show the results of phenylamide resistance monitoring of field crops in England, Wales and Scotland in 1993 and includes incidence of phenylamide resistant *P. infestans* from primary infection sources such as untreated crops and waste heaps.

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MATERIALS AND METHODS

Sample selection

Samples of potato blight were collected as actively sporulating leaf or stem lesion from infected crops of both early and maincrop potatoes or infected foliage found on discard heaps. Samples were packaged separately in clean, dry polythene bags which were lightly inflated and then sealed. They were then placed in a cool dark protective container to avoid damage during transit.

Phenylamide resistance tests

Up to five sporulating leaf or stem lesions were selected from samples from each site, each providing a spore population for a separate test. If necessary, sporulation was induced either by damp incubation at temperatures of 16-18°C or by re-activation following inoculation between potato tuber slices (cv King Edward).

Isolates of *P. infestans* were tested for sensitivity to phenylamide fungicides using metalaxyl (as Ridomil 25 WP). The test procedure used the modified floating leaf disc technique developed by Carter *et al.* (1982) and also described by Sozzi *et al.* (1992). Metalaxyl was used at concentrations of 2.0 mg/l and 100 mg/l and sporangial suspensions were not adjusted to a standard concentration because the quality of material received was too variable. Five leaf discs (14mm diameter) of cv King Edward, were used to test each isolate at each concentration of metalaxyl and in water alone. Each test was incubated for 9h at 15°C in the dark and 15h at 18°C during the day. All the potato tubers and leaf discs used in these tests were taken from plants grown from non-phenylamide treated seed tubers.

The leaf discs were examined microscopically after five days and the presence or absence of sporulation was recorded. No attempts were made to quantify sporulation or assess any tissue necrosis associated with lesion development. A site was defined as having resistance present if growth and sporulation of *P. infestans* was recorded at either 2.0 mg/l or 100 mg/l in at least one bulk-isolate. Isolates were defined as resistant if growth and sporulation was recorded at either 2.0 mg/l or 100 mg/l metalaxyl in at least one of the leaf discs at either concentration.

RESULTS

The results of the phenylamide sensitivity tests are given in the table. The incidence of phenylamide resistance has been classified according to the sample source, either untreated crop or discard heap or whether or not a phenylamide-containing fungicide had been used prior to sampling of a sprayed crop.

Samples of *P. infestans* were collected from a total of 145 sites. Phenylamide resistance was detected at 82.2% of sites which had not received any fungicide and also in 82.5% of samples from crops which had received at least one phenylamide fungicide. In sprayed crops, where phenylamides had not been used, 64.1% of sites contained resistant *P. infestans*.

Of the isolates tested (total 610), 69.3% from untreated crops and 76.3% from waste heaps contained phenylamide resistant *P. infestans*. This compares with 68.8% of isolates from phenylamide treated crops and 58.3% from non-phenylamide treated crops.

CONCLUSIONS

The results of the 1993 phenylamide resistance survey suggest an increase in the level of phenylamide resistant isolates of *P. infestans* collected from commercially grown fungicide treated potato crops from 46.3% in 1992 (Holmes, 1992) to 63.2% in 1993. Somewhat higher levels of phenylamide resistance were found in crops where phenylamide fungicides had been applied prior to sample collection compared with crops which had not been treated with a phenylamide fungicide. The incidence of phenylamide resistance in isolates of *P. infestans* from untreated crops or infected foliage found on waste heaps was also high at 69.3% and 76.3% of isolates tested respectively. The overall impression gained from these data is that a high level of phenylamide resistant *P. infestans* is present within the resident UK population. The apparent high incidence of resistance in important primary sources of infection is cause for concern and suggests that the resistant isolates are fit and able to overwinter in tubers that remain viable and survive the winter conditions. The phenylamide fungicides are marketed in mixtures with dithiocarbamates and therefore loss of performance due to resistence is difficult to judge in field crops.

This survey data should be viewed with caution and interpreted carefully. Williams & Gisi, (1992) draw attention to the difficulties encountered when carrying out a survey of this kind particularly in relation to late blight. They state that *'The nature and timing of the sampling, combined with the sensitivity testing used, can have a significant influence on the relevance of the sensitivity data obtained..... Testing of bulk populations (as described here) provide a qualitative answer that either resistance occurs or does not occur within the sample tested. A positive resistance reading is obtained with as little as 1% of the test population being resistant'.*

Undoubtedly, fungicide usage patterns are likely to play an important role in selecting for phenylamide resistance and FRAC guidelines should be followed (Urech & Staub, 1985). Nevertheless, it would be particularly beneficial to the industry if a quantitative assessment of phenylamide resistance in potato late blight programme could be made. The data presented here is almost certainly a worst case scenario, however even if as little as 1% of a test population is resistant a subsequent application of a phenylamide containing fungicide may leave the phenylamide resistant strains dominant (Clayton, 1993).

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Phenylamide resistance tests on *P. infestans* in 1993 - Proportion of sites and isolates where resistance was detected

Source or previous crop treatment	Number of sites (isolates) tested	Number and (%) of sites where phenylamide resistance* detected	Number and % Resistant isolates @ 100 mg/l	@ 2 mg/l + 100 mg/l
Untreated crop	35 (153)	29 (82.9)	72 (47.1)	106 (69.3)
Waste heap	22 (97)	18 (81.8)	64 (66.0)	74 (76.3)
Phenylamide ** treated crop	40 (170)	33 (82.5)	97 (57.1)	117 (68.8)
Non-phenylamide treated crop	39 (151)	25 (64.1)	71 (47.0)	88 (58.3)
Previous treatement not known	9 (39)	6 (66.7)	15 (38.5)	19 (48.7)
Totals	145 (610)	111 (76.6)	319 (52.3)	404 (66.2)

* Resistance detected at either 2.0 or 100 mg/l metalaxyl

** One or more treatments with a fungicide or mixture containing a phenylamide fungicide.

REFERENCES

Carter, G.A.; Smith, R.M.; Brent, K.J. (1982) Sensitivity to metalaxyl of *Phytophthora infestans* populations in potato crops in south-west England in 1980 and 1981. *Ann. appl. Biol* **100**, 433-441.

Clayton, R.C. (1993) Evaluation of different strategies for the development of phenylamide fungicides: effects on the development of phenylamide resistance in population of *Phytophthora infestans*. Proc., 12th Triennial Conference of the European Association for Potato Research 503-504.

Holmes, S.J. (1993) Potato Blight Control in 1993. In: *Proceeding of the Dupont Potato Seminar, NAC Stoneleigh.*

Sozzi, D.; Schwinn, F.J.; Gisi, U. (1992) Determination of the sensitivity of *Phytophthora infestans* to phenylamides: a leaf disc method. EPPO Bulletin 22, 306-309

Urech, P.A.., Staub, T. (1985) The resistance strategy for acylalanine fungicides. EPPO Bulletin **15** 539-543.

Williams, R.J.; Gisi, U. (1992) Monitoring pathogen sensitivity to phenylamide fungicides: principles and interpretations. EPPO Bulletin **22**, 299-306.

TOWARDS THE ISOLATION OF GENES DETERMINING INSENSITIVITY TO PHENYLAMIDE FUNGICIDES FROM *PHYTOPHTHORA INFESTANS*.

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ABSTRACT

A map-based (positional) cloning approach is being employed to isolate gene(s) from *Phytophthora infestans* that determine insensitivity to phenylamide fungicides. Crosses were performed to generate progeny segregating for the insensitivity phenotype. DNA markers linked to the gene are now being identified by screening the progeny for RAPD (random amplified polymorphic DNA) loci using bulked segregant analysis. So far, one potential linked marker has been identified. In the future, these markers will enable the molecular cloning of the gene(s) by chromosome walking.

INTRODUCTION

Insensitivity to the phenylamide, metalaxyl, occurred in potato late-blight (Phytophthora infestans) isolates soon after its commercial introduction in 1979. Outbreaks of late-blight were reported in metalaxyl-treated European crops of potato in 1980 and insensitive isolates are now widespread (Shattock *et al*; 1990). Recently it has been suggested that metalaxyl insensitivity, among other factors, may be contributing to the displacement of the "old" clonal populations of P.infestans by "new" genotypes (Fry *et al*., 1993, W.E. Fry, unpublished data).

Three phenotypes can be found among field isolates of late-blight, namely metalaxyl-sensitive, -insensitive and -intermediate. Where inheritance studies have been carried out by mating highly insensitive phenotypes with sensitive isolates, mostly intermediate phenotypes were obtained (Shattock, 1988). Patterns of segregation in some subsequent backcross and sib-matings suggest single gene control with sensitive and incompletely dominant insensitive alleles. In other cases, however, more complex patterns of segregation have been observed and are not consistent with simple inheritance (reviewed by Shaw, 1991).

A long-term goal of this project is to identify the molecular basis of insensitivity to the phenylamides in Phytophthora. The gene conferring insensitivity could be a mutated or amplified version of the target of the fungicide, or may be an entirely different locus. Several studies have suggested that phenylamides inhibit the synthesis of RNA by RNA polymerase I. One likely explanation for insensitivity is an altered target for the fungicide, and binding studies have indicated

that extracts of insensitive isolates had a reduced affinity for phenylamides (Davidse, 1988). However, as the machinery of transcription in eukaryotes is complex the precise target of the phenylamides cannot be deduced from this data.

A map-based (positional) cloning approach is being employed to isolate gene(s) from *P. infestans* that determine insensitivity to phenylamide fungicides. This species has been chosen for these studies since it is amenable to both classical and molecular genetic analysis (Shaw and Shattock, 1991; Judelson *et al.*, 1991). Crosses were made to generate progeny segregating for insensitivity and DNA markers linked to the gene are now being identified by screening the progeny using RAPD (random amplified polymorphic DNA) loci (Williams et al., 1991).

CROSSES

A cross was initially made between metalaxyl-insensitive A1 isolate 88.1.1 (United Kingdom) and -sensitive A2 isolate E13a (Egypt). Subsequently a cross was made between metalaxyl -sensitive A1 isolate Ca65 (California) and A2 isolate 2.16 (a F1 progeny from cross 88.1.1 x E13a), an intermediate phenotype.

From cross Ca65 x 2.16, 100 single oospore progeny were characterized for mating type and metalaxyl sensitivity. A ratio of 5 : 1 for A1 : A2 mating types was observed. On rye agar with and without 10 μg/ml metalaxyl, most progeny could be classified as either sensitive or intermediate phenotypes in a ratio close to 1 : 1. Twenty three progeny were selected representing approximately equal numbers of parental and recombinant phenotypes for metalaxyl sensitivity and mating type.

Since *P. infestans* is bisexual, the progeny of crosses may potentially contain selfs of the parents, which would complicate the genetic analysis. To verify that the progeny used in this analysis were not selfs, their genotypes were examined by scoring RAPD loci, as described below. All of the progeny of the 88.1.1 x E13a cross and 22 of the 23 progeny of the Ca65 x 2.16 cross were confirmed to be outcrossed individuals.

SCREENING FOR RAPD MARKERS

RAPD loci linked to the insensitivity gene are being identified using polymerase chain reaction (PCR) assays using 10-mer oligonucleotide primers (Williams *et al.*, 1991). Due to the small size of the primers and the low annealing temperatures employed, the primers bind to many sites within genomic DNA. This results in the amplification of bands when the binding sites are nearby and in the opposite orientation. Sequence variation between isolates results in polymorphic banding patterns, which are scored as genetic loci.

Five each of the most metalaxyl -sensitive and -insensitive progeny of the Ca65 x 2.16 cross were selected and used to screen for RAPD polymorphisms linked to the insensitivity gene by bulked segregant analysis (Michelmore *et al.*, 1991). The bulked segregant analysis technique simplifies and accelerates the screening process. Individual 10-mer primers were used to prime amplification reactions using 2 ng of template DNA pooled from five insensitive or five sensitive progeny. This approach places bands (loci) unlinked to phenylamide insensitivity

in both pools. Consequently, bands appearing only in one reaction will be enriched for those linked to the targeted gene (Fig. 1).

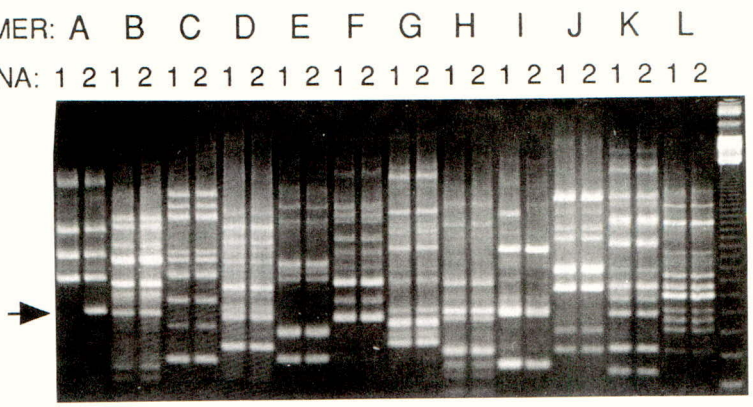

FIGURE 1: Screening for RAPD polymorphisms by bulked segregant analysis. Two bulks of DNA (1 and 2) were prepared that contained DNA pooled from five progeny of each genotype. This DNA was then used as template in PCR amplification reactions using different RAPD primers (A to L). Indicated in the left margin is the position of a polymorphism detected between bulks 1 and 2 using primer A. The right-hand lane contains a 123 bp ladder.

Preliminary RAPD reactions performed on the Ca65 and 2.16 parents indicated that each primer resulted in the amplification of about seven bands, with 0.75 polymorphic bands detected per primer. Approximately 1800 RAPD primers are available within our collection. Therefore, our assays are expected to test 1350 loci for linkage to the insensitivity gene. So far, approximately 520 primers have been tested by bulked segregant analysis of progeny DNA. Primers resulting in differences between the sensitive and insensitive pools were retested in amplification reactions using the DNA of individual progeny. One loosely linked marker, 19 cM from the insensitivity gene, has been identified, although the significance of this linkage (LOD score of 2.1; Ott, 1991) needs to be tested further by scoring additional progeny.

DISCUSSION

We have begun to construct a genetic map of the region containing gene(s) determining insensitivity to phenylamide fungicides. One potentially interesting linkage has been identified which needs to be studied further by scoring additional progeny. Additional RAPD primers will also be screened to identify additional linked markers. When a detailed genetic map of the region is available, a physical map will be constructed by long-range restriction mapping. This will reveal the

maximum physical distance between the markers and indicate the feasibility of isolating the locus by chromosome walking.

In future, these markers will enable the molecular cloning of the genes by serving as probes for screening cosmid libraries. Cosmid clones absolutely linked to the insensitivity phenotype will be introduced into a sensitive isolate by transformation. If the targeted gene is present on the cosmid, the recipient isolate should be converted to insensitivity. Cosmids conferring this phenotype change will be characterized in detail to identify the insensitivity gene.

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REFERENCES

Davidse, L.C. (1988) Phenylamide fungicides: mechanism of action and resistance. In Fungicide Resistance in North America, C.J. Delp (Ed), St Paul: American Phytopathological Society Press, pp. 63-65.

Fry, W.E.; Goodwin, S.B.; Dyer, A.T.; Matuszak, J.M.; Drenth, A.; Tooley, P.W.; Sujkowski, L.S.; Koh, Y.J.; Cohen, B.A.; Spielman, L.J.; Deahl, K.L.; Inglis, D.A.; Sandlan, K.P. (1993) Historical and recent migrations of *Phytophthora infestans*: chronology, pathways, and implications. Plant Disease 77, 653-661.

Judelson, H.S.; Tyler, B.M.; Michelmore, R.W. (1991) Transformation of the oomycete pathogen, *Phytophthora infestans*. Molecular and Plant Microbial Interactions, 4, 602-607.

Michelmore, R.W.; Paran, I.; Kesseli, R.V. (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating polymorphisms. Proceedings of the National Academy of Sciences, USA, 88, 9828-9832.

Ott, J. (1991) Analysis of Human Genetic Linkage. Baltimore: John Hopkins University Press.

Shattock, R.C. (1988) Studies on the inheritance of resistance to metalaxyl in *Phytophthora infestans*. Plant Pathology, 37, 4-11.

Shattock, R.C.; Shaw, D.S.; Fyfe, A.M.; Dunn, J.R.; Loney, K.H.; Shattock, J.A. (1990) Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988 : mating type, response to metalaxyl and isoenzyme analysis. Plant Pathology, 39, 242-248.

Shaw, D.S. (1991) Genetics. In: *Phytophthora infestans*, The Cause of Late-Blight of Potato, D.S. Ingram and P.H. Williams (Eds), London: Academic Press, pp. 131-170.

Shaw, D.S.; Shattock, R.C. (1991) Genetics of *Phytophthora infestans* : the Mendelian approach. In: *Phytophthora*, J.A. Lucas, R.C. Shattock, D.S. Shaw and L.R. Cooke (Eds), Cambridge: Cambridge University Press, pp. 218-230.

Williams, J.G.K.; Kubelik, A.R.; Livak, K.J.; Rafalski, J.A.; Tingey, S.V. (1991) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research, 18, 6531-6535.

MINIMISING PHENYLAMIDE RESISTANCE - A SUCCESSFUL STRATEGY

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ABSTRACT

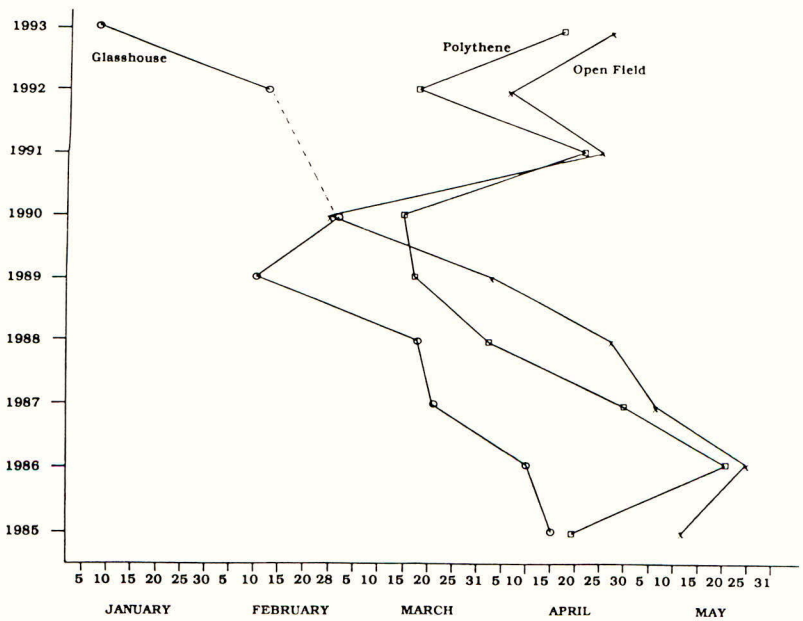
In an area where inoculum pressure is high due to the intensive cultivation of potatoes phenylamide resistance has been contained between 14% (28%) to 39% (62%) with strategic use of the relevant fungicides. Annual monitoring of all potato blight outbreaks from 1985 to 1993 has revealed no dramatic change in the susceptibility of populations of ***Phytophthora infestans*** to the phenylamide fungicides.

INTRODUCTION

The inoculum pressure and risk of resistance developing to the phenylamide fungicides by ***Phytophthora infestans***, the fungus causing late blight of potatoes, is intensified in Jersey where potatoes are grown nearly 12 months of the year. This includes glasshouse, polythene tunnel, early mulched, maincrop and autumn crops being harvested from November through to October.

The cultivar grown International Kidney - "Jersey Royal" is also extremely susceptible to blight infection. Due to marketing strategy and the history of the product there is no scope to change to a cultivar with higher resistance to blight.

Graph 1. First Outbreaks of Potato Blight Recorded in Jersey 1985 - 1993



METHOD

Laboratory assays using the leaf disc method (Sozzi, et al., 1992), of survey samples collected by the Department's Inspection Service have been carried out for 9 years (1985-1993). Isolates have been tested from crops grown under protection (glasshouse or polythene tunnel) and field crops, either polythene covered or open field. The increase in use of polythene mulches has encouraged earlier development of late blight symptoms in the field crop. In 1989, after an exceptionally mild winter, blight symptoms were first recorded in a field crop on 28th February as soon as the polythene mulch was lifted. More usually the blight epidemic occurs throughout April and early May.

RESULTS

The level of resistance found in Jersey has remained relatively steady over the period of testing. Even in bad blight years, eg. 1987-89, the level of resistance did not exceed 39% (62%). More recently in a moderate season resistance levels have dropped to 14% (28%) of fieldsamples tested. (Table 1, Graph 2).

However, in 1993 of the samples tested, 86 were sensitive at 2 ppm and 100 ppm, and 30 resistant at 100 ppm, with 11 "partially resistant" ie. sensitive at 100 ppm, but resistant at 2 ppm. The relevance of this category remains a point of discussion in the context of the sampling procedure available.

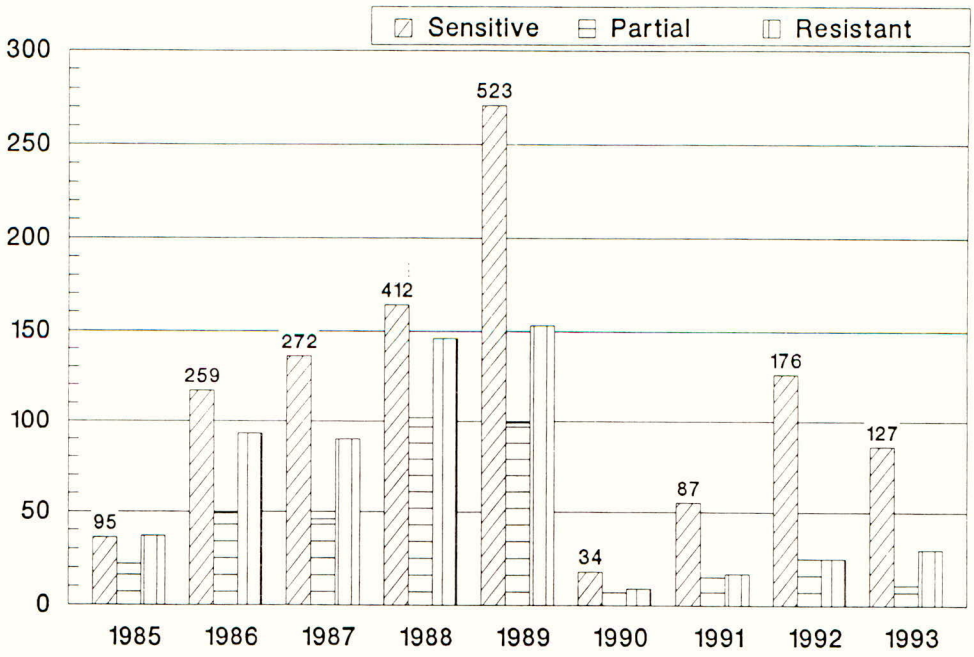
Table 1. Potato Blight - Phenylamide Group Resistance

Year	Total	Sensitive (%)	Partial Resistance %	Resistance (%)	Total
1985	95	36 (38%)	22 (23%)	37 (39%)	59 (62%)
1986	259	117 (45%)	49 (19%)	93 (36%)	142 (55%)
1987	272	136 (50%)	46 (17%)	90 (33%)	136 (50%)
1988	412	164 (40%)	102 (25%)	146 (35%)	248 (60%)
1989	523	271 (52%)	99 (19%)	153 (29%)	252 (48%)
1990	34	18 (53%)	7 (21%)	9 (26%)	16 (47%)
1991	87	55 (63%)	15 (17%)	17 (20%)	32 (37%)
1992	176	126 (72%)	25 (14%)	25 (14%)	50 (28%)
1993	127	86 (68%)	11 (8%)	30 (24%)	41 (32%)

The pattern of usage of phenylamide products has varied little over the years. Between 23% to 28% of fields tested had used systemic products at the beginning of their spray programme.

The disease is statutorily controlled under the Blight Disease (Jersey) Order, 1982, which allows for a fairly high degree of supervision in the growing of the potato crop. This has included the development of a comprehensive strategy for the use of phenylamide fungicides, which will be adhered to by the growers. The consequences of failure to control the disease is the issue of a Statutory Scorching Order by the Department of Agriculture Inspectors, for the focus of infection to be destroyed and removed. It is considered that this policy has reduced the potential risk of a build-up of resistant inoculum.

Graph 2. Potato Blight
Phenylamide Group Resistance Results Jersey 1985 - 1993
(figures above columns are the total tested)



Jersey Strategy for the Use of Systemic Fungicides 1994

1. **NOT** to be applied to crops grown under permanent protection ie. glass or polythene tunnels.
2. **NOT** to be applied to crops grown for seed or non export ware.
3. A maximum of 3 sprays may be applied to any single crop, the last of which must be before 30th June.
4. **NOT** to be applied curatively.
5. Crops under polythene covers must be treated immediately on removal of film provided no blight infection present.
6. Only to be used on field grown export crop of Jersey Royals.
7. Spraying interval advised 10-14 days.

CONCLUSIONS

The field performance of the fungicides under discussion have remained good despite some growers apparently losing confidence in the phenylamides for a time. The occasional problems of lack of disease control identified by the Extension Pathologist appear to be more related to application technology, and timing. These are the crucial factors to be addressed. Continued monitoring of the pathogen population is regarded to be essential.

REFERENCES

SOZZI, D, SCHWINN, F.J. and GISI, U (1992)
Determination of the sensitivity of ***Phytophthora infestans*** to phenylamides: a leaf disc nethod EPPO Bulletin 22 306-309