PCR-BASED DETECTION OF *PHYTOPHTHORA* SPECIES IN HORTICULTURAL CROPS

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ABSTRACT

Accurate and rapid detection and diagnosis of Phytophthora diseases in horticultural crops is of key importance in the management of the disease. The minimising of disease spread via infected rootstock and a move away from prophylactic fungicide application are two major benefits of such a procedure. The Polymerase Chain Reaction (PCR) offers great potential as a highly specific tool to achieve this. This paper reports on the design and optimisation of such a PCR-based detection system based on ribosomal DNA sequences of several important *Phytophthora* species of horticultural crops. Specific detection of the fungus in infected root material has now been achieved.

INTRODUCTION

The genus *Phytophthora* represents an important group of plant pathogenic fungi which are responsible for large scale losses of tropical and temperate crops. They are a particular problem in vegetatively propagated horticultural crops, being spread on infected planting material.

The sustainability of both the propagation and cultivation stages of many horticultural crops is severely threatened by *Phytophthora* spp. In order to minimise disease problems in nurseries, plants are often treated prophylactically with high doses of fungicides which rather than solving the problem may actually exacerbate it. Many fungicides have been shown to suppress rather than kill the *Phytophthora* spp. (Duncan, 1985) so apparently healthy material may be widely distributed, furthermore *Phytophthora* which has been exposed to strong selection pressure increases the potential for the development of fungicide resistance. In order to minimise this threat it is important that a scheme for the accurate and specific detection and diagnosis of *Phytophthora* is established.

An example of this which represents an important problem to raspberry and strawberry growers is *Phytophthora fragariae* which is a major limiting factor in crop growth, requiring the application of fungicides which are expensive and potentially damaging to the environment. It is now recognised that worldwide spread of the strawberry and raspberry varieties of this pathogen has been brought about via the movement of infected rootstock (Duncan, 1993). In an effort to stem this it has been declared a quarantine organism in many countries, which means stocks must be guaranteed "disease free" before importation.

There are however many difficulties in detection and diagnosis of *Phytophthora* spp. The non-specific symptoms on the root, crown or stem base makes visual confirmation of the presence of *Phytophthora* difficult. Currently, detection and diagnosis of *Phytophthora* relies on visual inspection, bait testing (Duncan *et al.*, 1993), isolation of the fungus on

selective media or diagnostic kits based on polyclonal antibodies, each of these has its disadvantages compared to a PCR-based system.

THE USE OF RIBOSOMAL DNA SEQUENCES AS THE BASIS FOR PHYTOPHTHORA DETECTION.

The fundamental importance of the ribosomes in protein synthesis means DNA sequence homology can be seen in almost all forms of life. Despite the highly conserved "core sequences", there is some variation in other rDNA which allows phylogenetic separation at many levels from kingdoms through to genera (Bruns *et al.*, 1991). The advantages of using rDNA sequences as target sites for PCR primers is now widely recognised. There is an abundance of publications on rDNA sequence variation, sequences are rich in informative regions, mutation rates are known and many copies are present in each nucleus thus increasing the sensitivity of detection. Since one is looking at a very tightly defined region, species can be added to the analysis at any time resulting in an expanding sequence database.

Many 18S and 28S ribosomal subunit sequences have been published and variation between genera noted. Spacer regions of the ribosomal repeat unit which are not thought to play a functional role, are less conserved and have been reported to show interspecific variation in plants (Sun *et al.*, 1994) and fungi (Lee & Taylor, 1992; Zambino & Szabo; 1993, Sherriff *et al.*, 1994) suggesting their suitability for the purposes of molecular detection.

The following is brief description of recent progress at SCRI on the molecular variation of ITS1 and ITS2 regions of *Phytophthora* spp. and their utility in the diagnosis and detection of *P. fragariae* and other species. Specific details of the procedures used and primers sequences will appear in subsequent papers.

PRIMER DESIGN

Using a set of PCR primers designed for the amplification of fungal spacer regions (White *et al.*, 1990) ITS 1 and ITS 2 regions from many *Phytophthora* spp. were amplified and found to be approximately 220 and 400 bp long, respectively. The species sequenced so far are *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. cambivora*, *P. cinnamoni*, *P. megasperma*, *P. nicotianae*, *P. cryptogea*, *P. citricola*, *P. drechsleri*, *P. infestans*, *P. idaei*, *P. pseudotsugae*, and *P. cactorum*. The double stranded PCR products were manually sequenced and aligned using multiple sequence alignment software on Seqnet (Daresbury Laboratory). Both ITS1 and ITS2 regions were sufficiently conserved to allow an accurate alignment, some regions were identical in all species tested and others showed considerable variation.

Two distinct types of sequence were noted, those from non-papillate species sharing regions of homology not seen in papillate and semi-papillate species which formed a separate sub-group. This rich source of defined sequence allowed the design of PCR primers specific for a particular species. To date, primers have been designed for *P. fragariae*, *P. cambivora*, *P. cinnamomi* and *P. nicotianae*. There is however sufficient sequence variation to design primers for almost any given species.

PRIMER TESTING

Each of the specific primers was tested against pure DNA from the 13 *Phytophthora* species detailed above and three showed excellent specificity, resulting in amplification products from many isolates of that species but no amplification products from other species. The *P. nicotianae* primer also amplified a product from *P. infestans*, which as a papillate species, was shown to be closely related to *P. nicotianae* (unpublished results based on ITS sequence homology). Preliminary studies on the sensitivity of the procedure on pure DNA showed the lower limit of detection to be 100 fg, which may be improved with nested PCR procedures.

Improvements in DNA extraction procedures and PCR efficiency have resulted in the successful amplification of *P. fragariae* DNA from both raspberry and strawberry roots which represents a major step forward in the diagnosis of the disease from a procedure taking weeks to one which may be completed in a matter of hours.

DISCUSSION

We now have a system for the detection of specific species of *Phytophthora* in infected material which has great potential as a diagnostic tool. More rapid disease diagnosis forms an integral part of any integrated control package and should result in more timely application of fungicides and reduce numbers of unnecessary applications. A system of careful monitoring of propagation material and attention to improved phytosanitary conditions should result in an overall reduction in the incidence of *Phytophthora* in propagation stocks, the benefits of which would be passed on to growers.

In order to carry out larger scale detection programmes further work on the development of protocols for efficient amplification from zoospores will be necessary. Such work is now underway at SCRI and should result in a system of monitoring plant stocks via the trapping and detection of zoospores in drainage water from pots, water from recirculating irrigation systems or environmental monitoring of pathogen populations in streams or soil.

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THE EFFECTS OF ARABLE FIELD MARGIN MANAGEMENT ON THE ABUNDANCE OF BENEFICIAL ARTHROPODS

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ABSTRACT

We examine the extent to which the restoration and management of arable field margins can enhance their potential as habitat for Linyphiidae (Araneae) and for Staphylinidae (Coleoptera). In 1987, extended-width field margins were established around six arable fields at Wytham, Oxford. The field margins were subject to ten contrasting management treatments. Arthropods were sampled from the field margin swards using vacuum suction sampling, between 1987 and 1991. Linyphiid abundance was reduced both by mowing and by spraying with glyphosate herbicide. Staphylinid abundance was also reduced by mowing, but there was no significant effect of spraying. Effects of mowing on both groups differed with the timing of mowing and the date of sampling, with spring and summer mowing having the most severe effects on abundance. There was a tendency for staphylinid abundance in the crop to be greater adjacent to uncut field margin treatments. Neither linyphiid nor staphylinid abundance was significantly affected by establishing field margin swards with a grass and wild flower seed mixture, rather than by natural regeneration.

INTRODUCTION

Modern farming methods do not provide high quality habitats for the great majority of invertebrates. Many species of agricultural weeds, which formerly supported a rich and varied insect fauna, are now rare and associated primarily with field edges. Heavy use of pesticides within the crop has contributed to this loss of invertebrate interest by causing direct mortality. Field margins formerly provided more permanent refuges and reservoirs for invertebrates on farmland, but these too have suffered from agricultural intensification, with hedgerow removal, and deliberate and accidental pesticide and fertiliser applications, impoverishing the plant and animal assemblages.

In recent years, studies have shown that the manipulation of crop edge (Chiverton & Sotherton, 1991) and linear 'island' (Thomas *et al.*, 1992) habitats can increase polyphagous predator densities in arable systems. We have shown elsewhere that the restoration and management of field margins around arable fields can potentially enhance agricultural habitats for butterflies (Feber & Smith, 1995; Feber *et al.*, 1994). As well as having value for nature conservation, field boundaries can also harbour invertebrate predators of crop pests. In this paper we describe the effects of contrasting methods of field margin management on the abundances of two groups of invertebrates, the Linyphiidae (money spiders) and Staphylinidae (rove beetles) whose predatory lifestyles make them of potential benefit to farmers.

Linyphiidae are the dominant spiders in arable crops in Europe, and an important component of the polyphagous predator complex. They make horizontal webs both on and above the ground, depending on the species, and have been shown to have a significant impact on aphid populations early in the season (Sunderland *et al.*, 1986). Staphylinidae are common in grassland and agricultural habitats and also have significant impacts on aphid populations (Dennis & Wratten, 1991). Both groups have ecological characteristics which made them likely to respond to our manipulations of field margin sward structure and composition. We present results which show the effects on Linyphiid and Staphylinid abundance of mowing, sowing with a grass and wild flower seed mixture, and spraying with glyphosate herbicide. We consider the implications of our results for the biocontrol of invertebrate pests in arable systems.

METHODS

In autumn 1987 we created 2 m wide field margins around arable fields at the University of Oxford's farm at Wytham. These comprised the original margin, about 0.5 m wide (the "old" margin), and a fallowed extension of about 1.5 m on to cultivated land (the "new" margin). Swards were established on the fallowed strips either by allowing natural regeneration ("unsown" swards) or by sowing a mixture of wild grasses and forbs ("sown" swards). Plots 50 m long were established on both sward types and subjected to the following management regimes: unmown, or mown (with cuttings removed) in (a) summer only (b) spring and summer or (c) spring and autumn. Two further treatments were imposed on unsown plots only: (a) mown in spring and summer with hay left lying and (b) unmown, but sprayed with glyphosate in late June or early July. The plots were mown in the last weeks of April, June and September ("spring, "summer" and "autumn") respectively. Glyphosate (3 l/ha Roundup in 175 litres water) was first sprayed in 1989. The treatments were randomised in eight complete blocks, each block occupying a single field. From the time that they were fallowed, the field margins were protected from fertiliser and spray drift.

We used D-vac suction sampling to quantify the abundance of our target groups in six blocks of the experiment. The D-vac samples comprised five, 30-second "sucks" taken at 10m intervals along both old and new margins, within each plot. This approximated to a total sample area of 0.5 m^2 per plot in each of the old and new margins. In May and July of 1989 samples were also taken at distances of 2 m and 10 m into the crop, adjacent to the field margins. Each sample was transferred to a polythene bag and immediately cooled to reduce activity. The live invertebrates were extracted by pootering within a few hours of collection and stored in 70% alcohol until they were sorted. Sampling took approximately two days.

In 1987 and 1988 samples were taken in September only. In 1989, 1990 and 1991 samples were taken in May, July and September, after the spring and summer cuts, but before the autumn cut. In this paper we present data for Linyphiids and Staphylinids from the samples taken from the new margins between 1987 and 1991.

Analyses

All analyses of the effects of the experimental treatments on the measured variables were performed on appropriately transformed data by analyses of variance. Specific hypotheses,

implicit in the design of the experiment, concerning the relative effects of particular treatments, were tested using planned comparisons. We then split the treatment effect into the main effects of sowing and cutting, by excluding the treatment in which the mown hay was left lying and the sprayed treatment. We performed a three-way analysis of variance on these remaining treatments which formed a 2 x 4 factorial structure (i.e. sown or unsown x 4 types of cut x 6 blocks).

Univariate repeated measures ANOVA was used for this analysis since measures of the dependent variables made on different dates on the same plot could not be treated as independent. Significance levels for individual samplings are presented as asterisks on the figures.

RESULTS

Effects of sowing

Sowing with a wildflower seed mixture had no significant effects on the abundance of either the Linyphiidae ($F_{(1,83)}$=0.00, P=0.944) or the Staphylinidae ($F_{(1,34)}$=0.10, P=0.757).

Effects of mowing

Mowing resulted in an overall significant reduction in the abundances of both linyphiids $F_{(3,83)}$=13.6, P<0.001) and staphylinids ($F_{(3,83)}$=38.7, P<0.001). The effect differed with the timing of sampling for both linyphiids (cut x round, $F_{(3,83)}$=13.6, P<0.001) and staphylinids (cut x round, $F_{(18,498)}$=7.4, P<0.001). The effects of the mowing regimes on each group are described below and mean abundances are shown in Table 1.

Mowing in summer only

Significantly fewer linyphiids were recorded from treatments which were mown in the summer, relative to those which were left unmown ($F_{(1,105)}$=23.8, P<0.001), over the three years. There was, however, a significant interaction between year and mowing, with the most substantial effects recorded in 1990 and 1991. In each year, linyphiid abundance was severely reduced following the summer mowing. Under this regime, numbers tended to recover by the autumn and increased further by the following spring (Figure 1). Mowing in summer only resulted in consistently higher abundances of linyphiids in spring than the other mowing regimes (Figure 1).

Staphylinids also performed poorly on the plots mown in summer only ($F_{(1,43)}$=22.3, P<0.001). Abundances were significantly lower on the mown than the unmown treatments in the summer samples in both 1990 and 1991, and the autumn samples of 1989 and 1990 (Figure 1).

Mowing in spring and summer

This mowing regime resulted in a more persistent and severe reduction in linyphiid abundance than mowing in summer only ($F_{(1,105)}$=31.6, P<0.001). Abundances in both spring

TABLE 1. Mean abundances of Staphylinidae and Linyphiidae per m^2 on plots under different mowing regimes throughout the experiment. Su = mown in summer only, Sp + Su = mown in spring and summer, Sp + Au = mown in spring and autumn. See figures for significances.

	Not mown	Mown Su	Mown Sp + Su	Mown Sp + Au
		Staphylinidae		
Sep 87	13.6	-	-	-
Sep 88	9.4	18.6	8.0	13.4
May 89	17.0	-	20.6	-
Jul 89	19.6	-	20.0	-
Sep 89	19.6	18.6	26.4	8.0
May 90	37.0	27.0	31.4	26.0
Jul 90	15.6	7.2	15.4	20.0
Sep 90	22.6	14.6	11.4	21.4
May 91	72.0	50.6	36.0	38.0
Jul 91	15.6	6.4	4.6	10.4
Sep 91	30.6	24.0	30.4	31.4
		Linyphiidae		
Sep 87	12.4	-	-	-
Sep 88	60.0	67.0	68.0	53.4
May 89	22.6	-	9.4	-
Jul 89	89.4	-	24.5	-
Sep 89	50.0	45.5	46.4	66.4
May 90	24.0	10.4	9.0	8.0
Jul 90	26.0	7.0	7.6	24.0
Sep 90	41.4	44.6	25.4	34.4
May 91	25.6	19.0	5.0	7.6
Jul 91	18.6	4.6	5.4	18.6
Sep 91	82.2	83.5	96.5	193.0

and summer in 1989 and 1990 were significantly lower on mown than unmown treatments (Figure 1). Although numbers on the mown treatments tended to recover in the autumns of 1990 and 1991, in all years they were substantially reduced again by mowing in the following spring (Figure 1).

Spring and summer mowing also significantly decreased staphylinid abundance ($F_{(1,43)}$=21.1, P<0.001) although, in contrast to the linyphiids, its effects on this group were no more severe than those of mowing in summer only. In 1989, for example, there was no significant difference in staphylinid abundance between mown and unmown plots in any sampling round under this regime (Figure 1). In 1991, staphylinid abundance on mown plots had recovered sufficiently by September to be indistinguishable from unmown plots.

Mowing in spring and autumn

Mowing in spring and autumn significantly lowered linyphiid abundance ($F_{(1,105)}$=9.9, P=0.003), although, of all the mowing regimes, this generally had the smallest and least persistent effects (Figure 1). However, the low linyphiid abundance after the spring in this, and the spring and summer, mowing regime, in all years, has unfavourable implications for biocontrol (see Discussion).

Staphylinid abundance was also lowered by a spring and autumn mowing regime ($F_{(1,43)}$=7.1, P=0.012).

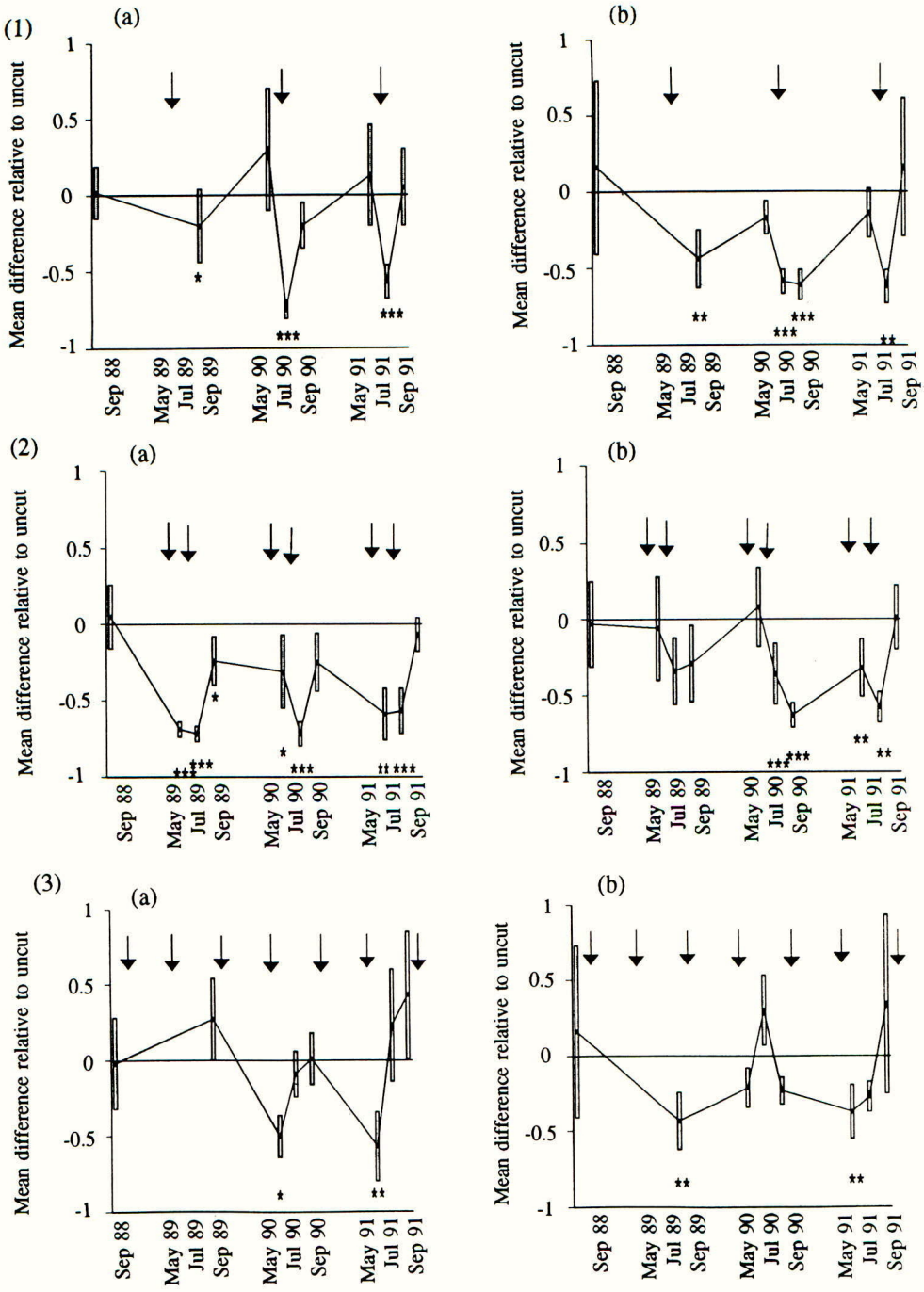

FIGURE 1. Mean proportionate differences between unmown plots and plots mown in (1) summer only (2) spring and summer and (3) spring and autumn for (a) Linyphiidae and (b) Staphylinidae. Arrows indicate time of mowing.

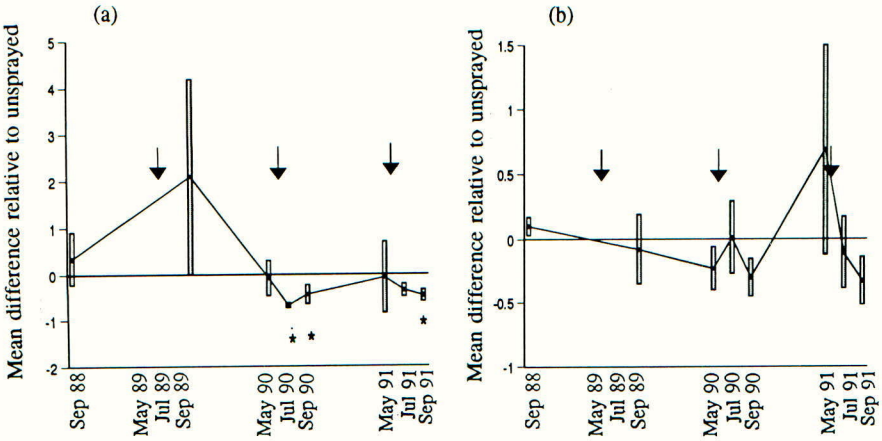

FIGURE 2. Mean proportionate differences between unmown plots and plots sprayed once-annually with glyphosate herbicide for (a) Linyphiidae and (b) Staphylinidae. Arrows indicate time of spraying.

Effects of spraying

Spraying once-annually with glyphosate significantly lowered linyphiid abundance overall ($F_{(1,105)}$=18.1, P<0.001; Figure 2).

By contrast to the Linyphiidae, staphylinid abundance was not significantly affected by glyphosate spraying ($F_{(1,43)}$=0.352, P=0.88).

Effects of treatment on abundances of Linyphiids and Staphylinids in the adjacent crop

Field margin treatment had no significant effect on the abundances of linyphiids sampled at 2 m or 10 m into the crop in either May ($F_{(4,20)}$=2.01, P=0.132 and $F_{(4,20)}$=1.60, P=0.213 respectively) or July $F_{(4,8)}$=0.98, P=0.471 and $F_{(4,8)}$=0.80, P=0.556 respectively) in 1989.

In May 1989, fewer staphylinids were associated with the plots cut in spring and summer than with those left uncut, at 2 m into the crop ($F_{(1,20)}$=4.05, P=0.058). The abundance of staphylinids associated with mown plots was also lower at a distance of 10 m into the crop ($F_{(1,15)}$=4.35, P=0.054). In July, there was a significant interaction between mowing and sowing, with sowing increasing abundance in the crop adjacent to mown plots ($F_{(1,6)}$=7.37, P=0.035). Abundance of staphylinids 2 m into the crop was also significantly lower adjacent to spring and summer cut plots than to unmown plots ($F_{(1,8)}$=7.38, P=0.026). There was no main treatment effect on staphylinid abundance at 10 m into the crop ($F_{(4,6)}$=0.94, P=0.499).

DISCUSSION

The ways in which the field margins were managed affected the abundances of both the Linyphiidae and the Staphylinidae. The dominant influences were those which altered the structural diversity of the sward. In general, factors which reduced the structural diversity of the sward also reduced the abundance of both groups. Thus, both were more abundant in the

absence of regular annual cutting. In each of the four years after establishment of the experiment, abundance of linyphiids and staphylinids was much higher on field margin plots which were left uncut than on those which were cut. However, there was no evidence for a cumulative deleterious effect of management, although this may not be apparent over our relatively short sampling period.

All of the mowing regimes lowered the abundances of the Linyphiidae and the Staphylinidae. Mowing in mid-summer (whether alone, or in addition to mowing in spring) had larger negative effects on the linyphiids than mowing in spring or autumn. However, although the effects of cutting in spring were short-lived under the spring and autumn regime, they may have had a disproportionate influence on the potential effectiveness of both the linyphiids and the staphylinids for suppressing aphids in the crop. Winder (1990) demonstrated that polyphagous predators were most important during the early stages of aphid population development.

Although unmown swards supported consistently higher numbers of linyphiids and staphylinids than mown swards, leaving swards unmanaged over the longer term decreases their plant species richness, with possible consequences for the invertebrate assemblage. In our experiment, by 1991, plant species richness was significantly lower on unmown pots than on those mown in spring and autumn (Smith *et al.*, 1993). This may eventually reduce the abundance of phytophagous species on which the predators depend, although such effects were not apparent over our sampling period.

Spraying once annually with glyphosate had deleterious effects on the abundance of linyphiids. Spraying affected the physical complexity of the vegetation as dead stems collapsed (Smith *et al.*, 1993), and may also have had indirect effects on the Linyphiidae through its influence on phytophagous prey species which depend on the plant species composition. There was no significant effect of spraying on the abundance of staphylinids, which are less dependent on the structure of the vegetation.

We were unable to detect any effects of sowing on the abundance of linyphiids or staphylinids on the new margin extensions. Differences in sward composition caused by sowing, rather than natural regeneration, such as an increase in plant species richness (Smith *et al.*, 1994), did not appear to be exploited by either of the two groups. The benefits to invertebrates of sown swards are likely to depend on their precise species composition. Sowing can have negative effects on some invertebrates by excluding plant species on which they depend; the common stinging nettle (*Urtica dioica*), for example, is an important host of polyphagous predators (Perrin, 1975) and its abundance was significantly reduced by sowing (Smith *et al.*, 1994). Conversely, plant species with specific characteristics might be included in a seed mixture to encourage other groups. Thus, for example, Thomas *et al.* (1992) showed that grass species which form dense tussocky growth provide a high density of overwintering sites for carabid beetles.

We found little evidence that different field margin management regimes affected the abundance of Staphylinidae or Linyphiidae in the crop adjacent to the margin in summer, although there was a tendency for staphylinids to be more abundant opposite unmown rather than mown plots. However, the length of the plots in relation to the dispersal abilities of the groups (Thomas *et al.*, 1991; Curry, 1994) may have partially masked any treatment effects

in the crop. Nonetheless, the magnitude of the differences between treatments provides strong evidence that the ways in which field margins are managed on a farm scale could potentially make a significant contribution to an integrated approach to crop protection.

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