SESSION 3A

SCLEROTINIA: ITS BIOLOGY AND IMPLICATIONS FOR DISEASE CONTROL

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THE EFFECTS OF ROTATION AND OTHER CULTURAL FACTORS ON *SCLEROTINIA* IN OILSEED RAPE, PEAS AND POTATOES.

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ABSTRACT

Field surveys and small plot trials have been used to assess the risk from *Sclerotinia* posed by a range of crop management practices. Populations of sclerotia in soil increase substantially following severe infections of susceptible crops, and in the absence of replenishment, decline with a half-life of *c.* 2.5 year. Apothecial production in autumn-sown crops is greatest where crop residues are burnt or chopped the previous season; and productivity is brought forward following minimal cultivations, and is delayed by spring nitrogen. In Britain two distinct epidemiological phases of the disease are seen, the first in May associated with winter crops, and the second 4-6 weeks later associated with spring-sown crops.

INTRODUCTION

Although widely recognised as a major pathogen overseas, especially in North America (Purdy, 1979; Dueck *et al.*,1983), *Sclerotinia sclerotiorum* has not, at least until recently, been regarded as a serious threat to crop production in Great Britain. Progressive post-war economic and technological changes in British agriculture resulted in a steady decline in the acreage of susceptible crops up to the late 1970s, and the threat presented by *Sclerotinia* accordingly declined in the increasingly cereal and grass dominated countryside. However, the last decade has seen a substantial reversal of the situation, necessitating a reappraisal of the risk from *Sclerotinia* posed by the cultivation of a wider range of potential hosts (Price & Colhoun, 1975; Purdy, 1979) over a greater area and often with shortened rotations (Jellis *et al.*,1984).

The current investigation was prompted by the factors above and the need to study the epidemiology of the pathogen under changing patterns of management, many of which threatened to transform *Sclerotinia* disease from a local to a national problem, a prediction finally borne out in 1991. Two main approaches to the investigation have been followed. Firstly, field surveys have been undertaken in known *Sclerotinia* "hot-spots" in an attempt to correlate disease outbreaks with particular combinations of husbandry or climatic factors. The second approach has been to use small-plot field trials to reproduce model systems of rotation and cultivation, and to examine the effects of these on pathogen survival, sporulation and infection.

FIELD SURVEYS 1988-1990

Farms on the Chichester plain and in the Romney Marsh area, selected on the basis of advice from local crop protection consultants, were

visited regularly during the 1988, 1989 and 1990 growing seasons. These areas have a lengthy history of Sclerotinia disease fostered by a favourable local climate and rotations which historically have included a high frequency of susceptible crops. Typically these include oilseed rape (OSR), peas, potatoes, horticultural Brassicas, field bean, in recent years linseed, and formerly Phaseolus bean.

The survey extended to some 35 fields over seven farms in the Chichester area and six fields on a single farm in Kent. The survey included assessments of sclerotial numbers in soil before and after cropping; apothecial production during the growing season; ascospore inoculum caught on rod spore traps (Jenkyn, 1974) or on petals detected by plating onto potato dextrose agar; and disease incidence estimated at a suitable stage prior to harvest. Fields received fortnightly visits between late March and the end of July, with weekly monitoring during part of April and May, which is the main infection period for winter OSR, the major crop in the survey.

Weather during the three years of the survey differed from the long term mean and was characterised by mild dry winters, dry springs and summers (not 1988) with periods of exceptional heat and drought. Crop growth stages were at times several weeks ahead of the long term average and ripening of some crops was up to a month early. Flowering of winter OSR was on average 12-14 day earlier than in a previous study in 1981-1983. These factors tended to restrict the incidence of Sclerotinia, especially on spring-sown crops and flushes of apothecial production tended to be short. Severe infection was restricted to lodged crops, and where isolated showers had provided surface moisture at a critical period. The following presents a summary of the main conclusions, supplemented by additional observations during 1991-92.

Survival of sclerotia

Numbers of sclerotia in field soils varied from zero up to 6.2 kg^{-1} air-dried soil (potatoes, Kent, 1990). In general, numbers declined under non-host or spring-sown crops without irrigation in the seasons of low rainfall. Numbers increased where disease occurred in the preceding crop. However the greatest increases were observed in the second crop following sowings in 1988 of pea, potato and sunflower (table 1). This delay reflects shallow sampling (designed to detect only sclerotia of significance in the current season) and the effect of soil inversion by ploughing.

TABLE 1. Numbers of sclerotia in air-dried soil following infection of a susceptible crop in the 1988 growing season

Crop	Location	Sclerotia kg^{-1} following cropping in			
		1987[a]	1988	1989[b]	1990
Pea	Chichester	0.80	1.20	3.0	2.57
Potato	Kent	1.20	0.80	1.51	-
Sunflower	Kent	-	2.60	3.60	1.60

a measured before cropping in spring 1988
b 1989 crops were non-susceptible

One site at Chichester at which no infection was seen in the only susceptible crop (peas, 1989) maintained high, though decreasing levels of sclerotia throughout (5.7 - 4.4 - 2.0 - 2.7 kg^{-1} soil). Other fields from the same farm where numbers of sclerotia were lower (c. 0.4 kg^{-1}) contained Coniothyrium minitans, a mycoparasite which attacks and degrades sclerotia.

Size of sclerotia varied widely between 1-3 mm from diseased linseed and OSR pods, up to walnut-sized (15-20 mm) in the case of sunflower. Sclerotial returns to soil are typically 5-7 per plant for OSR, and in the range 5-15 for potato. A severely infected (50% incidence) crop of vining peas in Norfolk was responsible for a mean sclerotial return of 88 m^{-2} (178 m^{-2} if the haulm was included), which in turn raised the soil reservoir to 1.20 kg^{-1} from an unknown baseline. Stems of single, severely infected sunflower plants (table 2) yielded up to 90 sclerotia, and a similar number, sometimes fused in groups, could be recovered from head lesions.

Incidence of apothecia

Numbers of apothecia under crops varied widely from zero to 89.3 m^{-2} (winter wheat, after sunflower). The average productivity at the peak over the years 1988-90 was 4.5 m^{-2}. In general, apothecial production was proportional to sclerotial load in soil, but with much weather-induced variation. The peak of apothecium production under winter OSR occurred seven days either side of mid-flowering, but apothecia tended to peak higher and be present for longer under winter cereals. The earliest apothecia were usually detected in winter wheat in late March/early April, although excavation of sclerotia in depots sometimes revealed stipes in January.

Apothecia under spring crops always appeared later, never before June, reflecting a requirement for a minimum period without soil disturbance of 6-8 weeks, and for a crop canopy to slow drying of the surface layers of the soil. This fortuitous delay ensures reasonable synchrony between ascospore release and the start of the susceptible period for spring crops.

The smallest sclerotia can support the production of only a single apothecium and must be in the surface 1-2 cm layer of soil for successful fruiting. In contrast those from sunflower may subtend up to 15 apothecia and stipes can reach the surface from a depth of 10-12 cm.

Ascospore dispersal

Ascospores were readily trapped on sticky rods and on petals of OSR. Although less quantitative, the latter were generally preferred for ease of use and for relevance to infection on OSR. Most spores were trapped at the peak of apothecium production, and fewest were seen on petals collected after fungicide sprays. However, these results could not be linked quantitatively to the potential for infection in the field. In some cases spores were trapped in fields, both cropped and fallow, in which no apothecia were seen, providing clear evidence for wind dispersal. Sclerotinia was rarely detected on petals of other crop hosts.

Disease incidence

In each year outbreaks of winter-kill by Sclerotinia on OSR were evident, but could not be ascribed with certainty either to mycelial or ascosporic infection in any year. It tended to occur in thick crops following lush growth in the mild autumns. Treatment with a benzimidazole fungicide prevented inter-plant spread of mycelium.

Summer infections over the three years 1988-90 were mostly at a low level, the mean incidence over all fields being 1.1% (sprayed winter OSR), 6.6% (unsprayed winter OSR), 1.7% (pea), 7.9% (potato), and 1.9% (spring OSR). The maximum levels seen in individual crops were very much greater (table 2), especially in 1991 when a wetter season provided greater opportunity for infection.

TABLE 2. Maximum disease incidence (percentage of plants with at least one lesion) seen in field surveys 1988-1992.

Year	Crop	Location	Disease incidence	Notes
1988	Winter OSR	Chichester	6.3	Sprayed
1988	Winter OSR	Chichester	16.7	Unsprayed
1988	Sunflower	Kent	17.9	-
1988	Pea	Norfolk	>50.0	Vining cv.
1988	Pea	Chichester	80.8	Combining cv.
1989	Potato	Kent	28.1(10.6)*	Main crop
1990	Potato	Kent	60.0(16.0)*	Main Crop
1990	Winter OSR	Chichester	47.1	#
1991	Winter OSR	Chichester	35.0	Unsprayed
1991	Spring OSR	Chichester	20.0	Unsprayed
1991	Linseed	Chichester	8.0	Dense crop
1992	Spring OSR	Chichester	15.0	Dense Crop

* figure in parenthesis is based on stems, not plants
\# localised infection following thunderstorm

A feature evident in each season was the presence of disease in crops of OSR in which apothecia were never seen despite considerable searching. After allowing for sampling errors it is clear that such crops could not generate sufficient inoculum internally to account for the disease levels seen, up to 20% in 1991. Additionally, some crops exhibited a disease gradient indicating the likely source of inoculum which in some cases must have carried up to 200m.

Except on sunflower where stem base infections from sclerotia were reasonably common, virtually all other summer infections clearly originated from wind-dispersed ascospores. On OSR, infections could invariably be traced to abcissed petals attached to petioles and stems (Kruger, 1974; McLean, 1958). Save for head infection of sunflower via senescent ray florets, floral organs seem to be less important in other crops. Peas can become infected via petals, but senescent lower foliage is also much involved. Petals of linseed are ephemeral and show little tendency to lodge on the plant: most infections originate at the axils of senescent lower leaves or are attributable to mechanical damage. Lesions on potato mostly originate from senescent leaflets and petioles.

Attempts to link disease incidence with pre-determinable risk factors have been thwarted by the generally low levels of infection of the first three years. Disease incidence in winter OSR and the sclerotial load in fields were not (1988-89) or were only weakly (1990) correlated. Similarly the association between infection and the Sclerotinia load on petals was weak except in 1990 when a correlation (r=0.89) was found across a sample of nine fields.

Alternative sources of inoculum

Although Sclerotinia was noted on a range of common weed species in surveyed crops, at no time was infection above trace levels. The only exception was infection of nettles (Urtica urens) in potato in 1990 and in a dense crop of linseed in 1991. Further, infection has rarely been seen on plants on field margins or in hedgerows. The overwhelming conclusion is that wild plants are an insignificant source of Sclerotinia inoculum: at best the have a role in perpetuating the pathogen at a low level.

On a single farm, a field margin strip of Jerusalem Artichoke (Helianthus tuberosus) grown as game cover was found carrying significant infection (lesions on 10% of plants). This was possibly the source of inoculum for a severe disease outbreak on peas in an adjacent field. Such specialist crops and horticultural holdings can locally provide inoculum, as more generally can contamination of seed stocks by sclerotia for all crops except potato.

FIELD TRIALS 1988-91

A number of small-plot trials was established during this period at Silwood Park, Berkshire to examine the effects of different regimes of crop management on Sclerotinia survival and development. Soils were infested with sclerotia either produced in vitro (Mylcreest & Wheeler, 1987) or recovered from infected crop debris from commercial farms. Never, in any of these trials was there any evidence of host specificity: infection of any of the crop hosts, or weeds, seemed to be achieved as readily by one isolate as another. Occasionally differences were seen between isolates in the time of apothecial production, but this seemed to be random inter-strain variability as much as any genuine crop specificity.

Trials established in 1988 and 1989 suffered severely from drought conditions and because of this a number of aims were not realised. The major conclusions of the trials are summarised below.

Sclerotial returns and survival in soil

Separate trials of combinable pea, cv Solara became severely infected during the 1988 growing season. Disease incidence ranged up to 41.5% (table 3). After harvest the haulm was chopped, the debris returned to the soil surface and sclerotia in random quadrats counted. Table 3 also records the resulting sclerotial load in soil sampled from the seedbed of the following crop (winter OSR or winter wheat). Also shown are the calculated sclerotial return to the soil surface for each 1% infection (pea only) and the sclerotial numbers in surface debris needed to load the cultivated layers of the soil by 1 propagule per kg.

Yields of sclerotia from individual pea plants in Trial 1 averaged 6.3, rather more than was typical of individual lesions on OSR (4.5). Averaged over all trials peas gave an increase in sclerotia of 52.0% over those present at drilling and OSR an increase of 31.2%.

When investigating the effect of management practices, greater numbers of sclerotia were detected in the seedbed of the following crop where rape straw was chopped prior to ploughing and where wheat straw or stubble was unburnt prior to incorporation by ploughing or minimal cultivation.

TABLE 3. Sclerotial returns to soil from infected crops of pea.

Trial	Disease[a]	Soil surface[b]	Bulk soil[c]	D	E
I	41.5	92.0	2.50	2.2	36.8
III	25.4	44.0	2.02	1.7	21.8
IV	25.4	48.0	1.63	1.9	29.4
Mean				1.9	29.3

a percentage disease incidence
b soil surface sclerotial numbers after harvest (m^{-2})
c sclerotia per kg air-dried soil in subsequent samples
D sclerotial return to soil surface for each 1% infection
E calculated sclerotial load on soil surface needed to raise bulk soil population by 1 sclerotium per kg

Longevity of sclerotia in soil has been examined over the medium term by soil sampling: table 4 shows representative data. Over the shorter term sclerotia buried in nylon mesh bags have been recovered from soils and assessed for viability. Both numbers recovered, and the capacity for myceliogenic germination of those remaining, typically decline by about 10% over six months. However in two trials capacity for carpogenic germination dropped much more rapidly, typically showing declines of 50% over the same period.

TABLE 4. Numbers of sclerotia sampled from trial plots.

Year	Trial 1 sclerotia kg^{-1}	after	Trial III sclerotia kg^{-1}	after
1988	2.45	pea	1.83	pea
1989	1.49	WW	2.60	WOSR
1990	1.02	WOSR*	0.94	WW
1991	-	-	2.61	pea

* no infection
WW winter wheat
WOSR winter oilseed rape

Apothecial production in relation to management practices

Averaged over all trials there was a broad positive correlation between numbers of sclerotia in soil and ensuing apothecial production. Table 5 shows examples of apothecial productivity and also reveals that winter wheat provides a better cover crop than rape for early season apothecia. This was a general observation made over several years: compared with OSR, under winter wheat apothecia tended to be more prolific, first appeared up to two weeks earlier, and the peak in production was also earlier.

Apothecium production was also influenced by the way in which previous crops residues were treated. In 1990 OSR plots, productivity was greatest where wheat straw had been burnt the previous season, and earliest where minimal cultivations, not ploughing, had been used to prepare the seedbed. Wheat following OSR had higher numbers of apothecia where straw had been chopped, a distribution also reflected in numbers of sclerotia, suggesting that chopping was liberating sclerotia from crop debris and possibly bringing forward fruiting by one season.

TABLE 5. Sclerotia in soils and apothecial productivity.

Sclerotia in soil[a]	Cover crop[b]	Peak apothecia[c]	Mean apothecia[d]
2.50	WOSR	5.30	1.47
2.50	WW	20.31	5.73
2.02	WOSR	6.70	0.54
1.63	WW	8.01	3.85

a number of sclerotia per kg soil
b for key see table 4
c apothecia m^{-2} recorded at the peak of production
d apothecia m^{-2} averaged over entire span of production

In one trial in 1988 apothecium production in OSR was delayed by high spring nitrogen, confirming earlier observations of Mylchreest (1985).

Infection of weeds by Sclerotinia

In 1988 a trial area was seeded with sclerotia and allowed to develop natural weed cover over the growing season. Apothecia were found first at the end of May and were present in large numbers through to July 5th. Despite the rapid development of ground cover and generally suitable conditions for infection (disease incidence in a nearby pea crop was 41.5%) little disease developed. Of the 20 plant species recorded in the 135 m^2 trial only six became infected, and the majority of lesions were accounted for by a single species (wild radish, Rhaphanus raphanistrum: 64 infected plants). Other hosts, in declining frequency of infection were Chamomilla recutita, Conyza canadensis, Cirsium arvense, Capsella bursa-pastoris and Polygonum aviculare (single plant). Sclerotial returns to soil were minimal, the smaller hosts averaging one to two per plant, and Cirsium and Conyza no more than four.

On separate occasions at Silwood Park, wild radish, mayweeds and

cudweed (Gnaphalium sylvaticum) were found infected, but despite considerable searching, Sclerotinia was never observed on larger members of the Compositae or Umbelliferae, such as burdock and hogweed, which would have the capacity for larger scale production of sclerotia.

DISCUSSION

In several respects the results of the field survey and small plot experiments reinforce each other, and a number of general conclusions can be drawn. Numbers of sclerotia in soils generally changed in a predictable way provided due allowance was made for the irregularities introduced by sampling variation. Infected pea crops in particular, caused a marked increase in soil population levels (table 3), the trial data providing two guideline figures; that each 1% infection will contribute approximately two sclerotia m^{-2}, and that to raise the bulk soil population by one sclerotium kg^{-1} approximately 30 sclerotia m^{-2} are needed on the surface. Sclerotial returns to soil of this magnitude are in line with field experience, for example Norfolk 1988, and with rises in the soil population seen on commercial farms (table 1). The highest farm figure of 6.2 sclerotia kg^{-1} must reflect the accumulated residue of many years of serious disease.

The high degree of gearing implicit in the ratio between soil surface and bulk soil numbers of sclerotia highlights the risks of relying too greatly on the error-prone and laborious task of washing soil through sieves. Using the ratio of table 5, an error of one sclerotium either way in a count could cover a range of nil to 50 in terms of the soil surface population.

Both trials and surveys indicate that infected crops of OSR return rather fewer sclerotia to soils than do peas, although maximum levels can be similar, for example Mylchreest (1985) reported a 38%-infected crop of cv. Bienvenue which returned 75 sclerotia m^{-2} to the soil surface. Of those crops less widely grown or less commonly infected, sunflower and potato can provide the greatest sclerotial returns. Given equivalent levels of infection, we believe the risk to succeeding crops is of the order; sunflower > potato = pea > winter OSR > spring OSR > linseed.

As would be expected, numbers of sclerotia in soils declined in the absence of disease or in sequences of non-host crops. The general trend was for numbers to halve approximately every 2.5 year. This crude measurement of numbers gives no indication of viability which may decline more rapidly. We have preliminary evidence that capacity to undergo carpogenic germination, a key factor in epidemiology, may be lost more quickly than would be suggested by cruder measures of viability. This phenomenon requires verification and the whole topic of sclerotial degradation in soils is a fruitful one for further study.

Under British conditions apothecial production occurs in two main flushes. The first in autumn-sown crops covers the period April-May, and the second, mostly taking place in June and July takes place in spring-sown crops. There are thus two distinct epidemiological phases which overlap only slightly, and there are obvious combinations of donor and receptor crops for inoculum. Winter wheat is an obvious source of spores for winter OSR and potentially for winter beans, although these are rarely

if ever seen infected with S. sclerotiorum (Jellis et al., 1990). Evidence from both survey and trial all indicates that winter wheat is a marginally better cover crop than OSR for sporulation of Sclerotinia; and such crops, on land that has a history of the disease should always be considered a risk factor for nearby winter OSR. Not only are apothecia seemingly earlier and more abundant in winter wheat (table 5), but the short and, prior to flag leaf emergence, relatively open crop canopy is conducive to efficient spore dispersal. Likewise, spring cereals are a potential source of inoculum to spring-sown host crops.

In field surveys clear evidence was seen for infections resulting from both internally and externally generated inoculum. Wind dispersal was also a factor in trials and it repeatedly confounded attempts, when using small plots, to correlate apothecium productivity with spore trapping data, with disease incidence or with yield. It is our experience that ascospores can be dispersed in infective quantities certainly in the range up to 200 m, a view that broadly parallels North American experience (Abawi & Grogan, 1979; Williams & Stelfox, 1979).

Attempts to correlate petal infestation by Sclerotinia in OSR with the resulting levels of disease have been only partially successful in these studies, and we doubt that it could be used alone as the basis of a forecasting system as is proposed in Canada (Turkington et al., 1991). Certainly it is a more reliable guide to OSR crops immediately at risk than counting apothecia or sclerotia, but overriding all other factors is the effect of weather. In each of the three years under study crops escaped infection because the warm dry conditions prevented the substantial period of surface wetness needed for infection by Sclerotinia (Abawi & Grogan, 1975).

There is little evidence from our studies that infection of weed or hedgerow plants provides other than an incidental source of inoculum. Notwithstanding isolated observations to the contrary (Hims, 1979), it would seem that non-cultivated plants are not a major reservoir of the disease in Britain, and the results of the natural infection trials provide little encouragement for those who advocate the use of Sclerotinia for the biological control of weeds (Riddle et al., 1991).

Experiments designed to investigate the effects of various post-harvest treatments have been rendered somewhat academic now that stubble burning is no longer an option. Broadly, the choice should be between attempting to exhaust the pathogen as quickly as possible, and trying to bury it. For the former approach, applicable in the absence of nearby susceptible crops, we would recommend if practicable, chopping the straw of any infected crop, followed by minimal cultivation, and sowing to winter cereals which are both resistant to the disease and very conducive to apothecium formation. In the second approach deep ploughing in the first year, followed by minimal cultivations for as many years as soil conditions allow, should be the goal. Neither approach is likely to be wholly successful however due to the incomplete nature of soil inversion during ploughing and the essentially random nature of sclerotial decline. In the special case of severe infection on sunflower the potential for return of inoculum to the soil is so great that cutting and removal of individual plants is probably warranted despite the expense.

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RELEASE AND DISPERSAL OF *SCLEROTINIA* ASCOSPORES IN RELATION TO INFECTION

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ABSTRACT

From 1988 to 1991 a plot of sunflower plants (cv. Sunbred 246) was inoculated with sclerotia of *Sclerotinia sclerotiorum* in February or March and disease development was monitored on each plant during the summer. The concentration of airborne ascospores corresponded roughly to the number of apothecia but the relationship differed between years. The seasonal timing of ascospore production and the concentrations found also varied between years. Ascospores were released predominantly during the day, mostly around 1200 BST. Disease symptoms were observed, between 25 and 40 days after ascospores were first found. In 1988 the number of plants per week with new symptoms was roughly proportional to the average ascospore concentration measured 5 weeks previously. Laboratory studies suggested that ascospores were released in response to decreases in relative humidity. The observations suggest that severity of disease may be related to ascospore concentration during the infection period.

INTRODUCTION

Oilseed crops have become more important in U.K. agriculture over the last few years, for example oilseed rape (*Brassica napus* var. *oleifera*) is now the third largest arable crop grown. Interest in other oilseed crops, such as linseed (*Linum usitatissimum*) and sunflowers (*Helianthus annuus*), is also increasing. All three oilseed crops are hosts to the pathogen *Sclerotinia sclerotiorum* which can cause several yield reducing diseases.

Although much is known about the environmental conditions required for sclerotia survival and apothecial development in *S. sclerotiorum* (eg. Coley-Smith & Cook, 1971; Mitchell & Wheeler, 1990) there appears to be little information available on ascospore release and dispersal and the relationship between ascospore production and potential disease development. In this paper we report some results of a four year study of ascospore release and disease progress in a sunflower plot at I.A.C.R., Rothamsted.

METHODS

Field experiments

Experiments were done in an 8 x 8.8m plot enclosed by concrete walls sunk 2m into the ground (McCartney & Lacey 1991). The plot was divided into 32 sub-plots, 1.0 x 2.2m each separated from its neighbour by a concrete or wooden partition also sunk 2m into the soil. The sub-plots were arranged in four rows of eight, with the short side of

each sub-plot parallel to rows and orientated North-South. In February or March in each year sclerotia of *S. sclerotiorum* were planted about 1cm deep in the central eight sub-plots (Table 1). In early May (Table 1) each sub-plot was planted with sunflower seedlings (cv Sunbred 246), about 30cm apart, in a 3 x 7 grid pattern. During periods of dry weather in May and June overhead irrigation was used to prevent the surface soil drying out.

The numbers of apothecia of *S. sclerotiorum* in each sub-plot were counted at weekly intervals during June and July. From June onwards, every week each plant in the plot was assessed for the presence of lesions caused by infection of *S. sclerotiorum*. Collapsed plants were removed from the plot and sclerotia formed within the plant collected. In September all remaining diseased plants were harvested, allowed to dry and sclerotia collected.

Concentrations of airborne ascospores were monitored in the centre of the plot using a seven day recording volumetric spore trap (Burkard Manufacturing Co. Ltd, Rickmansworth, UK.). The trap was operated from late May until the end of August in all four years (Table 1). The ascospore deposit on the trapping surface was counted using a light microscope and 24h average concentrations of ascospores estimated (McCartney *et al.*, 1986). Hourly average ascospore concentrations were also assessed on selected days.

On occasion, ascospore concentration was measured, at different heights above the ground, using roto-rod spore samplers (McCartney *et al.*, 1986).

Hourly average values of soil temperature, air temperature and humidity were recorded within the plot and air temperature, solar radiation and wind speed at 2m above the ground and rainfall were measured about 10m from the plot edge.

Laboratory experiments

The effect of changes in humidity on ascospore release was studied in a miniature wind tunnel 56cm long with 8x3cm cross section (McCartney & Lacey, 1990). Air was drawn through the tunnel using a Burkard spore trap, which also trapped spores released in the tunnel. Apothecia were placed in the wind tunnel for periods of up to a week and exposed to different sequences of "high" (close to saturation) and "low" (between 60 and 70%) relative humidity.

RESULTS

Ascospore release

The miniature wind tunnel experiments showed that ascospores were discharged in response to decreases in relative humidity. Very few ascospores were discharged when apothecia were exposed in air close to saturation. Apothecia were placed in the wind tunnel and exposed to alternating periods of "high" (>98%) and "low" (about 70%) relative humidity. The lengths of the periods of each were the same and lasted from 1 to 6 hours. After about a week the tunnel was opened to ambient air and the apothecia allowed to dry. In these experiments ascospores were usually discharged at the onset of

the drying periods with very few ascospores caught during periods of high humidity. This pattern was found with "high" and "low" humidity cycles of 1 to 6 hours duration. Ascospore discharge continued for periods of up to 72h. Large numbers of ascospores were often discharged at the end of the test as the apothecia dried. Similar patterns of discharge were found when the periods of low humidity were reduced to about 10 min. Apothecia were exposed to changes in periods of light and dark (12h light, 12h dark) in addition to changes in humidity. Light (artificial fluorescent tubes) appeared to have little effect on ascospore discharge.

Ascospore discharge was also observed in the field. Laboratory grown apothecia were placed in the field plot overnight and ascospore discharge monitored by collecting ascospores on microscope slides held over the apothecia. The slides were change at hourly intervals or after a "puff" of ascospores was observed. Apothecia were observed to discharge ascospores in "puffs" and most were discharged between 1000 BST and 1300 BST. Individual apothecia continued to release ascospores for up to six hours. The time between "puffs" was variable but could be as short as 10min.

TABLE 1. Sclerotia sowing dates, crop planting date, trapping dates, apothecia numbers, average weather parameters and total numbers of infected plants for 1988, 1989, 1990 and 1991.

	1988	1989	1990	1991
Sclerotia sowing date	16/2	28/2	18/3	29/3
Weight of sclerotia (g)	416	503	205	278
Planting date	13/5	3/5	9/5	15/5
Ascospore trapping dates	27/5-6/9	25/5-17/6	25/5-19/8	30/5-13/8
Maximum number of apothecia (date)	860 (6/7)	74 (22/6)	460 (24/7)	1500 (16/7)
Mean air temperature* (°C)	14.5	16.0	15.9	17.2
Mean soil surface temperature* (°C)	15.4	15.6	15.4	16.6
Total rainfall, including irrigation* (mm)	167	157	136	192
Mean daily radiation total* (MJ m^{-2})	15.1	19.6	18.6	16.7
Infected plants (total)	449	93	33	46
Infected heads (total)	6	0	7	30

* Averaged over ascospore production period.

Ascospore dispersal

Apothecia were found in the central eight sub-plots by the first half of June in all four years. The numbers of apothecia increased rapidly reaching a maximum by late June or mid July (Table 1) after which they declined. Apothecia numbers differed between years the largest number recorded was in 1991 (1500) and the smallest in 1989 (74).

The seasonal pattern ascospore release was similar for all four years (Fig 1). Although there was considerable day to day variation in ascospore numbers, concentrations tended to increase with time reaching a maximum and then declining. The highest 24h average spore concentration (about 7000 m^{-3}) was measured in 1988, but, in the other years were much lower (Fig. 1) The timing of spore release also varied from season to season; peak concentrations were measured in late June in 1989, mid July in 1988 and late July in 1990 and 1991. In 1988 spores were produced for about 55 days compared with between 40 and 45 days in 1990 and 1991, while in 1989 ascospore production lasted only about 25 days.

Ascospore concentrations appeared to follow the increase and decline in apothecia numbers. In 1988 and 1990 ascospore concentration was roughly proportional to the number of apothecia found in the plots when averaged over a week: concentration increased by about 4 ascospores m^{-3} per apothecium. In 1989 apothecia numbers were low and although ascospore concentrations were also small there were about 13 ascospores per apothecium. In contrast in 1991 when apothecia numbers were large ascospore concentrations were similar to those found in 1989.

Hourly average ascospore concentrations in the centre of the plot showed a marked diurnal periodicity with the largest concentrations found around midday (see McCartney & Lacey, 1991). Generally few ascospores were caught during the night, although occasional peaks were found at night, often following rain. Ascospore concentration usually increased sharply after about 0800 BST and had decline again by 1600 BST.

Several measurements of ascospore concentration at different heights above the ground were made at the downwind edge of the plot during the period of peak spore production. The spore traps were exposed for about 4 hours in the morning and early afternoon. Ascospore concentrations decreased with height above the ground. The rate of decrease depended on crop height: for example in 1988, the concentration at crop height (between 0.6 and 0.8m) was about 0.4 of the value near the ground, while in 1991 it was about 0.1 when the crop was about 1m tall.

Disease development

The relationship between ascospore concentration and subsequent disease for 1988 and 1989 seasons has been discussed elsewhere (McCartney & Lacey, 1991). The results for 1990 and 1991 confirmed some of the earlier observations: the first disease symptoms were found between 25 and 40 days after ascospore concentration exceeded about $50m^{-3}$; no new diseased plants were found about 40 to 50 days after ascospore production had effectively stopped. The total number of diseased plants which developed in the plot depended roughly on the number of ascospores released. Disease was severest in 1988 with about 67% of the 672 plants in the plot infected. In 1989, 1990 and 1991 only 14,

5 and 7% of the plants developed disease. In 1988 and 1989 few sunflower heads became infected (Table 1) while in 1990 and 1991 the proportion of infections found on heads was larger (21 and 86% respectively) although the total number of plants infected was small. In 1990 and 1991 the peak in spore production occurred in late July when the crop had reached full height (Fig. 1) and was in full flower. In contrast in 1988 and 1989 most of the ascospores were dispersed towards the end of June or early July before the crop had reached full height (Fig. 1). Indeed, in 1989 when ascospore dispersal was earliest no disease was found on the heads. In three of the four years (1988, 1989 and 1990) about 5% of the plants developed lesions near their base, which may also have been caused by mycelial infections. In 1990 only four plants developed base lesions.

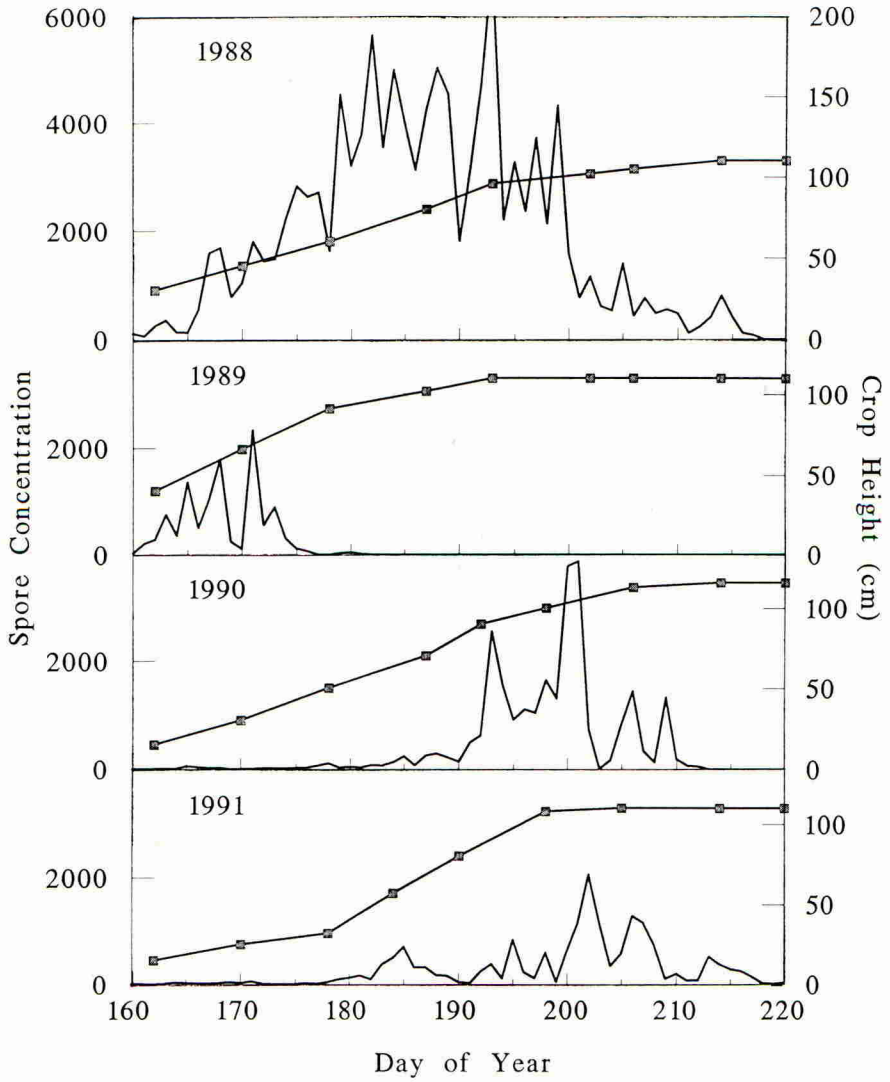

Figure 1. Twenty-four hour average concentration of ascospores measured in the centre of the plot from 9 June till 8 August for 1988 to 1991. Crop height (■) is also plotted.

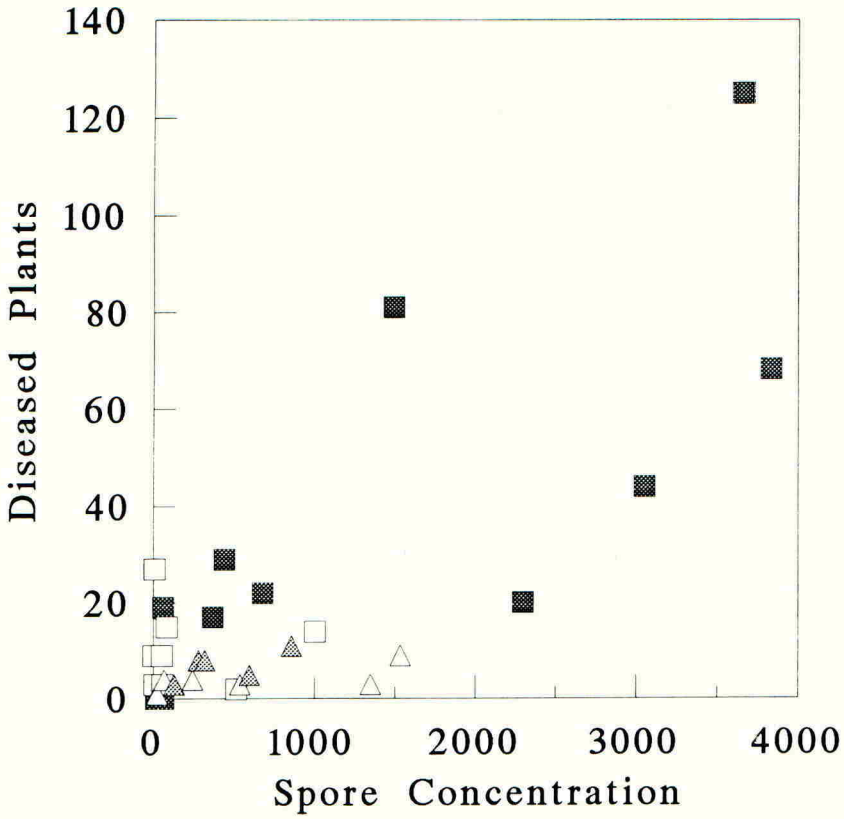

Figure 2. Relationship between the concentration of ascospores in the air and disease. 24h mean ascospore concentration averaged over weekly intervals plotted against the total number of new diseased plants five weeks later in 1988 (■), six weeks later in 1989 and 1991 (□, ▲) and seven weeks later in 1990 (△).

DISCUSSION

The results of these experiments show that the incidence of stem and head rot can be highly variable. The numbers of apothecia which developed in the plots also varied between seasons. Apothecia did not develop until ground cover was achieved in the central infected plots. It is not clear why there were such large differences in apothecia production as similar numbers of sclerotia were sown each year (in 1989, 1990 and 1991 uncollected sclerotia from previous years would also have been present): average soil temperatures for June and July for all years were similar (Table 1); rainfall, including irrigation was larger in 1988 and 1991 than in the other two years; solar radiation and air temperature were slightly larger in 1989, 1990 and 1991 than in 1988. Low soil moisture can inhibit apothecial formation (Teo et. al. 1989) thus the drier soil surface in 1989 and 1990 may have reduced the potential for apothecia formation in those years.

In three of the four years ascospore concentrations in the air within the crop appeared to be related more to the numbers of apothecia present than weather variables. However, the relationship between apothecia and ascospores was not consistent between years. Harthill (1980) found little relationship between weather variables and ascospore concentration above tobacco crops in New Zealand as long as there was sufficient rain to moisten the soil and promote apothecia formation.

The release of ascospore was more or less confined to the middle of the day; only a few spores were released at night or in the early morning. Harthill (1980) found a similar pattern in tobacco crops. Wind tunnel tests suggested that changes in humidity are implicated in ascospore discharge. Dijkstra (1964) observed puff discharge in response to exposure to gusts of cold air. In the field, Kruger (1975) found that airborne ascospores were more common when the weather was dry and windy than when it was wet. Decreases in relative humidity and increases temperature and wind speed were often observed within the plot in the morning which may have triggered ascospore discharge. Ascospore dispersal will be most effective if they are released during the middle part of the day, when wind speed and turbulence levels tend to be larger. The results of the roto-rod traps suggest that substantial numbers of ascospores may escape from the crop. *S. sclerotiorum* ascospores have been shown to be dispersed considerable distance by wind (Williams & Stelfox, 1979).

The first disease symptoms were found between 25 and 40 days after spore production began, suggesting that it takes between 30 and 40 days from initial infection before visible symptoms appear on the plants. The maximum number new infections per week occurred about five weeks after the highest spore concentrations were measured in 1988, six weeks in 1989 and 1991 and seven weeks in 1990. In 1988 the number of new diseased plants per week was roughly proportional to the average ascospore concentration measured five weeks before (Fig. 2). Because of the low levels of disease in the other years the relationship between spore concentration and subsequent disease was less clear. The 1988 results suggest that in years when the weather is favourable for infection airborne ascospore concentration may be a useful indicator of potential disease incidence. However, the variability in the efficiency of infection (ie the number of infections per unit of ascospore concentration) may make setting practical ascospore concentration thresholds difficult. Studies on other crops suggest that disease may not necessarily correlate well numbers of apothecia formed in the crop (Morrall & Dueck 1982, Boland & Hall 1988). However, Gugel & Morrall (1986) showed that, in oilseed rape crops in Canada, petal infestation by *S. sclerotiorum* ascospores, which may be closely related to ascospore concentration, was a good predictor of subsequent disease.

In 1988 and 1989, when ascospore release occurred largely before full flower, mostly stems became infected, whereas in the other two years a larger proportion of heads were infected when ascospore concentration peaked later. This suggest that sunflowers may be susceptible to attack throughout the growing season.

Severe attacks by *S. sclerotiorum* in sunflower and other oilseed crops can cause severe damage. Unfortunately, little can be done to save the crop after disease symptoms appear. Disease incidence varies greatly from year to year, therefore, it would be useful to be able to predict the potential of disease epidemics developing so that adequate control measures could be taken. These results suggests that apothecia production or

ascospore concentration may be a useful indicator of disease potential. However, further work is needed to increase our understanding of the biological and environmental constraints governing the production of apothecia and ascospores in the field if practical methods of disease forecasting are to be developed.

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RESISTANCE TO SCLEROTINIA SCLEROTIORUM IN LINSEED, OILSEED RAPE AND SUNFLOWER CULTIVARS, AND ITS ROLE IN INTEGRATED CONTROL

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ABSTRACT

Artificial inoculation with mycelium and ascospores of Sclerotinia sclerotiorum produced significantly different levels of infection on linseed cultivars but not on oilseed rape cultivars in NIAB tests. Field trials at different locations in England developed different levels of disease on cultivars. The differences in infection levels in linseed cultivars correlated with those found in artificial inoculation. The levels of disease in oilseed rape cultivars were correlated with earliness of flowering and were inversely correlated with cultivar height. The resistances found by other workers in oilseed rape and sunflower are currently being developed in breeding programmes. The prospects for exploiting resistance and changes in morphological characters which reduce susceptibility are discussed.

INTRODUCTION

Stem rot of oilseed rape caused by Sclerotinia sclerotiorum is a very important disease in a few areas in the south of England and occurs sporadically and occasionally at significant levels in other regions (Jellis et al, 1984). The last two seasons in the UK have resulted in widespread inspections at high levels due to the occurrence of warm wet weather at the onset of flowering which favour apothecia production and ascospore infection (Lamarque, 1983).

In Eastern and Southern Europe the incidence of S.sclerotiorum in oilseed rape and sunflowers is high, resulting in extensive fungicide usage to prevent crop losses (Regnault & Pierre, 1984). In linseed and flax, stem rot incidence is generally low and crop losses have not been reported (Mitchell et al., 1986).

This paper discusses studies of the resistance to stem rot in some oilseed rape (Pope et al, 1992) and linseed cultivars (Pope & Sweet, 1991) submitted to NIAB for National List and Recommended List testing in 1987-1991. Resistance studies in sunflowers, oilseed rape and linseed conducted by other workers are reviewed and the prospects for exploiting different types of resistance are discussed.

LINSEED

A range of linseed varieties entered for NIAB Descripive List trials were tested in artificial inoculation tests and in field trials. The incidence of infected stems two weeks after inoculation in 8 trials inoculated with mycelium and two trials inoculated with ascospores is shown in Table 1. In addition the length of the stem lesions was recorded on 25

infected plants per plot in two of the trials (Table 2). After 2 weeks secondary spread occurred to previously uninfected stems, and recording was terminated.

The inoculated tests consistently induced different levels of infection on cultivars and the severity of infection, recorded as stem lesion length, showed similar cultivar differences, suggesting that cultivars differ in both their resistance to primary infection and to the development of the disease. Artificial inoculation with ascospores demonstrated that S. sclerotiorum ascospores can infect linseed in the absence of applied exogenous organic material (ie. petals) under certain conditions of high humidity, in contrast to the normal requirements for infection by ascospores of oilseed rape (Lamarque, 1983).

A field trial at Chichester in 1990 developed appreciable levels of stem rot (Table 1) in early August, following flowering from the 22nd June. Very little stem rot was observed in other trials. Inoculation of field plots with mycelial suspension induced very little infection (below 0.1% infection) so that data from field trials are still very scarce. However the field trial result at Chichester produced similar results to the inoculated tests (Table 1) and it thus appears that inoculated tests produce results that indicate field resistance (Figure 1). However, additional field trials are required to confirm this finding.

Sclerotinia stem rot has only occasionally been reported in linseed, though as the crop is becoming more widely grown in both UK and mainland Europe, the disease may become of greater significance. Linseed is a crop which flowers in June and matures in July - September in the UK. It tends not to form a dense canopy and the combination of later growth and more open habit reduces the humidity within the crop in comparison with oilseed rape. The conditions favoured by Sclerotinia for infection thus occur less frequently than in rape (Lamarque, 1983).

Though the NIAB studies detected resistance in linseed, it is not thought likely that exploitation of this resistance by breeders will occur unless the increasing area of linseed becomes subject to greater levels of infection.

OILSEED RAPE

Inoculated tests using both mycelium and ascospores (Pope et al, 1992) produced high but similar levels of infection in all tested cultivars with no significant differences being apparent, so that current varieties appear to have little or no resistance to Sclerotinia.

Field trials had very variable levels of Sclerotinia stem rot at different sites in different seasons (Table 3), reflecting the influence of climatic conditions on disease incidence. In trials where appreciable levels of disease developed, significant differences in disease incidence were recorded between cultivars. Significant differences in height and time of flowering were also recorded. Correlation between height, and incidence of stem rot were $r = 0.41$ in all the trials, $r = 0.85$ in the 1989 trials (Figure 2) with higher levels of infection, and $r = 0.63$ in the Rosemaund 1991 trial. The correlation coefficient between earliness of

TABLE 1. The percentage infection of linseed plants following inoculation with mycelial suspension or ascospores of S. sclerotiorum and in a field trial in 1990.

Cultivar	Inoculated Mean Infection	No. of Experiments	Field Infection
Amazon	46.79 a	10	2.00 a
Blue Chip	35.15 ab	10	1.50 ab
Vimy	34.95 abc	5	-
Atlante	34.29 bc	5	1.50 ab
Lidgate	32.06 bc	2	-
Tadorna	31.67 bc	10	-
McGregor	29.04 bcd	5	0.50 cde
Linda	28.00 bcd	5	0.75 cd
Beryl	27.03 bcd	5	1.00 bc
Norlin	23.23 cd	10	0.00 de
Antares	18.40 cd	5	0.25 de
Mean	30.96		0.94
L.S.D. (P=0.05)	11.92		0.74

All data angularly transformed. Cultivar means adjusted by fitted constants analysis and ranked.

Figures followed by the same letter cannot be separated statistically. (Duncans Multiple Range Test). LSD calculated from average S.E. (diff) = 5.93.

TABLE 2. Mean length of stem lesions on linseed cultivars inoculated with S. sclerotiorum.

Cultivar	Lesion length (mm)
Amazon	133.67
Blue Chip	100.59
Tadorna	98.86
McGregor	93.34
Atalante	92.84
Linda	60.50
Beryl	60.30
Norlin	50.06
Antares	37.73
Mean	80.88
SED	95.4

Fig. 1. Relationship between Sclerotinia infection levels in linseed cultivars in inoculated tests and a naturally infected field trial in 1990.

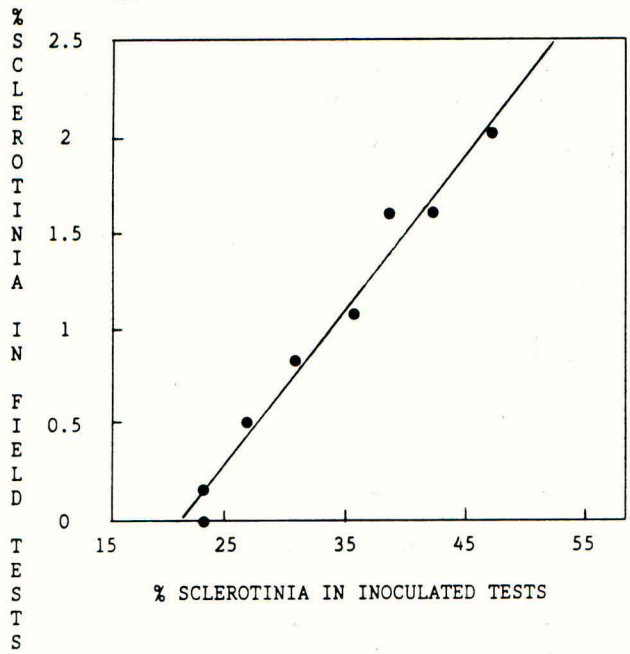


Fig. 2. Relationship between Sclerotinia infection levels and height of oilseed rape cultivars in 1989 trials.

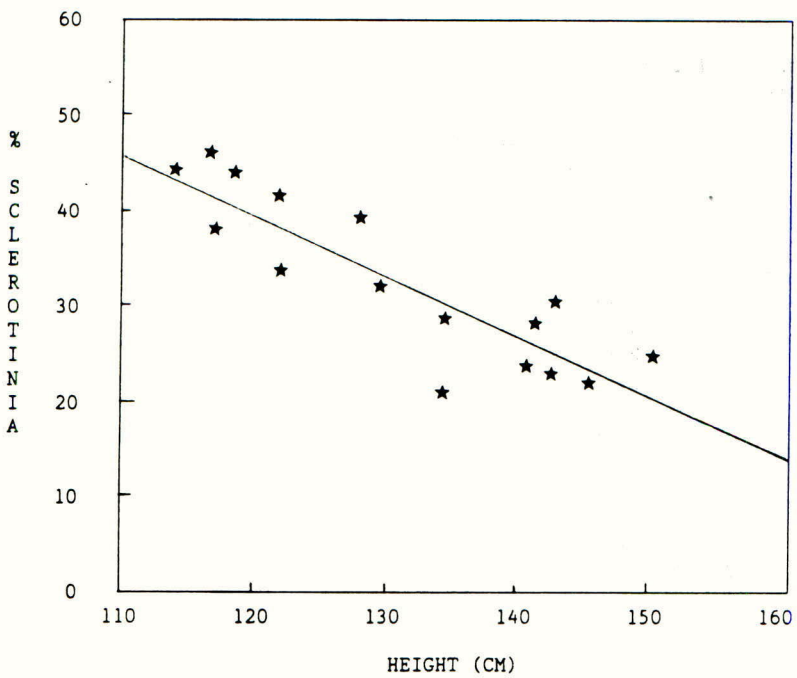


TABLE 3. Sclerotinia stem rot incidence (%) and plant height at flowering in 6 oilseed rape trials in 1987, 1988 and 1989.

Cultivar	Mean % infection	Mean height (cm)
Rafal	21.7	118.3
Bienvenu	4.8	125.0
Ariana	17.6	141.8
Mikado	25.6	114.2
Pasha	18.6	120.0
Libravo	14.0	141.3
Cobra	16.9	137.1
Karma	5.7	133.0
Lictor	15.5	142.1
Liquanta	5.6	147.0
Doublol	8.9	133.0
Payroll	2.9	120.7
Corvette	23.9	118.5
Score	22.7	128.5
Capricorn	8.7	123.7
Susana	14.9	150.0
Link	2.8	153.7
Tapidor	30.7	116.6
Liborius	14.6	146.8
Semudnk 1023	16.0	139.4
Samourai	26.1	124.7
Falcon	23.7	132.5
Mean % plants affected	25.3	134.6
SED	4.5	3.8

Fig. 3. Relationship between Sclerotinia infection levels and time of flowering in oilseed rape cultivars in 1989 trials.

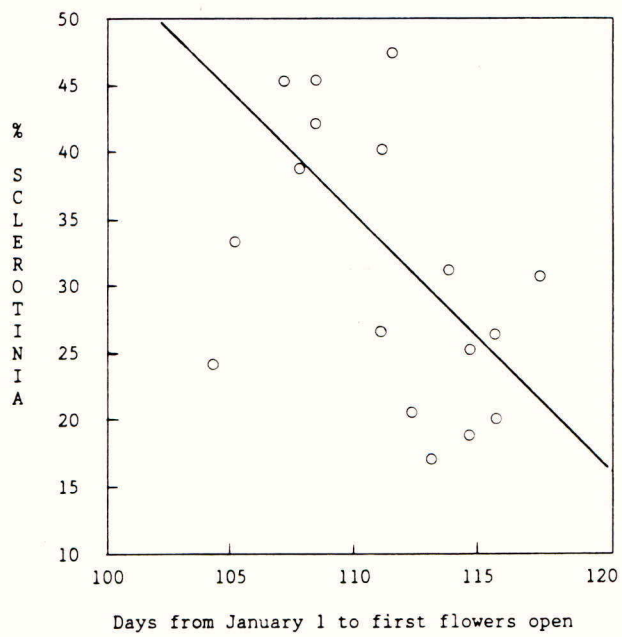


TABLE 4. Flowering, height and Sclerotinia stem rot incidence in winter oilseed rape trial, Rosemaund, 1991.

Cultivar*	Index of Flowering 1-9 (9=early)	Height at flowering (cm)	% Plants with Sclerotinia at pod ripening
Libravo	4.2	159	42.5
Cobra	3.8	155	50.5
Tapidor	4.4	139	98.7
Falcon	4.0	152	72.5
Lictor	2.9	162	39.2
Samourai	7.5	142	87.5
SE	3.1	156	53.0
Eurol	6.8	148	92.5
Idol	6.6	150	83.7
Envol	6.2	149	70.0
Aztec	4.1	143	53.2
Lincoln	4.0	159	40.0
Limerick	3.5	161	30.0
Rocket	5.4	140	75.0
Dragon	6.4	149	92.0
29	3.4	154	50.0
PR	5.1	152	58.7
AN	6.4	153	53.7
Bristol	5.7	150	71.2
AS	3.5	161	30.0
NP	4.5	146	55.0
LI	2.5	161	19.0
Lineker	2.8	165	30.7
B7	3.4	162	47.5
LD	3.6	144	48.0
AR	3.4	161	50.5
Cobol	3.2	160	28.0
DI	2.3	162	51.7
R9	5.8	151	51.2
IN	5.0	145	61.2
Apache	4.0	151	72.5
CP	1.5	159	18.0
LSD (P=0.05)	0.76	6.6	8.6

* Coded varieties are not on the UK National List.

flowering and stem rot incidence were -0.62 in the 1989 trials and -0.63 in the trial at Rosemaund in 1990. There was also a close negative correlation between height and earliness of flowering (r = 0.82 in the 1989 trials and r = 0.51 at Rosemaund).

Thus the incidence of infection in field trials varied between cultivars and appeared to be correlated with height and time of flowering of cultivars (Figs 2 and 3). Souliac (1991) demonstrated that incidence of Sclerotinia increased when oilseed rape was treated with growth regulators that reduced height without affecting flowering time. It could therefore by hypothesised that shorter cultivars are more susceptible due to their reduced height resulting in closer proximity of flower petals to ascospores released by apothecia on the ground. However, since most short cultivars are also earlier to flower, it could also be hypothesised that earlier flowering cultivars have more petals exposed to infection when ascospore production is at its greatest. Susceptibility of oilseed rape cultivars could thus be associated with a combination of reduced height and earlier petal exposure. Further studies are required which differentiate the effects of height from time of flowering.

Incidence of sclerotinia stem rot was correlated with yield (r=0.52) and reduction in yield (r=0.64) in the 1991 variety trial at Rosemaund (Figure 4, Table 5).

Where plots lodged or plants had been pushed down during tractor passes, stem contact occurred between lodged or flattened plants, allowing infection to spread by mycelial growth from stem to stem. This often resulted in very high levels of secondary infection.

Since time of flowering, lodging and height markedly influence levels of disease in oilseed rape and petals are a primary source of infection, plants that have significantly reduced petal and sepal sizes and duration of flowering, flower later and stand up well, but are of above average height, will have lowest levels of infection in many seasons. However, since Sclerotinia incidence is very dependent on the weather at the flowering period, high risk crops will often merit fungicide treatment to avoid potentially high yield losses.

Newman and Bailey (1987) described the resistance occurring in certain Japanese selections of spring rape and breeders have incorporated this resistance into winter oilseed rape breeding lines. Resistant cultivars are now being evaluated and will come into NIAB trials in the next two years.

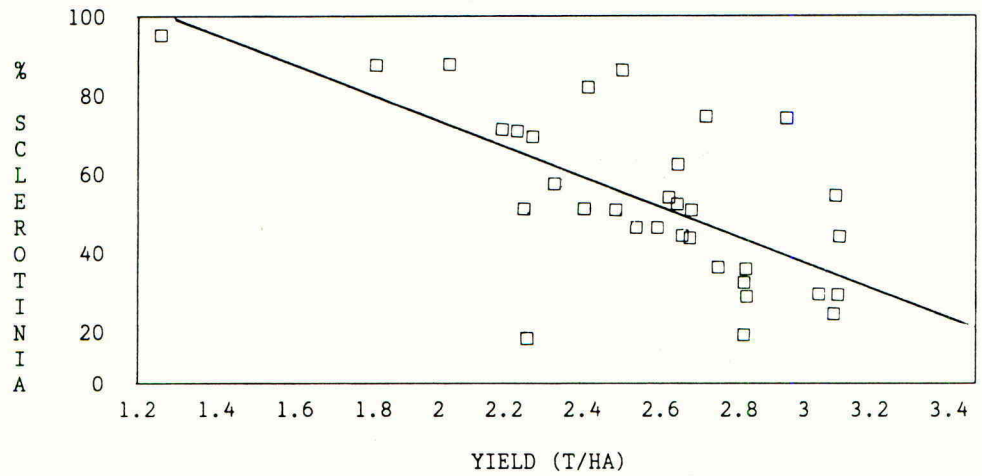
SUNFLOWERS

Resistance to S. sclerotiorum in sunflower cultivars has been described in Europe (Tourvieille & Vear, 1986), North America (Gulya, et al, 1989) and many other parts of the world. Infection occurs at the stem base, in leaf axils, or in the flowering head, from early bud development to complete development of the capitulum post flowering. Individual tissues in a sunflower cultivar differ in susceptibility and tests have shown that inoculation of the different tissues will discriminate cultivars (Castano et al, 1989). Peres et al (1989) showed that ascospore inoculation of the terminal bud at the 5-12 leaf growth stage resulted in the most severe levels of infection.

TABLE 5. Effects of Sclerotinia stem rot on the yield of some oilseed rape cultivars at Rosemaund compared with National average yields in 1991.

Cultivar	Yield at Rosemaund t/ha	Mean yield over all sites t/ha	% Reduction in yield at Rosemaund	% Plants with Sclerotinia
Libravo	3.04	3.19	4.7	42.5
Cobra	2.60	3.35	22.0	50.5
Tapidor	1.28	3.19	60.0	98.8
Falcon	2.20	3.42	36.0	72.5
Lictor	2.84	3.32	14.0	39.2
Samourai	2.55	3.45	26.0	87.5
Eurol	2.08	3.48	40.0	92.5
Idol	2.42	3.39	29.0	83.7
Envol	2.77	3.49	21.0	70.0
Aztec	2.52	3.39	26.0	53.2
Lincoln	2.75	3.45	20.0	40.0
Limerick	3.03	3.26	7.0	30.0
Rocket	2.22	3.39	34.5	75.0
LSD (P=0.05)	0.24	0.16	-	8.6

Fig. 4. Relationship between Sclerotinia infection and yield of oilseed rape cultivars at Rosemaund in 1991.



Studies of resistance mechanisms in sunflower have revealed that plants produce both phytoalexins (Martinson et al, 1988) and phenolic compounds (Bazzalo et al, 1985) in response to inoculation with S.sclerotiorum. In addition terpenoids with antifungal properties have been identified in sunflowers (Picman et al, 1990). Plant breeders are now selecting breeding material possessing enhanced levels of these compounds.

S. sclerotiorum produces oxalic acid from hyphae which assists with cell wall and membrane degradation and thus the necrotrophic development of the pathogen in the host. Oxalic acid acts as the elicitor for phytoalexin production and the subsequent production of phenolic compounds. Molecular markers that discriminate between resistant and susceptible sunflower cvs on the basis of their response to oxalic acid are being developed (Mouly & Esquerre-Tugaye, 1989) which can subsequently be used to assist with the selection of resistant material.

EXPLOITING RESISTANCE IN INTEGRATED CONTROL SYSTEMS

Resistance to Sclerotinia in all three crops appears to be multigenic, with resistance gene combinations occuring 'naturally' in some material. However, resistance is partial in the three crops, so that where environmental conditions favour severe epidemics, losses are still likely to occur. Thus at present, it appears that resistance will be sufficient in seasons of low inoculum potential or low risk, whereas additional strategies are required in high risk seasons. These could involve using growth regulators or lower seed rates to keep crops standing and to avoid secondary spread from stem contact and high humidity in the crop; use of cultivars with combinations of resistance and reduced petal size or petal numbers, and reduced length of flowering period to minimise disease levels. However, it seems likely that the use of fungicides applied at early and mid-flowering on sunflower and rape respectively will continue to provide cost effective disease control in high risk situations, and thus the present emphasis for good disease control is on environmental monitoring and risk evaluation, combined with the strategies described above.

ACKNOWLEDGEMENTS

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BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM* IN GLASSHOUSE CROPS

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ABSTRACT

Coniothyrium minitans and three *Trichoderma* isolates were examined for their ability to control Sclerotinia disease in sequential celery and lettuce crops over a six-year period. Depending on disease level, preplanting applications of *C. minitans* to soil naturally infested with *Sclerotinia sclerotiorum*, gave significant control of disease in lettuce but relatively poor control in celery. *C. minitans* decreased sclerotial survival over moist autumn fallow periods, survived for over one year and spread to infect sclerotia in other plots. The three *Trichoderma* isolates had little or no effect on disease or sclerotial viability, even though they survived in the soil.

INTRODUCTION

Sclerotinia sclerotiorum is a pathogen of more than 360 species of plants worldwide (Purdy, 1979). In the UK it regularly causes crop losses of between 1-10% in lettuce, celery, oil seed rape and potatoes but these can be considerably more. Losses in the glasshouse are generally kept low by prophylactic spraying with fungicides and, if the disease builds up, soils are fumigated with chemicals such as methyl bromide or sterilized with steam. However, some fungicides such as Ronilan (vinclozolin) have been withdrawn for use on glasshouse lettuce and soil sterilization is being carried out less frequently due to environmental concerns over the fumigants and the high cost of these treatments. Consequently, alternative disease control methods are urgently required.

Over the last six years at HRI-Littlehampton, biological methods of controlling Sclerotinia disease on celery and lettuce in the glasshouse, have been investigated using preplanting soil applications of fungal antagonists. Initial studies by Lynch & Ebben (1986) demonstrated that use of wheat grain inoculum of *Coniothyrium minitans* before each of three consecutive lettuce crops, gave 85% disease reduction or greater when 30-50% disease was present in control plots, comparable with fortnightly sprays of Ronilan. This paper reviews subsequent experiments involving the use of *C. minitans* and three *Trichoderma* isolates with proven biocontrol activity against sclerotial pathogens, in sequential celery and lettuce crops (Whipps *et al.*, 1989; Budge & Whipps, 1991) and indicates possible directions for future commercial development.

EXPERIMENTAL

Two series of glasshouse experiments were carried out using soil applications of maizemeal-perlite preparations of *C. minitans* and the *Trichoderma* isolates. Firstly, one

summer crop of celery was followed by one winter crop of lettuce (Whipps *et al.*, 1989) and secondly, one summer crop of celery was followed by one winter-spring and one summer-autumn crop of lettuce (Budge & Whipps, 1991). In the first experiment, *C. minitans* and *T. harzianum* were applied before the celery crop but then no further additions were made and in the second experiment, *C. minitans*, *T. harzianum* HH3 and *Trichoderma* sp. B1 were applied before the celery and first lettuce crops only. Numbers of sclerotia present in the soil, their viability and infection by antagonists were assessed at harvest and following a three-month fallow period. Survival of the antagonists in the soil was also monitored.

RESULTS

In the celery crop in Experiment 1, *C. minitans* treatment resulted in 24% control of disease when 25% disease was present in the control plots but in Experiment 2, no control was found when 83% of plants in control plots were infected (Table 1). After application of *C. minitans* to the first lettuce crop in Experiment 2, 47% control of disease was obtained when 90% of control plants were infected, but there was no significant control in the lettuce crops with no preplanting application of *C. minitans*. None of the *Trichoderma* applications significantly controlled disease. Prophylactic sprays with Ronilan almost completely controlled disease in Experiment 1.

TABLE 1. Effect of *Coniothyrium minitans* and *Trichoderma* species on Sclerotinia disease in sequential celery and lettuce crops.

Treatment	Disease (%) and disease reduction (%)					
	Celery		1st Lettuce		2nd Lettuce	
	Disease[c]	Disease Red.	Disease	Disease Red.	Disease	Disease Red.
Experiment 1[a]						
Control	25[x]		50[x]		-	-
C. minitans	19[y]	24	36[x]	28	-	-
T. harzianum	23[x]	8	47[x]	6	-	-
Ronilan	1[z]	96	1[y]	98	-	-
Experiment 2[b]						
Control	83[x]		90[x]		59[x]	
C. minitans	80[x]	4	48[y]	47	50[x]	15
T. harzianum HH3	79[x]	5	63[x]	31	56[x]	5
Trichoderma sp. B1	94[y]	-13	77[x]	14	46[x]	22

[a] Values from 9 plots of 100 celery and 130 lettuce plants. Antagonists incorporated into soil before planting celery only.

[b] Values from 5 plots of 56 plants of celery and lettuce. Antagonists incorporated into soil before planting celery and 1st lettuce crops only.

[c] For each experiment, values in columns followed by different letters are significantly different at 5% level estimated from 2 x SED of plant data.

Following soil incorporation of *C. minitans*, sclerotial populations in the soil at harvest in both the celery and lettuce crops were significantly lower than in control plots, but not in lettuce crops where preplanting applications were not made (Table 2). *Trichoderma* sp. B1 in the first lettuce crop in Experiment 2 was the only other antagonist to achieve lower sclerotial populations at harvest. Following moist fallow periods in the celery and second lettuce crops in Experiment 2, there was a downward trend in numbers of sclerotia recovered in all plots. However, *C. minitans* was the only antagonist to reduce the number recovered significantly during these periods in both crops. There were no differences in the number of sclerotia recovered following the fallow period after either of the first lettuce crops when the soil was dry.

TABLE 2. Effect of *Coniothyrium minitans* and *Trichoderma* species on production and survival of sclerotia of *Sclerotinia sclerotiorum* in sequential celery and lettuce crops.

Treatment	Number of sclerotia[e,f]					
	Celery		1st Lettuce		2nd Lettuce	
	Harvest[a]	Fallow[b]	Harvest	Fallow	Harvest	Fallow
Experiment 1[c]						
Control	10.1^{x}	ND[g]	82.1^{x}	87.4^{x}	-	-
C. minitans	3.1^{y}	ND	84.6^{x}	65.3^{y}	-	-
T. harzianum	8.2^{x}	ND	88.5^{x}	79.6^{x}	-	-
Ronilan	$<0.1^{z}$	ND	$<0.1^{y}$	$<0.1^{z}$	-	-
Experiment 2[d]						
Control	31.6^{x}	26.2^{x}	84.8^{x}	95.0^{x}	48.6^{x}	10.2^{x}
C. minitans	14.9^{y}	6.6^{y}	24.4^{y}	21.1^{z}	54.0^{x}	18.2^{x}
T. harzianum HH3	44.8^{x}	28.6^{x}	64.2^{x}	67.3^{y}	60.4^{x}	28.0^{y}
Trichoderma sp. B1	20.4^{x}	12.0^{y}	34.9^{y}	50.4^{y}	36.8^{x}	9.8^{x}

[a] Sclerotia recovered at harvest
[b] Sclerotia recovered after 3-month fallow period
[c] Antagonists incorporated into soil before planting celery only
[d] Antagonists incorporated into soil before planting celery and 1st lettuce crops only
[e] Values are the mean number of sclerotia per 500 cm^{-2} except for lettuce in Experiment 1 where values are the mean number of sclerotia per plant
[f] For each experiment, values in columns followed by different letters are significantly different at the 5% level estimated from 2 x SED of log transformed data
[g] Not determined

C. minitans reduced viability of sclerotia at all sampling times, except harvest in the second lettuce crop (Table 3). There were no further significant reductions in viability after the fallow period. No *Trichoderma* isolate had any effect on sclerotial viability.

TABLE 3. Effect of *Coniothyrium minitans* and *Trichoderma* species on percent viability of sclerotia of *Sclerotinia sclerotiorum* in sequential celery and lettuce crops.

Treatment	Viability (%) of sclerotia[f]					
	Celery		1st Lettuce		2nd Lettuce	
	Harvest[a]	Fallow[b]	Harvest	Fallow	Harvest	Fallow
Experiment 1[c]						
Control	ND[e]	ND	98.9[x]	96.1[x]	-	-
C. minitans	ND	ND	82.8[y]	77.6[y]	-	-
T. harzianum	ND	ND	98.6[x]	96.4[x]	-	-
Experiment 2[d]						
Control	96.3[x]	97.7[x]	98.0[x]	92.0[x]	86.0[x]	92.9[x]
C. minitans	75.7[y]	82.9[y]	60.0[y]	48.3[y]	78.7[x]	78.5[y]
T. harzianum HH3	95.7[x]	96.0[x]	98.7[x]	95.0[x]	84.3[x]	94.6[x]
Trichoderma sp. B1	98.0[x]	96.3[x]	99.7[x]	97.0[x]	87.6[x]	89.5[x]

[a] Sclerotia recovered at harvest
[b] Sclerotia recovered after a 3-month fallow period
[c] Antagonists incorporated into soil before planting celery only
[d] Antagonists incorporated into soil before planting celery and 1st lettuce crops only
[e] Not determined
[f] Values derived from samples of 30 sclerotia per plot where possible. For each experiment, values followed by different letters are significantly different at the 5% level estimated from 2 x SED of logit transformed data.

C. minitans was recovered from sclerotia from all treatment plots, except the fallow sample of *Trichoderma* sp. B1 from celery in the second experiment, and increased with time in the control and *Trichoderma* treatments in Experiment 2, to maximum values of 67 and 53% infection respectively (Table 4). The highest levels of infection of sclerotia (86%) were in plots treated with *C. minitans*. *Trichoderma* isolates were detected in a maximum of only 5% of sclerotia from plots treated with *Trichoderma* isolates, even though all antagonists survived in the soil in treated plots at levels of 10^4 colony forming units (c.f.u.) cm^{-3} one year after incorporation. *Trichoderma* counts in control plots were generally about 10^2 c.f.u. cm^{-3}.

DISCUSSION

The *Trichoderma* isolates had relatively little effect on disease or sclerotial viability in comparison with *C. minitans*, even though they survived in soil and infected sclerotia in the laboratory (Davet, 1986; Davet & Camporota, 1986). The relatively high temperature (28°C) used for the original infection tests could explain part of this, as soil temperatures at 2 cm depth in the glasshouse rarely exceed 18°C during the summer. Subsequent laboratory experiments have shown poor infection by these *Trichoderma* isolates at 18°C, whereas *C. minitans* infects well (Whipps & Budge, 1990).

TABLE 4. Occurrence (%) of *Coniothyrium minitans* in sclerotia of *Sclerotinia sclerotiorum* from soil in sequential celery and lettuce crops.

Treatment	Celery		1st Lettuce[f]		2nd Lettuce	
	Harvest[a]	Fallow[b]	Harvest	Fallow	Harvest	Fallow
Experiment 1[c]						
Control	ND[e]	ND	1.0[x]	1.8[x]	-	-
C. minitans	ND	ND	46.2[y]	41.3[y]	-	-
T. harzianum	ND	ND	1.6[x]	6.0[z]	-	-
Experiment 2[d]						
Control	18.3[x]	9.0[x]	17.0[x]	22.3[x]	67.3[x]	35.0[x]
C. minitans	56.7[y]	39.2[y]	81.7[y]	86.7[y]	72.0[x]	74.0[y]
T. harzianum HH3	10.0[x]	7.0[x]	2.3[x]	12.7[z]	53.0[x]	23.6[x]
Trichoderma sp. B1	11.3[x]	0[z]	3.7[x]	16.0[x]	40.0[x]	29.0[x]

[a] Sclerotia recovered at harvest
[b] Sclerotia recovered after a 3-month fallow period
[c] Antagonists incorporated into soil before planting celery only
[d] Antagonists incorporated into soil before planting celery and 1st lettuce crops only
[e] Not determined
[f] Values derived from samples of 30 sclerotia per plot where possible. For each experiment, values followed by different letters are significantly different at the 5% level estimated from 2 x SED of logit transformed data.

Application of *C. minitans* before planting gave significant control of Sclerotinia disease on lettuce in these and previous experiments (Lynch & Ebben, 1986) but the longer cropping period of celery and its higher leaf canopy allowing greater dispersal of pathogen spores, may limit the use of *C. minitans* in this crop. At low disease levels, *C. minitans* can provide disease control in lettuce equivalent to prophylactic sprays of Ronilan (Lynch & Ebben, 1986) but this level of control cannot be maintained by *C. minitans* as the disease pressure increases, even though significant disease control is obtained. When used, Ronilan has always given disease control greater than 95% in all our experiments. However, *C. minitans* was able to survive and infect sclerotia at all times of the year, decreasing sclerotial numbers when the soil was moist and inhibiting viability and apothecial production (see Whipps *et al.*, 1989 for latter data). It was also able to infect sclerotia at a distance from application and consequently shows potential for long term efficacy.

Taken together, these experiments and those of Lynch & Ebben (1986) show that only when there is more than 40% disease in untreated naturally infected plots does *C. minitans* fail to give control of Sclerotinia disease equivalent to fungicide treatments. This is a far higher disease level than commercial growers would accept before steaming or sterilizing the soil. Consequently, preplanting applications of *C. minitans* appear to be a realistic proposition for control of Sclerotinia disease in the glasshouse. However, there are still problems to overcome. For example, the bulky solid substrate treatments

may be too expensive or inconvenient to use in the existing growing systems, therefore other culture and application systems need to be investigated. Further, because of the risk of *Botrytis cinerea* or other diseases, prophylactic fungicide sprays may still be required. A search for strains of *C. minitans* compatible with these and other chemicals routinely used in the glasshouse would seem of value. Similarly, if more active isolates could be obtained at the same time, the period to commercial trials could be reduced.

ACKNOWLEDGEMENTS

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SESSION 3B

TOXICOLOGY: MODERN METHODS FOR RISK ASSESSMENT

CHAIRMAN PROFESSOR C. L. BERRY

SESSION ORGANISER MR P. H. ROSE

INVITED PAPERS 3B-1 to 3B-4

MECHANISMS OF CHEMICAL CARCINOGENESIS: APPLICATION TO SAFETY ASSESSMENT OF PESTICIDES

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ABSTRACT

Cancer risk assessment for any chemical, including pesticides, requires an understanding of the mechanism of the chemically-induced neoplastic process. The evolution of a neoplasm is a complex multi-event and multi-stage process, which proceeds through two sequences: the conversion of a normal cell to a neoplastic cell and the development of neoplastic cells into tumors. Pesticides can affect the carcinogenic process in a variety of ways, both by altering the genome and by providing a growth advantage for neoplastic cells. The risk assessment model should reflect these differences in cancer mechanism. For epigenetic carcinogens, the use of a model requiring a minimum dose (threshold) for cellular responses, essential for neoplastic development, greatly affects the assessment of risk at low exposure levels in humans. For DNA-reactive carcinogens, the current understanding of neoplastic processes warrants the use of a linear-at-low-dose (non-threshold) model in most instances.

INTRODUCTION

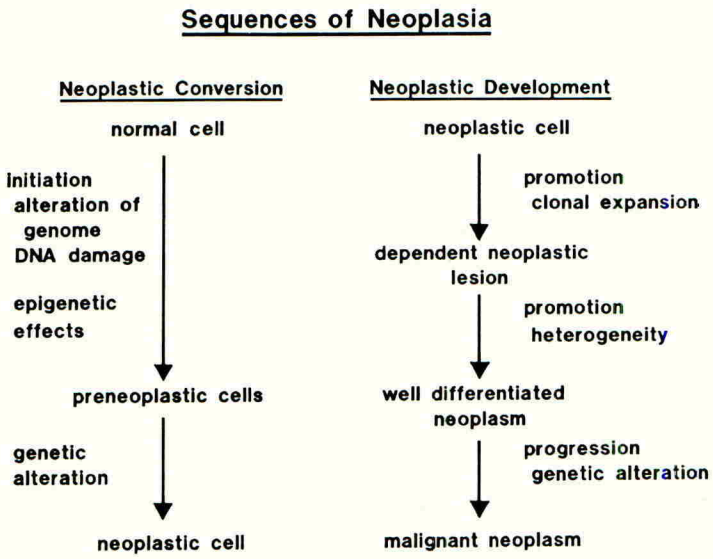
The pathogenesis of chemically-induced cancer is highly complex, consisting of a series of events. The process can be divided into two distinct sequences, neoplastic conversion, involving changes in the genetic apparatus of cells leading to generation of a neoplastic cell (often by way of altered preneoplastic cells) followed by neoplastic development, in which the neoplastic cell evolves into a tumor. In experimental models, pesticides can influence both sequences, either by enhancement or inhibition (Williams, 1990; Williams, 1984).

The tumorigenic effects of carcinogens can be exerted by several mechanisms. Evidence of this is provided by observations that, although many carcinogens give rise to chemically reactive species that damage DNA, some animal carcinogens, notably certain pesticides, do not have these properties. In recognition of this fundamental difference, a mechanistic categorization of carcinogens into two main types, DNA-reactive and epigenetic, has been developed (Weisburger & Williams, 1981) and applied to a wide spectrum of chemical carcinogens (Williams & Weisburger, 1991). The distinction between DNA-reactive or genotoxic carcinogens and the epigenetic type has, in turn, provided a basis for the selection of the type of model used for risk assessment by some regulatory agencies (Whysner and Williams, 1992).

THE NEOPLASTIC PROCESS

The neoplastic phenotype is transmitted to the progeny of neoplastic cells as a consequence of a change in the structure or expression of genetic information. DNA-reactive carcinogens are capable of effecting such an alteration directly through a mutational event, either in gene structure or

arrangement. In contrast, epigenetic agents may act either by facilitating expression of a preexisting abnormal genome or by inducing an abnormal genome through: 1) spontaneous mutation during increased levels of induced cell proliferation; 2) induced mutation through impairment of the fidelity of DNA polymerases; 3) induction of a stable altered state of gene expression; or 4) generation of intracellular reactive species such as activated oxygen, which are in turn genotoxic. The sequence of events by which chemicals produce cancer from their action on normal cells is outlined in Figure 1.



Formation of DNA reactive species

Some DNA-reactive carcinogens have chemical reactivity inherent in their structures. Apart from these, most DNA-reactive carcinogens require biotransformation into reactive metabolites. Biotransformation results from the operation of enzyme systems usually involved in the metabolism of endogenous substrates, but which can also operate on xenobiotics. The principal enzymes that biotransform chemicals are part of the cytochrome P-450 dependent monooxygenase system associated with the endoplasmic reticulum. For all classes of genotoxic and epigenetic carcinogens, except nitrosamines, most metabolic steps generate detoxified, water-soluble metabolites, which can be excreted usually in the form of conjugates. Thus, the metabolism of carcinogens to activated forms is a minor byproduct of biotransformation.

A variety of chemicals modify biotransformation processes (Williams, 1984). Among these are a number of organochlorine pesticides, such as DDT, which induce liver enzyme activities. In general, such pesticides inhibit the carcinogenicity in experimental animals of activation-dependent carcinogens by inducing detoxification systems.

The ultimate reactive form of organic direct-acting carcinogens and activated metabolites of procarcinogens is an electrophilic reactant. For epigenetic carcinogens, it is also possible that reactive species could be generated from normal cellular constituents. For instance, a class of epigenetic carcinogens has the characteristic of inducing proliferation of the cytoplasmic organelles peroxisomes. Peroxisome proliferators appear to lead to generation of reactive oxygen species as a result of production of H_2O_2 during oxidation of lipids in peroxisomes (Rao & Reddy, 1987). A member of this class is the herbicide lactofen.

The ultimate electrophilic reactants of carcinogens can bind to all four bases of DNA as well as to the phosphodiester backbone. The base adducts are formed at several sites, with the most susceptible site appearing to be the purine nitrogen. Considerable evidence now indicates that binding to DNA is a critical reaction of carcinogens. The particular regions of DNA i.e., the specific genes whose modification is essential to initiation of the carcinogenic process are beginning to be identified. Several carcinogenic pesticides are known to be DNA-reactive, for example, ethylene dibromide. Most, however lack this activity (Blair *et al.*, 1990).

If the damage to DNA by a DNA-reactive carcinogen is not repaired, or is repaired incorrectly, and the affected region is used as the template for synthesis of new DNA, a permanent mutation can be introduced through mispairing of bases. Because repair does not occur immediately, rapidly proliferating tissues and those stimulated to proliferate are highly susceptible to carcinogens. Several types of damage to DNA are now known to be promutagenic. For example, alkylation of the 0^6 position of guanine results in base pairing with thymine rather than cytosine.

Cells are endowed with a variety of elements that protect them against DNA-reactive chemicals. As mentioned, detoxification predominates over activation. Reactive species undergo reaction with abundant cellular nucleophiles such as protein, glutathione, etc., which limit reaction with DNA. The small amount of reactive agents that reach the nucleus may undergo random reaction with portions of the genome that, unlike oncogenes and tumor suppressor genes, do not lead to neoplasia. Damage to DNA arrests DNA replication, perhaps through induction of anti-proliferative factors, allowing for DNA repair. Finally, DNA repair is highly efficient, commencing almost instantly after DNA damage and proceeding rapidly with high fidelity. All of these elements, individually and in combination function to diminish the carcinogenicity of DNA-reactive agents. Depending upon the particular agent, the resultant effect at low doses may be negligible. In some cases, qualitative differences in effect at low doses may result in no carcinogenic effect. The presence of a threshold dose, even for a DNA-reactive substance, could occur where detoxification is complete at low doses. Such may be the case for the oxidation products caused by peroxisome proliferators in the presence of abundant amounts of antioxidant. The antioxidants neutralize (reduce) the oxidants before they become capable of causing DNA damage.

Neoplastic cells

Neoplastic cells have an altered genome, which may include DNA and chromosomal mutations. The essential biological abnormality in neoplastic cells is a loss of growth control. Certain lines of evidence suggest that the neoplastic state is a homozygous recessive condition, which may reflect inactivation of anti-oncogenes. Other studies point to the activation of dominant cellular oncogenes. Oncogenes code for factors involved in cellular growth, whereas tumor suppressor genes code for factors that restrain proliferation.

Neoplastic cells occur spontaneously or can be induced by chemical or other agents. They may persist in a dormant state for long periods in spite of genetic alterations, or, depending upon host conditions, grow to form a neoplasm. The elements that prevent expression of initiated cells as neoplasms are poorly understood, but may involve factors, such as chalones, which regulate growth and differentiation. Large molecules can be exchanged between cells through specialized membrane structures known as gap junctions. In this way, transmission of regulatory factors from normal to initiated cells may effect control of the initiated cells. The cells of fully developed neoplasms are deficient in gap junctions and possess other membrane abnormalities, indicating a limitation in their ability to receive regulatory signals.

Promotion

The classical definition of promotion is the enhancement of the carcinogenicity of an agent by a second agent, not carcinogenic by itself under the test conditions, acting after exposure to the first has ended. In experimental animals, promotion has been shown to occur in most organs, including skin, liver, stomach, colon, breast, and bladder. Promoters are capable of eliciting tumor formation, albeit in small yield, when administered alone under conditions of prolonged exposure at high levels. Under these conditions, promoters presumably act on initiated cells that "normally" occur. Therefore, promoters do not cause neoplasia but enhance the neoplastic process. This is probably the basis for the tumorigenicity in the liver of certain organochlorine pesticides shown in Table 1.

Table I. Liver Neoplasm Promotion by Organochlorine Pesticides

Pesticide	Species	Effect	Reference	
DDT	rat	+	Peraino *et al.*	(1975)
	mouse	+	Williams & Numoto	(1984)
	hamster	-	Tanaka *et al.*	(1987)
Chlordane	mouse	+	Williams & Numoto	(1984)
heptachlor	mouse	+	Williams & Numoto	(1984)
lindane	mouse	+	Schroter *et al.*	(1987)

Promoters could also give rise to tumors by inducing cell proliferation (hyperplasia), and, thereby, a variety of effects have been suggested to underlie the promoting action of chemicals (Williams, 1984). Since initiated cells can remain dormant in tissues for many months, it seems evident that these altered cells, in the absence of promotion, are being kept under some type of growth regulation. Non-covalent binding to components of cell membranes, including various receptors, may underlie the action of epigenetic carcinogens that operate as tumor promoters. For the organochlorine pesticides that act as tumor promoters, such effects on cell membranes may be critical.

As described above, cells exchange molecules through membrane gap junctions. If this kind of intercellular exchange is involved in the regulation of differentiation and growth, then interference with this process could release dormant tumor cells for growth into neoplasms. Thus, one mechanism for tumor promotion may involve interference with the growth control suppression of latent tumor cells. A large number of tumor promoters have now been demonstrated to have the ability to inhibit

intercellular communication (Tong & Williams, 1987), and therefore, this effect assumes importance as one basis for tumor promotion.

The enhancement of cell proliferation is a characteristic of epigenetic carcinogens. Chemically induced cell proliferation may be a primary event affecting mitotic rate (mitogenesis), a secondary event following cell necrosis (toxicity), or a selective effect on initiated cells (promotion). The selectivity of the chemical may result from a differentially high stimulation of mitosis of initiated cells, a greater toxicity for normal cells, or another effect. It is not clear whether non-selective mitogens or toxins can affect promotion.

Regardless of the underlying cause, tumor promoters release the initiated cell from growth control, resulting in proliferation of the initiated cell line. The result is an increase in the number, or clonal expansion, of initiated cells. These mechanisms of tumor promotion all require high and sustained levels of the agent to elicit the effect. This is presumably due to the necessity for a cellular response which overcomes growth control mechanisms, resulting in cell division. Also, the agent must affect a large number of cells, and the effect must be of sufficient duration to allow neoplastic cells to achieve a state of clonal expansion that cannot be brought under homeostatic regulation in the absence of the chemical.

The biologic effects that are required for release of control mechanisms would not be predicted to occur from trivial amounts of chemicals. For example, if cell damage and death are required for a proliferative response, then toxic amounts of chemical are required for tumor promotion. Likewise, a large dose would be required to disrupt receptor-mediated mechanisms, such as those involving hormones, which are under homeostatic feedback control. An example of a pesticide affecting hormonal control is amitrole, which produces thyroid tumors in rodents.

MECHANISM-BASED APPROACHES FOR RISK ASSESSMENT OF PESTICIDES

Ethylene dibromide as an example of a genotoxic carcinogen

Ethylene dibromide (EDB or 1,2 dibromoethane) was used as a fumigant for stored crops from 1948 until 1983, when it was banned in the U.S. by the Environmental Protection Agency (EPA) after ostensibly hazardous levels were discovered in finished grain products. EDB has been found to be carcinogenic in rats and mice by different routes of exposure and in multiple target organs including forestomach, lung, liver, connective tissue, spleen, mammary gland, and nasal cavity (IARC, 1987). Genotoxicity testing has been mostly positive in bacterial and mammalian systems including DNA binding and unscheduled DNA synthesis.

Guengereich *et al.*, (1987) found that EDB was activated to an electrophilic species by conjugation with glutathione and that this conjugate reacted with DNA through an episulfonium ion intermediate. They identified the major DNA adduct as S-[2-(N7-guanyl)ethyl]glutathione. Evidence that EDB is a DNA-reactive carcinogen raises the possibility that even low exposure doses could modify the genome, thereby resulting in neoplastic conversion. Such initiated cells have a finite probability of escaping homeostatic control and progressing to neoplasms under circumstances that provide a growth advantage. It is possible that adequate protective mechanisms, as discussed above, are operative in the case of EDB to prevent a tumorigenic effect at low exposure. However, until more is known about processes that could mitigate the carcinogenic potential

of EDB, the use of a conservative linear-at-low-dose extrapolation is warranted. EPA has, thereby, calculated oral and inhalation cancer potency factors of 85 and 0.76 $(mg/kg/day)^{-1}$, respectively.

Lindane as an example of an epigenetic carcinogen

Lindane is a polychlorinated hydrocarbon pesticide that exemplifies an epigenetic carcinogen. It has been found to induce benign tumors of the liver only in certain strains of mice (IARC, 1987). Numerous studies of genotoxic effects have been negative in both bacterial and mammalian systems (IARC, 1987). Most importantly, tests for DNA binding and unscheduled DNA synthesis in mouse and rat liver have been negative, indicating that lindane is not a DNA-reactive carcinogen in the target organ. Lindane has been found to promote but not initiate tumors of the liver, and other studies such as the inhibition of cell-cell communication substantiate that tumor promotion is the mechanism of tumorigenicity. Consequently, lindane appears to enhance the incidence of spontaneous liver tumors in these mice due to the promotion of spontaneously transformed cells.

The finding that lindane acts by enhancement of tumorigenicity rather than by causing a neoplastic conversion should be the determining factor in the choice of a risk assessment model. Consequently, lindane does not "cause" cancer in rodents but acts by modifying an inherent neoplastic process. Tumor promotion is associated with a cellular response that is reversible and would be expected to have a threshold dose as in the cases of other toxic effects involving cell growth (mitogenesis), cell damage (necrosis), or programmed cell death (apoptosis). These cellular effects have been associated with the ability of a chemical to promote initiated cells into neoplasms. The risk assessment for such effects involves establishing a relevant no-observed-effect-level (NOEL) and dividing by an uncertainty factor. This results in determination of an acceptable daily intake (ADI). The magnitude of the uncertainty factor depends upon many considerations, but most commonly, uncertainty factors of 100 to 1,000 are used. This is the risk assessment model recommended for pesticide residues in food by the International Programme on Chemical Safety (IPCS, 1990).

One method of determining a NOEL for the tumorigenic effects of lindane is by transforming a large number of cells in the liver (initiation) using a proven carcinogen, and determining the dose-response for promotion of these transformed cells. Utilizing the data of Schroter *et al.* (1987) the NOEL for lindane was found to be 0.5 mg/kg/day for the development of altered foci in liver initiated with N-nitrosomorpholine. Altered foci are preneoplastic lesions of the liver, a small fraction of which progress to tumors (Williams, 1980). Consequently, the calculated ADI for lindane would be 5µg/kg/day utilizing an uncertainty factor of 100.

The NOEL calculation above for promotion of altered foci is much lower than the lowest dose of 160 mg/kg/day, which induced benign tumors in mice. Utilizing this NOEL for enhancement of preneoplastic lesions, the derived acceptable daily intake (ADI) will include an additional margin of safety compared to a neoplastic end-point. In contrast, in the past the U.S. EPA has used the no-threshold, linear-at-low-dose risk assessment model for lindane (EPA, 1991). The use of this model has developed a cancer potency factor of 1.8 $(mg/kg/day)^{-1}$. Therefore, the regulatory limit associated with a lifetime cancer risk of 10^{-6} would be 0.0005 µg/kg/day. (It should be noted that the EPA is currently re-evaluating its carcinogenic assessment of lindane and has in some instances used a safety factor approach).

CONCLUSIONS

Most chemicals that have caused cancer in humans are of the DNA-reactive type (Williams, 1987). Given sufficient exposure, it seems likely that any experimental carcinogen of this type would produce cancer in humans. Many animal carcinogens have not been proven to cause cancer in humans in spite of significant exposures (IARC, 1987), which may be due to the defense mechanisms (i.e. chemical detoxification and DNA repair processes) with which humans are endowed. Regardless, pesticides of this type such as EDB should be regarded as qualitative hazards (Williams, 1987). Alternatively, an approach such as that utilized by the U.S. EPA can be used which assumes no threshold and results in the development of a linear-at-low-dose cancer potency factor.

Few carcinogens of the epigenetic type have been associated with cancer in humans, and these are mainly hormones or immunosuppressants which are used therapeutically at tumorigenic doses (Williams & Weisburger, 1991). Humans have been exposed to many pesticides known to cause cancer in experimental animals, but none has been definitely linked to cancer in humans (IARC, 1990). The absence of effects in humans has been suggested to be due to the fact that exposures of humans are below the threshold for the biological effect, which includes cell proliferation response. In addition, some effects may be specific to the rodent test animal and not at all relevant to human. Lindane has been used as an example of an epigenetic pesticide, in which the dose-response information indicates that the NOEL for the development of neoplastic foci is 0.5 mg/kg/day. The use of an uncertainty factor of 100 for this preneoplastic end-point would indicate that exposure to 5 µg/kg/day would not pose a carcinogenic risk in humans. In contrast, an acceptable risk of 10^{-6} risk (one additional chance of cancer in a million over a lifetime) would be associated with a dose 10,000 times less using the linear-at-low-dose extrapolation.

The identification of carcinogenic mechanism for a pesticide in order to distinguish which risk assessment model should be used is recommended for regulatory procedures. A DNA-reactive pesticide such as EDB, which has been shown to produce tumors at multiple sites, in different species, and by different routes of administration, should be regulated more stringently than the epigenetic tumorigen lindane, which enhances benign tumors in mice. The linear-at-low-dose risk assessment method results in similar cancer potency factors for EDB and lindane, considering them similarly hazardous to humans. Likewise, the use of a safety factor approach for both pesticides, would have a similar result although the acceptable doses would be much higher.

Due to the current understanding of the role of cancer mechanism to predict human risks, DNA-reactive carcinogens should be treated as qualitative hazards and eliminated, or the risks should be determined by a non-threshold model in most cases. In contrast, the use of a safety factor approach for epigenetic carcinogens will adequately protect humans against potential hazards due to these pesticides.

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PROGRESS IN THE INTERNATIONAL HARMONISATION OF METHODS TO INVESTIGATE THE NEUROTOXICITY OF CHEMICALS.

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ABSTRACT

There are marked differences between the requirements of the UK and the US Regulatory Authorities with regard to the assessment of the neurotoxicity of chemicals. In the UK it is believed that careful observation of animals in routine toxicity studies will allow the identification of essentially all compounds with neurotoxic potential. If warranted, effects can then be characterised by studies designed to investigate specific neurotoxic effects. The particular studies appropriate in a given instance need to be identified on a case-by-case basis. The EPA however have published comprehensive guidelines on the testing of chemicals for neurotoxicity. These include a neurotoxicity screening battery (based on a 'Functional Observation Battery', motor activity studies and neuropathology) together with guidelines to investigate specific aspects (eg peripheral neuropathy, scheduled-controlled operant behaviour, and developmental neurotoxicity). Attempts to harmonise these approaches based on proposals for OECD test guidelines are considered.

INTRODUCTION

There are significant differences in the requirement of Regulatory Authorities in different countries for the testing of chemicals in order to assess their potential for neurotoxicity. These are particularly marked as regards the US Regulatory Authorities, principally the EPA, and the UK and EC Member States in general. This paper will focus on the differences and will cover attempts to harmonise the requirements through the OECD test guidelines programme.

In this regard the OECD programme is particularly relevant. The OECD comprises 24 member countries including USA and Canada, the EC and Nordic countries, Japan and Australia. Under the mutual acceptance of data (MAD) agreement member countries agree to accept toxicity data on chemicals provided that they are generated to an OECD test guideline and in compliance with the OECD principles of Good Laboratory Practice (OECD 1981). During the period 1981-6 the OECD agreed numerous guidelines covering all the relatively routine methods in toxicology. Such international harmonisation has been important in reducing the needless duplication of studies, with minor variations, for different regulatory authorities and has played a significant role in reducing the number of animals used in toxicity testing.

There is however no agreement as to the total package of testing required by different regulatory authorities, the MAD agreement only

covering methodology. It is here that there are at present major differences between the UK and the USA with regard to neurotoxicity testing requirements.

DEFINITION OF NEUROTOXICITY

It is pertinent to first consider what it meant by a neurotoxic substance. The simple definition is that it is a chemical that produces an adverse change in the structure or function of the nervous system. Adverse effects are treatment related changes which interfere with normal function and compromise adaption to the environment. However to be of value this definition needs to be refined by considering primary as opposed to secondary effects. Some of the classical signs of neurotoxicity (ataxia, convulsions, behavioural changes etc) will clearly be produced by all chemicals if given at sufficiently high enough dose levels and hence all chemicals could be regarded as being potentially neurotoxic. For practical purposes chemicals should be considered neurotoxic if this is a primary event, rather than being secondary to general systemic toxicity.

UK APPROACH TO TESTING CHEMICALS FOR NEUROTOXICITY

In the UK there are no regulatory requirements specifically for investigating neurotoxicity other than the investigation of delayed neurotoxicity of organophosphorus compounds (OPs) in the hen. This is very much a special case, the animal model (hen) used being specific for one type of end-point (delayed neurotoxicity) induced by a specific class of chemical (OPs) (OECD Guideline No. 418). The hen is given a single high dose of compound (together with atropine to protect against the acute cholinergic effect of the compound if appropriate) and observed clinically for behavioural abnormalities, ataxia and paralysis. At autopsy selected neural tissues are examined histologically. This model is a sensitive method for detecting delayed neurotoxicity produced by OPs but it is not sensitive to delayed neuropathy produced by other chemicals such as n-hexane, acrylamide and carbon disulphide.

The approach adopted in the UK to detect compounds with neurotoxic potential is based on a careful clinical examination of animals in routine acute and repeated dose toxicity studies. This should be carried out at least once a day and should include observations for signs of toxicity on skin, eyes, fur, mucous membranes, the respiratory, circulatory, autonomic and central nervous systems, somato-motor activity and behavioural pattern. Such observations provide a comprehensive basis for the initial assessment of effects on the nervous systems eg changes in arousal state (hyperactivity or lethargy) motor function (disturbances in gait, abnormal posture or muscle tone) or pharmacological effects (sedation). Effects on the autonomic nervous system can be assessed from observations on functions such as salivation, lachrymation, urination or defecation.

In this regard there is a need to consider both acute toxicity (single dose) data and repeated dose studies. Some chemicals produce marked neurotoxicity after a single dose (eg trimethyltins), whereas in other cases, for example acrylamide or carbon disulphide induced peripheral neuropathy, prolonged exposure is needed and 90 day studies are essential for detection of these effects.

In addition to the clinical (cage-side) observations, in the repeated dose studies full autopsies will be carried out with histopathological examination of representative tissues of the nervous system (brain, spinal cord, peripheral nerve etc) together with many other tissues/organs.

It is considered important that the neuropathological data are integrated with the clinical observations; an overall assessment using expert judgement is then made as to whether the compound has neurotoxic potential.

It is the UK view that such studies can identify those compounds with significant neurotoxic potential. If further in-depth testing is warranted to characterise these effects, and to provide more information on mode of action, the appropriate studies need to be identified on a case-by-case basis. Additional studies that may be warranted in a given instance include additional behavioural studies, detailed investigation of sensory function, electrophysiological studies, neuropathology (perfusion fixation of tissues in situ and possibly examination by electron microscopy) and neurochemical studies.

APPROACH ADOPTED BY EPA (USA)

The EPA adopt a somewhat different approach. Detailed guidelines for the testing of pesticides for neurotoxicity were first published in 1982. Their guidelines were comprehensively updated in 1991 and now cover both chemical submissions (under TOSCA) and pesticide submissions (under FIFRA) (EPA 1991).

The current guidelines are comprehensive and include a neurotoxicity screening battery of tests together with specific guidelines covering the following aspects:-

Delayed neurotoxicity of OPs (hen model).

Developmental neurotoxicity (to provide data on effects on the nervous system which might arise following exposure during pregnancy and lactation).

Tests to investigate peripheral nerve function (substances believed to produce peripheral neuropathy).

Scheduled-controlled operant behaviour (designed to evaluate effects on rate and pattern of response under schedules of re-enforcement and intended to be used for compound that have shown neurotoxic signs such as CNS depression or stimulation in other toxicity studies).

Hence the major difference between the US approach and the UK is the need for a specialised 'neurotoxic screening battery' (described in detail later) to identify compounds with neurotoxic potential, rather than the use of the repeated dose toxicity study for this purpose.

POSSIBILITIES OF HARMONISATION : OECD ACTIVITIES

The US presented proposals to the OECD for an OECD test guideline

entitled 'Neurotoxicity Screening Battery' in 1988; these were co-sponsored by the Netherlands. They were based essentially on the EPA screening tests and comprised the inclusion of the following in both single and repeated dose studies:-

A functional observational battery (FOB). This is a set of non-invasive procedures designed to detect gross functional deficits in animals and to better quantify behavioural or neurological effects detected in other studies. In addition to a careful clinical examination of the animals outside the home cage the following additional studies were included. Forelimb/hindlimb grip strength - initially using a semi-quantitative measure but followed-up by quantitative methods when abnormalities were seen (Meyer OA et al 1979); this test is designed to detect compounds that induced peripheral neuropathy. Tests for sensory function to detect gross sensory deficits (sight, hearing) (Marshall JF and Teitelbaum 1974; Tupper DE and Wallace RB 1980; WHO/IPCS 1986).

Motor activity tests were also recommended using an automated device capable of detecting both decreases and increases in activity.

At autopsy enhanced examination of the nervous system was required, and specifically perfusion fixation in situ of tissues from at least 6 high dose animals and controls.

Although it was recommended that these tests were combined routinely with acute and repeated dose toxicity studies, this would not be possible without using considerably more animals than in the 'conventional' toxicity studies.

In addition the proposals raised concerns about the interpretation of the data generated particularly the motor activity studies. Such activity is not a simple function, but the combination of many different activities, walking, rearing, sniffing, grooming etc (Rice DC 1990). There are a large number of methods available, many automated (Reiter LW 1978, Evans HL et al 1986). In addition to the need to distinguish primary effects from secondary effects due to general toxicity, the results are markedly affected by complexity and novelty of the environment, nutritional and hormonal status, biological rhythm, age and social setting (Reiter LW 1978). Interpretation of any data generated is thus very difficult.

RECOMMENDATIONS OF AN OECD EXPERT WORKING GROUP

The US proposals were considered by an ad hoc OECD expert working group on neurotoxicity testing at a meeting hosted by the EPA in Washington in 1990.

The recommendations of this working group were that the FOB part of these proposals should be made as compatible as possible with the OECD systemic toxicity guidelines. It was known that the OECD had recognised that the existing guidelines for a 28 day repeated dose oral toxicity study (Guideline 407) and the corresponding 90 day study (Guideline 409) needed updating, and the UK were preparing proposals for consideration by the OECD. It was agreed that the recommendations from the Washington neurotoxicity meeting should be considered when preparing the proposals for updating the 28 day study. The concerns from the US for a greater emphasis

on the need for careful clinical observation of the treated animals with some minimal testing for neuromuscular and sensory function, were recognised.

Regarding neuropathology, the expert working group recommended that perfusion fixation techniques should be regarded as a second-tier procedure, for investigation of a suspect or proven effect. It was recognised that this was not an appropriate 'primary surveillance' method and it should not be required in routine toxicity testing.

PROGRESS ON UPDATING OECD REPEATED DOSE (28 DAY) ORAL TOXICITY (GUIDELINE 407)

Proposals from the UK for updating this guideline which included greater emphasis on the need for careful clinical observation, and also studies of hindlimb grip strength, sensory function and with the option of measuring motor activity together with somewhat enhanced (conventional) histopathology have been circulated to OECD member countries for a first round of commenting. Comments received were considered by a small expert working group meeting in Paris in February 1992. It was agreed to recommend the following for the critical 'observations' section of the guideline.

> "Clinical observations should be made once daily and signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (eg lachrymation, piloerection, pupil size, usual respiratory pattern). Changes in gait, posture and reactivity to handling as well as presence of clonic or tonic movements, stereotypes (eg excessive grooming, repetitive circling) or bizarre behaviour (eg self-mutilation, walking backwards) should also be recorded. Observations should be made outside the home cage in a standard arena. Observations should be detailed and carefully recorded preferably using explicitly defined scales. In the fourth exposure week sensory reactivity to stimuli of different modalities (eg auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment should be conducted. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment".

PROPOSALS FOR OECD NEUROTOXICITY GUIDELINE

The OECD are now in the process of preparing a Neurotoxicity Guideline as a second tier study which could be used when there is cause for concern based on the results of previous studies eg the updated Guideline 407. This is likely to incorporate parts of the EPA neurotoxicity guideline and will be a further aid to harmonisation.

CONCLUSIONS

If these proposals are accepted in the second commenting round, they will be adopted by the OECD in an updated Guideline 407. Similar changes are then likely to be agreed for the sub-chronic (90 day) Guideline (guideline 409). This should enable the US regulatory authorities to obtain sufficient information to identify compounds with neurotoxic potential from the routine repeated dose toxicity studies.

More detailed Tier II studies should then only be used to characterise the neurotoxic effects of compounds already identified as having neurotoxic potential.

Some degree of harmonisation of testing requirements may thus be achievable. This would have benefits for industry as the need for additional specific studies for certain regulatory authorities would be reduced.

Note: The views expressed in this paper are those of the author and in no way commit the Department of Health.

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HAZARD ESTIMATION FOR PESTICIDE APPLICATORS

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ABSTRACT

Worker exposure studies are used to measure the external contamination of pesticide applicators and toxicology studies to predict the consequences of an absorbed dose. There is, however, no rule of thumb which allows exposure on the skin to be converted to internal dose. This presentation is intended to show that several methods exist to calculate the extent of absorption from simple *in vitro* bridging from rodent to human skin to kinetic modelling and biomonitoring. A stepwise approach should be adopted according to the available data and levels of concern surrounding the toxicology.

INTRODUCTION

In recent years it has become clear that the assessment of hazard to pesticide applicators is at least as important as the significance of crop residues to consumers. It is indisputable that the applicator has greater risk of exposure by virtue of his contact with the concentrated product, whereas many pesticides leave very small or undetectable residues in food crops. This is not to say that the applicator is necessarily at significant risk of intoxication, as many other factors come into play such as :

a) the application method
b) the amount and concentration applied
c) formulation and packaging technology
d) skin penetration
e) the toxicity of the active ingredient.

A risk assessment compares toxicity with either a predicted or measured value for absorbed dose. Clearly there is a need to progress from estimation to measurement of unknowns where the toxicity gives greatest concern. Hence a tiered approach to exposure estimation would progress from modelling based on conservative assumptions, at the level of least concern, to field measurement of actual absorbed dose (biomonitoring) at the other extreme.

This presentation is intended to demonstrate some of the techniques which can be used in estimation of the absorbed dose and from this the margin of safety with respect to the toxicity of the compound.

This presentation is limited to a consideration of the dermal route of exposure as it is generally considered to be the most important. It should be noted, however, that oral ingestion and inhalation may be significant routes of exposure under certain circumstances.

The parameters of hazard estimation for pesticide applicators

The important parameters in hazard estimation for pesticide applicators are conventionally considered to have the following relationships.

$$MOS = \frac{NOAEL}{Da}$$

$$Da = Ed\ Ca$$

NOAEL = No Observed Adverse Effect Level
MOS = margin of safety
E_d = dermal exposure
C_a = coefficient of absorption
D_a = absorbed dose

The NOAEL originates from toxicology studies in animals or less often from human data. The margin of safety is arbitrary and while conventionally set at 100 may vary according to the perceived quality of the data or seriousness of the effect.

Dermal exposure is the value most readily obtained from field studies either by patch or whole suit methods. This type of data lends itself to generic treatment as it is usually a function of the applicaton method, rather than the properties of the active ingredient. Skin absorption, on the other hand, varies according to the product in question and cannot easily be treated generically. For modelling without actual data it is necessary to adopt some conventional and usually pessimistic, value for skin penetration.

Exposure models

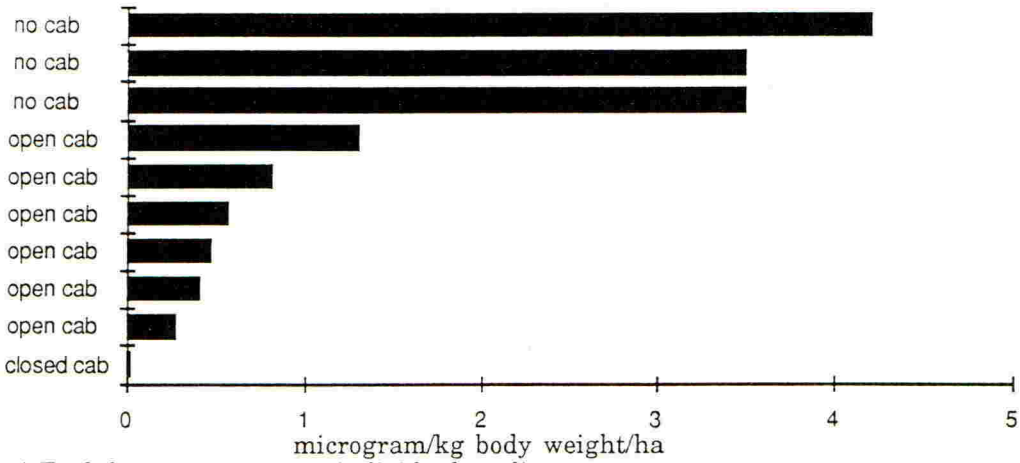
Provided generic data bases are subject to quality control with respect to their contents and updated to reflect changes in technology it should be possible to extract a value for dermal exposure (E_d) for the equations shown.

There are now four models or more correctly generic data bases for the estimation of applicator exposure : these are from U.K. (Martin, 1990), Germany (Lundehn *et al*, 1992), The Netherlands (van Hemmen, 1992)) and North America (PHED, 1991). This means that for common application techniques there now exists sufficient data on exposure that it is probably not worthwhile to
further duplicate this data. Although it remains necessary to confirm that sufficient data exists and that it can meet criteria for quality before this conclusion is made final. However, it may be useful to do exposure studies to demonstrate the efficiency of technological advances (eg. engineering controls, formulation, technology, etc). As a simple illustration, fig. 1 demonstrates the protection from exposure when applying a maize herbicide in spring which is afforded by a cab, even an open cab.

This leaves dermal absorption as the principal unknown. Skin penetration should normally be expressed as a rate constant, which is the only true basis of comparison between compounds. However, such a constant is hard to use directly as the area of skin exposed is unknown and for various

practical reasons is bound to stay unknown. The pragmatic approach normally taken is to express skin penetration as a percentage of the material available for absorption (i.e., on the skin) for the actual assessment.

FIGURE 1. Herbicide application on corn
Actual contamination according to the type of tractor cabin.*



* Each bar represents an individual applicator

Measurement of skin penetration

Skin penetration can be measured in a number of ways each with advantages and disadvantages. The traditional approach is with radiolabelled compound applied to the skin of a rat or rabbit. Usually the area of skin exposed is proportionately greater than expected for most human exposures during normal agricultural operations. Furthermore as rodent skin is dissimilar to human skin in structure and physiology it may provide an unreliable model.

More recently techniques have been developed to measure penetration through epidermal membranes which can be prepared from rat, rabbit and human skin. Such *in vitro* systems are subject to the normal criticism of being artificial and in this case also of having no blood supply to the receptor surface. For this reason the choice of receptor medium may influence the permeation of the solute in a way which is not necessarily representative of the *in vivo* situation. However, such techniques are very useful as a basis of comparison between compounds and between species (table 1).

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situation. However, such techniques are very useful as a basis of comparison between compounds and between species (table 1).

TABLE 1. In vitro skin penetration (8 h) = mg/cm^2/h

		Rat	Rabbit	Human
Herbicide (dry) formulation neat		0.68		0.42
3.3 g/litre	Dilution	2.85		0.61
2.0 g/litre	Dilution	2.71		0.93
Insecticide formulation neat		1.92	1.68	0.18
4.0 g/litre	Dilution	0.07	0.33	0.003
0.2 g/litre	Dilution	0.02	0.35	0.02
Fungicide formulation neat		15.50		2.10
4.0 g/litre	Dilution	1.00		1.20
0.4 g/litre	Dilution	0.20		0.20

From the examples given here of studies done with the same system it can be seen that rat and rabbit skin are not necessarily predictive of one another. It is also clear that rodent skin usually overestimates skin penetration relative to humans but in a way that is not necessarily predictable ; the ratio varying from about 0.8:1 to around 18:1 in the illustration given.

The third approach which is clearly the method of choice from the scientific point of view is measurement of dermal absorption in human volunteers. This approach is however constrained by ethical considerations or even forbidden in some countries. Assuming that the ethical requirements can be met and that techniques exist to analyse the compound or metabolites with sufficient sensitivity to be useful, this approach can give very valuable information.

In an example given below with triclopyr (work done at Dow Chemical : Carmichael *et al*, 1989) data were obtained on oral and dermal pharmacokinetics of triclopyr which is excreted in urine, by animals and man, as the unchanged compound. Modelling was performed in the first instance by the oral route and confirmed by dermal application. This showed that triclopyr kinetics could be modelled with a two comportment model with a fast (alpha) elimination phase and a slower (beta) elimination phase (see table 2). Relative to elimination the speed of dermal penetration (half-life abs) was quite slow. The modelling gave a good correspondence between the predicted amount of triclopyr absorbed and that found in urine and demonstrated that approximately 1-2 % of applied dose was absorbed following 8 hours exposure.

TABLE 2. Triclopyr pharmacokinetic parameters

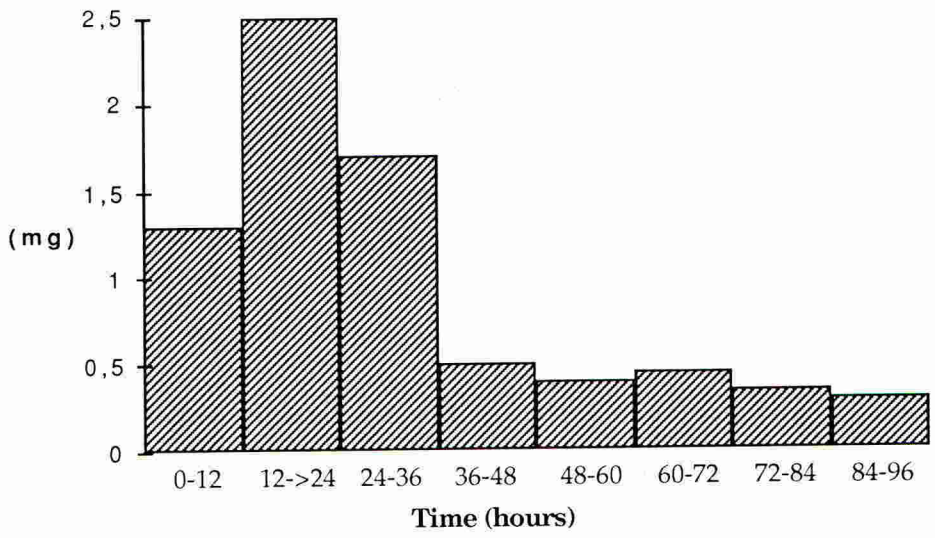
Parameters	3.7 mg/kg dermal dose
Body weight (kg)	73.0
Half-life$_{abs}$ (h)	16.8
Half-life$_{elim}$ alpha (h)	1.3
Half-life$_{elim}$ beta (h)	5.0
Urine, % dose found (96 h)	1.37
Urine, % dose predicted (96 h)	1.58
Dermal absorption corrected for recovery %	1.65

Biomonitoring

The approach described above for measuring dermal absorption in humans is directly applicable to calculations of hazard based on actual or theoretical values for exposure. The only major assumption to be made is that the absorption study employed a skin surface 1 cm2/kg body weight similar to that which would be exposed in the field.

However, with much smaller assumptions this data could be applied to applicator biomonitoring studies where no exposure measurement is made. The only requirement would be complete collection of urine (e.g., 48 hours). Fig. 2 illustrates, for the triclopyr example, the relationship between time and urinary excretion of the parent compound before modelling. The pharmacokinetic model is able to predict from the duration of the collection interval what proportion of the absorbed dose is excreted ; therefore a direct estimation of the absorbed dose can be made.

FIGURE 2. Herbicide X in urine - Dermal dose equivalent to 3.7 mg/kg

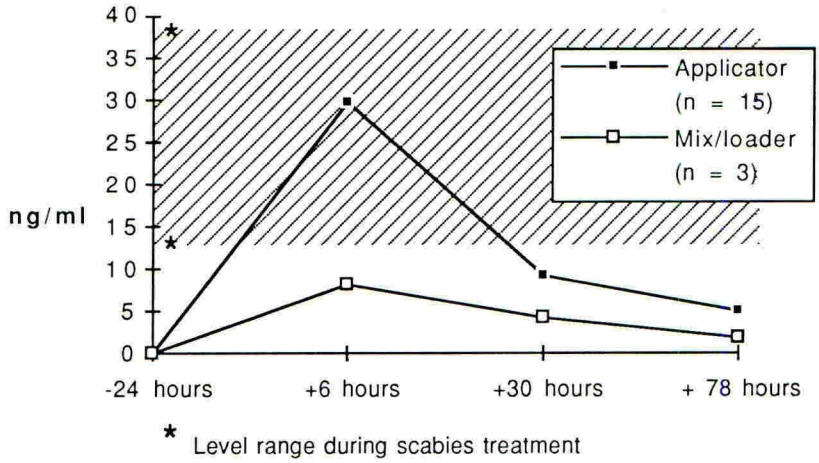


Effect biomonitoring

Another approach which can be used, if appropriate, is direct measurement of an indicator of exposure. In practice there are few indicators sufficiently sensitive for this approach. Blood cholinesterase depression is the best known example and can be measured with organophosphates, although it is not reliable with carbamates due to spontaneous reversion of the inhibition (Vandekar, 1980).

Occasionally a hybrid type of study can be envisaged as illustrated in fig. 3 with Lindane.

FIGURE. 3. Mist blower application on cocoa
Evolution of applicator and mixer-loader
Lindane blood level (ng/ml)



This is one of the rare case where sufficient human data exists to compare blood levels following exposure with those following therapeutic use. In this case with workers applying the product to cocoa in a situation where relatively high exposure is expected the maximum blood level attained was less than that typically found during treatment for scabies (Ginsburg, 1977; Lange, 1981) and far lower than any dose known to cause signs of intoxication. In addition it can be seen that elimination is rapid.

CONCLUSION

Various approaches can be applied to the estimation of operator hazard during application of pesticides. The establishment of international generic data bases can avoid the necessity of worker exposure studies in many cases where good quality generic data exists. For those data bases such as the U.K.

where the actual data are not open to peer review there remain doubts as to the validity of the generic values. These concerns are being addressed and are elaborated upon in the next paper by W. Chen. A tiered approach is suggested to increase the precision of skin absorption estimation according to the gap between the apparent margin of safety based on calculation and the margin of safety required. Very often the rodent skin *in vivo* system will provide sufficient reassurance that a substance is not a fast skin penetrant. *In vitro* measurement allows further refinement and comparison between animal and human skin, but remains a model system. It is clear that the least assumptions are made with human volunteer studies, but for ethical or practical reasons these are not always feasible. They are also expensive and time consuming and such precision is not always necessary.

Assuming that exposure data of a representative nature is available a tiered approach to exposure estimation also appears reasonable. The first stage being simple paper calculation. Conventional exposure studies are therefore only useful to demonstrate technological improvements in formulation, mixing, loading or application or where generic values of sufficient quality do not exist. If measurement of skin absorption and reference exposure values leave the issue unresolved the final resort is a pharmacokinetic study followed by biomonitoring.

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THE HARMONISATION OF OPERATOR EXPOSURE RISK ASSESSMENT

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ABSTRACT

In connection with the development of Uniform Principles, an ad hoc working group on operator exposure was formed, consisting of specialised experts from European Community (EC) countries. The aims of the working group are to establish the scientific basis for: (1) A new and harmonised EC protocol for the estimation of operator exposure by modelling. (2) To build an European operator exposure database. (3) To develop a new EC protocol for the measurement of operator exposure. (4) To define a tier risk assessment process for field applicators.

INTRODUCTION

The European Communities (EC) Registration Directive 91/414/EEC provides a harmonisation regime for the authorisation by Member States of plant protection products in accordance with Community rules, namely the Uniform Principles.

In connection with the development of Uniform Principles, an ad hoc EC working group on operator exposure was formed, consisting of specialised experts from France, Germany, U.K., The Netherlands and Ireland. The objectives of the working group are to establish the scientific basis for: (1) A new and harmonised EC protocol for the estimation of operator exposure by modelling. (2) To build an European operator exposure database. (3) To develop a new EC protocol for the measurement of operator exposure. (4) To define a tier risk assessment process for field workers.

Starting with the existing U.K. and German models and databases, the working group has developed the scientific basis for new and harmonised protocols for the estimation (modelling) and measurement of operator exposure. A tier operator risk assessment scheme has been defined incorporating these new protocols.

TOXICOLOGICAL ASSESSMENT

In assessing the risks to operators working with crop protection products, it is important to recall the fundamental principle of toxicology, namely "Dose Makes the

Poison". Risk is dependent on both toxicity and exposure. Therefore, in the operator risk (or safety) assessment, both toxicological assessment and exposure assessment must be taken into consideration.

The endpoint of the toxicological assessment of a crop protection product is the determination of an Acceptable Operator Exposure Level (AOEL), derived after a thorough evaluation of the toxicological profile of the product. This process involves:

(1) Examination of all the relevant toxicological data ensuring both high quality and compliance with the data and protocol requirements set forth in Annexes II and III of the EC Registration Directive (European Communities, 1991) and the study report on Uniform Principles by Dr. Mark Lynch (Lynch, 1992).

(2) Selection of the relevant No-observed-adverse-effect-level (NOAEL) which is based primarily on the subchronic toxicity and developmental toxicity studies [including reproductive effects on the parent (Fo) generation of a two-generation reproduction study]. These studies are selected because short-term repeated (subchronic) exposures are the ones experienced by field applicators using crop protection products.

(3) Application of the appropriate safety factor which is dependent on the toxicological profile of the product. Traditionally, a 10-fold safety margin is used for setting occupational exposure limits in the manufacturing plant. In the German model (Lundehn, 1992) for field exposure, a 25-fold safety factor is applied to general toxicological effects. A 100-fold safety factor is conventionally used for more severe toxicological endpoints.

(4) Finally, the AOEL is determined by applying the appropriate safety factor to the relevant NOAEL.

EXPOSURE ASSESSMENT

The Ad Hoc EC Operator Exposure Working Group has developed a tier scheme for the determination of operator exposure. This is summarised in the following.

Tier 1 Estimation of operator exposure

There is a general agreement among scientific experts that sufficient exposure data have been generated in recent years, which enables the development of a generic European operator exposure database. This database is built

according to application methods categorised as follows:

Boom Spray Applicators
Mixer and Loader Exposure
Air Blast Application
Hand Held Application
Bystander Exposure
Harvester (Re-entry) Exposure
Mediterranean Region Exposure
Glasshouse/Greenhouse Exposure
Seed Treatment Exposure
Grain Storage Products
Application of Solids, Granules and Dusts
Aerial Applications

Starting with the existing U.K. (U.K. Report, 1986) and German (Lundehn, 1992) models and databases, the Ad Hoc EC Working Group has developed the scientific basis for a new and harmonised EC protocol (model) for a first tier estimation of operator exposure.

In this new protocol (model), the exposures to mixer/loader and applicators are estimated using an European operator exposure database. Dermal, inhalation and oral routes of exposure are taken into consideration. The European operator exposure database is categorised according to application techniques and a large number of variables are taken into account, e.g. concentration of the active ingredient, formulation type, application rate, work rate, container type and size, clothing and gloves, etc.

Tier 2 Specific measurements of model parameters

It is important to recognise that the first tier estimation using the generic database and modelling is usually an over-estimation of the actual operator exposure. A number of conservative assumptions are generally used in the model calculations. In the event that the first tier estimation shows that the margin-of-safety (MOS) to operators applying the product is inadequate, then a second tier approach is used, employing actual data on specific model parameters such as dermal absorption and protective clothing penetration.

Both In Vitro and In Vivo methods for the measurement of skin absorption have been developed and submitted to the Organisation for Economic Cooperation and Development (OECD) for their validation and adoption as OECD/EEC test protocols.

Tier 3 Determination of operator exposure

Finally, as a third tier, actual field operator exposure studies can be conducted to determine more precisely the level of exposure to applicators. A state-of-art protocol, including the measurement of external exposure (contaminations) by dermal, inhalation and oral

routes, the performance of pharmacokinetics studies applicable to humans and the determination of internal dose by biomonitoring (urine, plasma, etc.), has been developed and discussed in the recent International Workshop on Risk Assessment for Worker Exposure to Agricultural Pesticides in Hague, The Netherlands (International Workshop, 1992). This test protocol will be submitted to OECD for their review and adoption as an OECD/EEC guideline.

A TIER PROCESS FOR ASSESSING OPERATOR SAFETY

A tier process for assessing operator safety has been developed by the Ad Hoc EC Operator Exposure Working Group (Figure 1). This tier process was presented at the recent International Workshop on Risk Assessment for Worker Exposure to Agricultural Pesticides in Hague, The Netherlands (International Workshop, 1992). This scheme was supported by scientific experts from Europe, United States and Canada attending the Workshop.

As a first tier of safety assessment, operator exposure can be estimated from the use of the EC operator exposure model and generic database. Certain assumptions for unknowns (e.g. dermal absorption to be 10%) would be used. If an acceptable margin-of-safety (MOS) to operators can be demonstrated, based on the estimated operator exposure level as compared with the AOEL (determined from the toxicological assessment), then the crop protection product can be approved without further investigation.

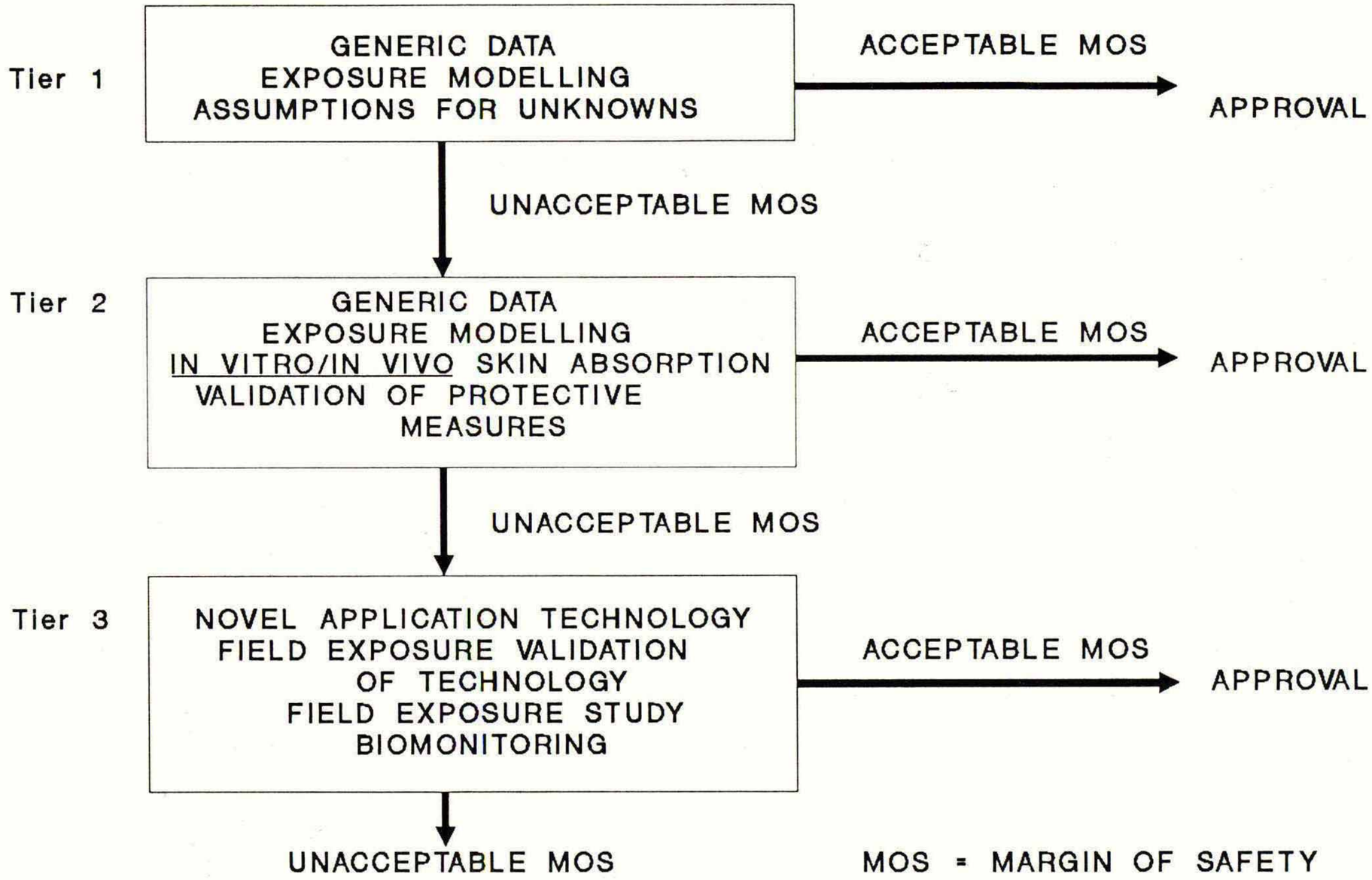
However, if the first tier estimation shows that the MOS is inadequate, then a second tier study would be performed, using actual data on specific model parameters e.g. skin absorption, protective clothing penetration, container size and design, etc. This would improve the risk assessment.

Finally, as the third tier, actual field operator exposure studies can be conducted to determine more precisely the operator exposure and safety assessment. Measurements of external exposure and the internal dose (by biomonitoring of urine, etc.) can be performed and the results used in the safety assessment.

CONCLUSION

The investigation and assessment of the toxicological profile of plant protection products will largely be completed for the inclusion of active substances in Annex I of the EC Registration Directive 91/414/EEC. Those assessments will include the selection of appropriate safety factors and the setting of Acceptable Operator Exposure Levels (AOEL) and Acceptable Daily Intake (ADI) levels for man. While AOEL is determined for the purpose of assessing operator safety, the ADI is established for consumer safety assessment.

Figure 1. TIERED REGULATION OF APPLICATOR HAZARD



Starting with the existing U.K. and German operator exposure models and databases, the Ad Hoc EC Operator Exposure Working Group has developed the scientific basis for the new and harmonised protocols for the estimation (modelling) and measurement of operator exposure. A tier operator risk assessment scheme has been defined incorporating these new protocols.

This work of the Ad Hoc EC Operator Exposure Working Group provides a number of significant contributions to the science of risk assessment and regulation as well as advantages to the industry. The development of the new EEC/OECD protocols for the estimation (modelling) and measurement of operator exposure would enable the European and international harmonisation of test guidelines. This would clarify with respect to data required and test guidelines and thus eliminating unnecessary repetition of testing. The collation of different operator exposure data into the European operator exposure database would improve the quality, accessibility and sharing of data internationally. Furthermore, through the building of the operator exposure database, exposure data gaps would be identified. This in turn will stimulate further scientific research and development. The tier risk assessment scheme developed by the Working Group would also enhance and improve the operator exposure risk assessment process.

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