Theor Appl Genet (1997) 94:1000–1006

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The release of genetically modified grasses. Part 1: pollen dispersal to traps in *Lolium perenne*

Received: 5 June 1996 / Accepted: 11 October 1996

Abstract As part of a series of experiments on determining the risk of introducing genetically modified wind-pollinated forage grasses an experiment on pollen dispersal was conducted and the use of theoretical descriptions to predict dispersal in model systems investigated. Pollen traps were placed around a central source of *Lolium perenne*. Traps were exposed with their sticky surfaces towards and away from the pollen source and also facing skywards during four stages of anthesis (early, mid 1, mid 2 and late). There was a great deal of variation in dispersal over time and to traps of different orientations. Twelve datasets were collected and used to comprehensively test Bateman's equations for the wind dispersal of pollen. The equations were not particularly useful for describing dispersal over distance and clearly need to be modified to take factors such as wind direction into account.

Key words Pollen dispersal · Genetic release · *Lolium perenne*

Introduction

There is concern that transgenes will be spread from genetically modified crop plants to related crop, feral or wild species (Raybould and Gray 1993). In this situation the transgenic construct would be introduced into new genetic backgrounds where its stability and expression are uncertain and pleiotropic effects unpredictable (Kareiva 1993). Consequent hybrids may have novel properties. A transgene previously unexpressed in pollen might give rise to new 'hay-fever' allergens, for example. If invasiveness is influenced there could be undesirable changes in the species balance of the natural ecosystem (Ellstrand 1992; Regal 1988) or increased weediness in agriculture (Crawley et al. 1993; Kareiva 1993; Raybould and Gray 1993).

Raybould and Gray (1993) identified several crops for which there is a high probability of gene flow, via pollen, to wild species resulting in native-crop hybrids. Experiments with wild and cultivated radishes (both *Raphanus sativus*), for example, detected extensive gene flow from crops to stands of weeds, including some to stands a kilometre away from the crop (Ellstrand et al. 1989; Klinger et al. 1991). Kirkpatrick and Wilson (1988) found 5% of progeny from wild gourds (*Cucurbita texana*) contained alleles specific to cultivars of *C. pepo* grown as crops 1300 m away.

The major wind-pollinated crops in the UK that are cross-compatible with native and feral populations and which are likely to flower on a wide scale are forage grasses. The ryegrasses (*Lolium perenne* and *Lolium multiflorum*) are widely cultivated outcrossing forage crops. They cross readily with wild and feral ryegrasses (*L. perenne* and *L. multiflorum*) and occasionally with fescue species. Lolium-fescue hybrids show some fertility and an ability to backcross with the parents. *Festulolium loliaceum* (*Lolium perenne* × *Festuca pratensis*) is the most common of these hybrids. Given the wind-pollinated nature of ryegrasses and the copious amounts of pollen produced it is pertinent to ask how much pollen is likely to be dispersed from transgenic ryegrass crops into wild *Lolium* or *Fescue* populations and hence the transfer of transgenes into plants that form part of natural ecosystems.

The first comprehensive theories of the mechanics of aerial spore dispersal were developed to describe the spread of fungal diseases (Gregory 1945). These were based on Sutton's descriptions of the dispersal of a smoke cloud from a source (Sutton 1932) and Taylor's theories of diffusion and turbulent motion (Taylor 1915, 1920). Bateman (1947) subsequently

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Communicated by P. M. A. Tigerstedt

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adapted the theories for pollen dispersal. Until this time pollen dispersal had been assumed to be normal. Bateman clearly demonstrated the leptokurtotic nature of dispersal and suggested equations for pollen dispersal in various situations (Bateman 1947; 1950). Since then there has been little attempt to verify, or challenge, these equations. Subsequent pollen-flow studies have tended to be descriptive with dispersal equations confined to empirical fits of particular and often small datasets.

In the experiment reported here sticky traps were used to determine the extent of pollen dispersal from a *Lolium perenne* source to a distance of 80 m. Three different orientations of traps were used and exposed at four times during anthesis. Bateman's equations were tested against the twelve datasets produced as the first step in searching for theoretical descriptions for predicting dispersal from transgenic forage grasses.

Materials and methods

The site

The experiment was conducted on Borth Bog in West Wales, UK (OS grid reference SN915625). This is a nature reserve and free of ryegrass. It faces the Irish Sea to the west. The nearest ryegrass (about 100 m away) is cropped short and heavily trampled by horses and did not flower during the course of our experiments. The risk of contamination from extraneous ryegrass pollen was further reduced by using the variety 'Aurora', which flowers earlier than other local ryegrasses.

Experimental design

Pollen traps were constructed by clipping microscope slides onto posts. Three microscope slides, each with one side smeared with Vaseline, were attached to each post at a height of 1 m from the ground. Each slide was orientated in a different position: 'forward' traps faced the pollen source, 'reverse' traps had their reverse sides towards the source and 'upper' traps had their faces to the sky. The posts were set out in concentric circles with radii of 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60 and 80 m, with as many posts in a circle as metres in the radius. Two posts were also placed in the centre.

Forty plants in 7-inch pots and 300 in 3-inch pots were set out in the centre of the circle, just prior to anthesis (and after the 'blank run', as explained below). Clean slides were clipped to the posts and changed four times during the period of anthesis (21–24 May, 24–27 May, 27–30 May and 30 May–02 June).

Preparation of slides for pollen counting

Pollen deposited on the surface of the slides was stained with aceto carmine and a coverslip then applied. Pollen density was determined by counting the number of pollen grains in a 10 μ × 10 μ area. The area of each sample was governed using a reticulated eyepiece. Forty counts were made from each slide.

Blank run

Traps were placed out for 2 days before the ryegrass plants were brought to the site. The slides were then removed, prepared (as above) and examined under a microscope. Two types of pollen were found. Both were completely distinct from ryegrass pollen.

Model fitting

Genstat (version 5.3; Genstat 5 Committee, 1987) and MLP(version 3.09; GJS Ross 1987) were used for statistical analysis, model and curve fitting.

Dispersal equations

An equation for describing spore deposition (p) at a distance (D) from a point source was first proposed by Gregory (1945):

$$p = \frac{ae^{-bD}}{D} \tag{1}$$

The constant a represents the total amount of pollen liberated into the pollen cloud. The exponential component of decline with distance represents deposition of a proportion b ($b \geq 0$) of the pollen per unit distance. The inverse linear component of decline represents a dilution factor, as the circumference of the circle over which the pollen is dispersed increases linearly with distance.

Bateman (1947) derived methods for analysing data collected under various conditions of wind and turbulence. The effect of turbulence is to decrease proportional deposition and increase dilution:

$$p = \frac{ae^{-bD(1-m)}}{D^{(1+m)}} \tag{2}$$

The dispersal equations are based on Sutton's theories of eddy diffusion in the atmosphere (1932, 1934). Sutton (1932) suggests that parameter m must be between 0.5 and 1 (in the original paper m is twice that of the m in Eq. 2, i.e. between 1 and 2). These theoretical limitations on the value of m indicate an upper limit on the power of the turbulence to influence dispersal and deposition. Bateman (1947) suggested, for pollen dispersal, $m = 0.62$ at a minimum turbulence and $m = 0.88$ at maximum turbulence.

Equation 2 is a special case of the model

$$p = \frac{ae^{-bD^{d}}}{D^{c}} \tag{3}$$

which will be referrred to as the line plus exponential model as it is the sum of a linear and exponential regression of log (p) on log (D).

Procedure for curve fitting

Initial exploratory data analysis was undertaken to determine the error distribution and identify a form of analysis that makes consistent assumptions. A separate set of analyses was then undertaken for each of the 12 (three orientations by four time periods) datasets to the following protocol.

Equation 3 was fitted for each dataset. The goodness-of-fit to the equation was tested against a one-way analysis of variance by distance. Where the residuals from the fit to Eq. 3 are significantly greater than those from the ANOVA, none of these equations can provide a good fit to the data. No further analysis was undertaken in this case.

Where the residuals from the fit to Eq. 3 are not significantly greater than those from the ANOVA, Eq. 2 might also provide a satisfactory fit. This was tested by an F-test comparing the fit to Eq. 2 with that to Eq. 3, which tests the theoretical expectation that parameters c and d are symmetrical about the value 1.

Similarly, if Eq. 2 does not give a satisfactory fit, nor can Eq. 1, so no further analysis was undertaken. Where Eq. 2 did give a satisfactory fit, Eq. 1 was fitted and its fit compared with Eq. 2, testing the need to allow for turbulence with parameter *m*.

Results

As expected pollen deposition declined with distance (Fig. 1). Deposition was initially small, built up to a peak during mid anthesis and declined in late anthesis (Fig. 2).

Most pollen was deposited on forward-facing traps, with smaller amounts onto skywards-facing traps and less again onto the reverse sides of traps (Fig. 2). Nevertheless, the general trend is not constant throughout anthesis, and deposition onto upwards-facing traps was more prominent during early anthesis.

Distribution of residuals

Variances from the three-way ANOVA of pollen counts, for time by orientation by distance, were highly heterogeneous and the residuals non normal (Bartlett's $\chi^2_{155} = 1453$ ($P = 0$), skewness $= -0.158 \pm 0.039$, kurtosis $= 16.17 \pm 0.078$). Variation in pollen counts was greater than that expected for a random Poisson process (Fig. 3) with significant heterogeneity of deviances.

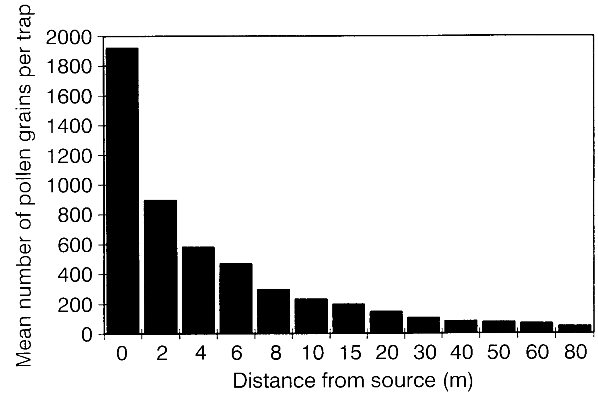

Fig. 1 Pollen deposition with distance

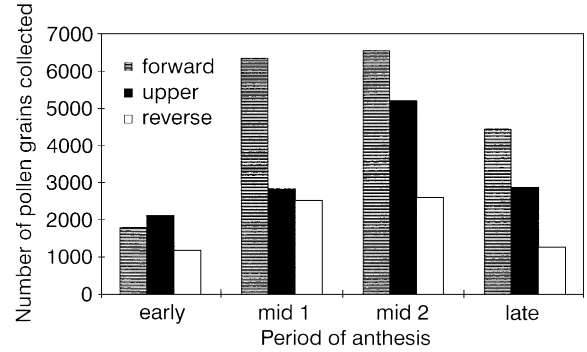

Fig. 2 Total number of pollen grains collected on each trap orientation throughout anthesis

Fig. 3 Log Poisson mean deviances of pollen counts with 95% confidence limits. Expectation of log mean deviance is zero for a random Poisson process

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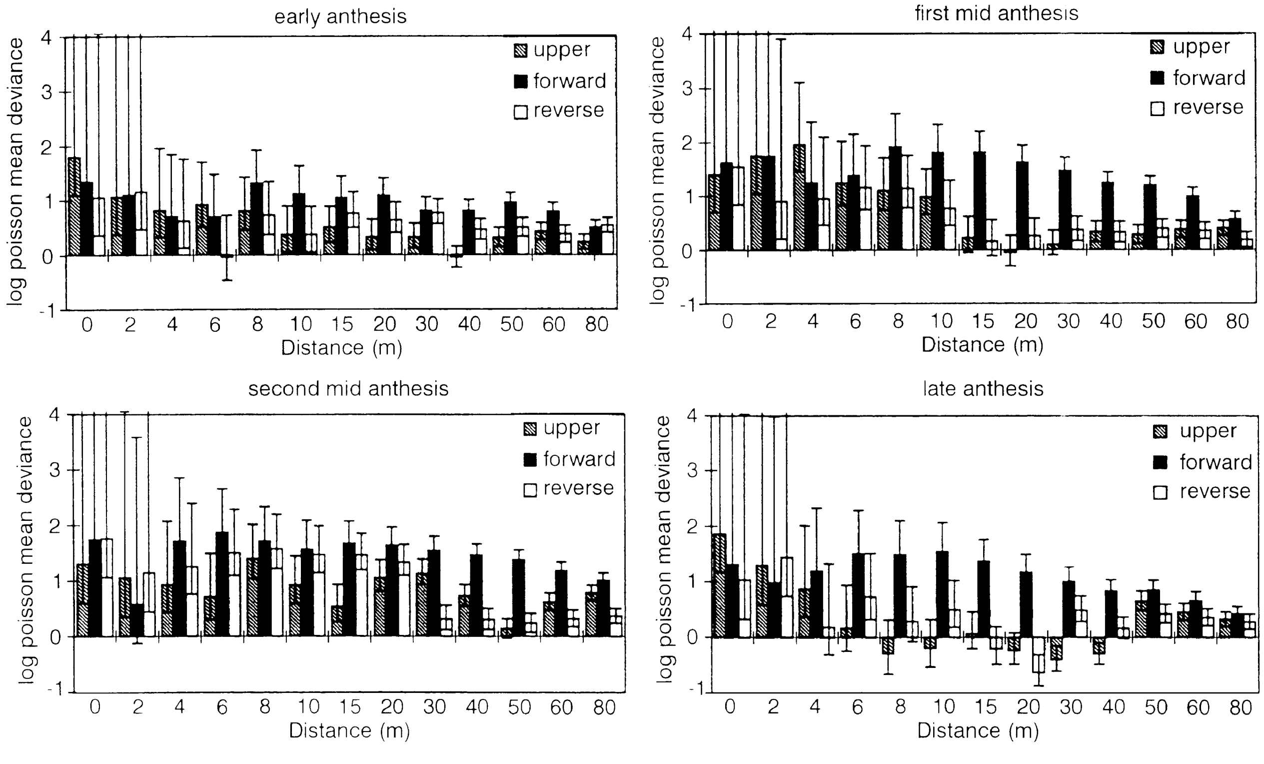

There was a tendency for the highest deviances to occur at middle distances and on forward-facing traps, but trends in deviance with distance varied with both time and orientation (Fig. 3). All subsequent analyses were therefore based on a Poisson distribution weighted to allow for variation in dispersion.

ANOVA

Patterns of variation with distance varied with time and orientation (distance and all interactions with time and orientation being highly significant, Table 1). Preliminary attempts to fit curves modelling the pollen trapped as a function of distance indicated that these patterns of variation with distance differed qualitatively for different times and orientations, requiring different forms of equation, not just different parameter values. Therefore, a separate analysis was applied to each of the 12 combinations of four times by three trap orientations.

Modelling pollen deposition with distance

Curve fitting

The simplest model (Eq. 1) did not provide a satisfactory description of the pattern of variation in pollen deposition with distance. The datasets fall into two categories in this respect.

For 10 of the 12 sets, including all upwards-, 3 reverse- and 3 forward-facing traps, there were significant deviations from an acceptable fit (Table 2).

For the remaining 2 datasets, i.e. from forward-facing traps during early anthesis and reverse-facing traps during second mid anthesis, the fit was statistically acceptable (Table 2) but not biologically acceptable, as the estimates of the coefficient b were negative (Table 3). Following the interpretation of b given above, this implies that the pollen deposited is a "negative proportion" of the pollen cloud, which is nonsensical. Furthermore, when the b coefficient is negative the fitted curve displays a minimum, such that there is a "turning point" – a distance beyond which the fitted value for pollen deposition increases (Table 3). This is implausible as a general response.

Table 1 Weighted Poisson ANOVA of the effect of time, trap orientation and distance on the number of pollen grains deposited on traps

Source of variation	d*f*	Mean deviance	*F*	*P*
Time	3	8 661	195.4	< 0.001
Orientation	2	40 784	919.9	< 0.001
Distance	12	28 273	637.7	< 0.001
Time × orientation	6	2 332	52.61	< 0.001
Distance × time	36	694.2	15.66	< 0.001
Distance × orientation	24	447	10.08	< 0.001
Time × orientation × distance	72	137.2	3.095	< 0.001
Residual	3768	44.33		

For the forward-facing traps during early anthesis the estimated turning point lies beyond the most distant traps used in the experiment (Table 3), and the observed data provide no evidence of a real turning point (Fig. 4). Even for the reverse-facing traps at

Table 2 F-tests of the adequacy of Eqs. 1, 2 and 3 in describing variation in pollen trapped with distance from the source. Tests for Eqs. 1 and 2 are not given where the equation they are tested against does itself not provide an adequate description. Values in bold indicate the simplest models giving a statistically acceptable fit

Regression equation to which goodness-of-fit test applies:		1	2	3
Regression equation or ANOVA whose residual was used to test goodness-of-fit:		2	3	ANOVA
Degrees of freedom for *F*-test:		1, 324	1, 325	9, 314
Trap orientation	Time interval			
Forward	Early	$F = 3.23$ $P > 0.05$	$F = 2.02$ $P > 0.05$	$F = 1.56$ $P > 0.05$
Forward	Mid 1		$F = 2.81$ $P > 0.05$	$F = 0.67$ $P > 0.05$
Forward	Mid 2		$F = 0.08$ $P > 0.05$	$F = 0.35$ $P > 0.05$
Forward	Late		$F = 0.87$ $P > 0.05$	$F = 0.97$ $P > 0.05$
Upwards	Early			$F = 13.26$ $P < 0.001$
Upwards	Mid 1			$F = 7.34$ $P < 0.01$
Upwards	Mid 2			$F = 2.56$ $P < 0.01$
Upwards	Late			$F = 8.94$ $P < 0.01$
Reverse	Early			$F = 25.50$ $P < 0.001$
Reverse	Mid 1			$F = 6.07$ $P < 0.025$
Reverse	Mid 2	$F = 3.14$ $P > 0.05$	$F = 1.11$ $P > 0.05$	$F = 1.64$ $P > 0.05$
Reverse	Late			$F = 3.74$ $P < 0.001$

Table 3 Summary of curve fits for the four datasets for which Eq. 1 gives a statistically acceptable fit. The 'turning point' represents the distance beyond which pollen deposition is predicted to increase

Dataset details	Parameter estimates		Turning point (m)	Percentage of variance accounted for
	a	*b*		
Early, forward	110.60	– 0.007	133.50	52.4
Mid 2, reverse	52.63	– 0.018	55.69	34.8

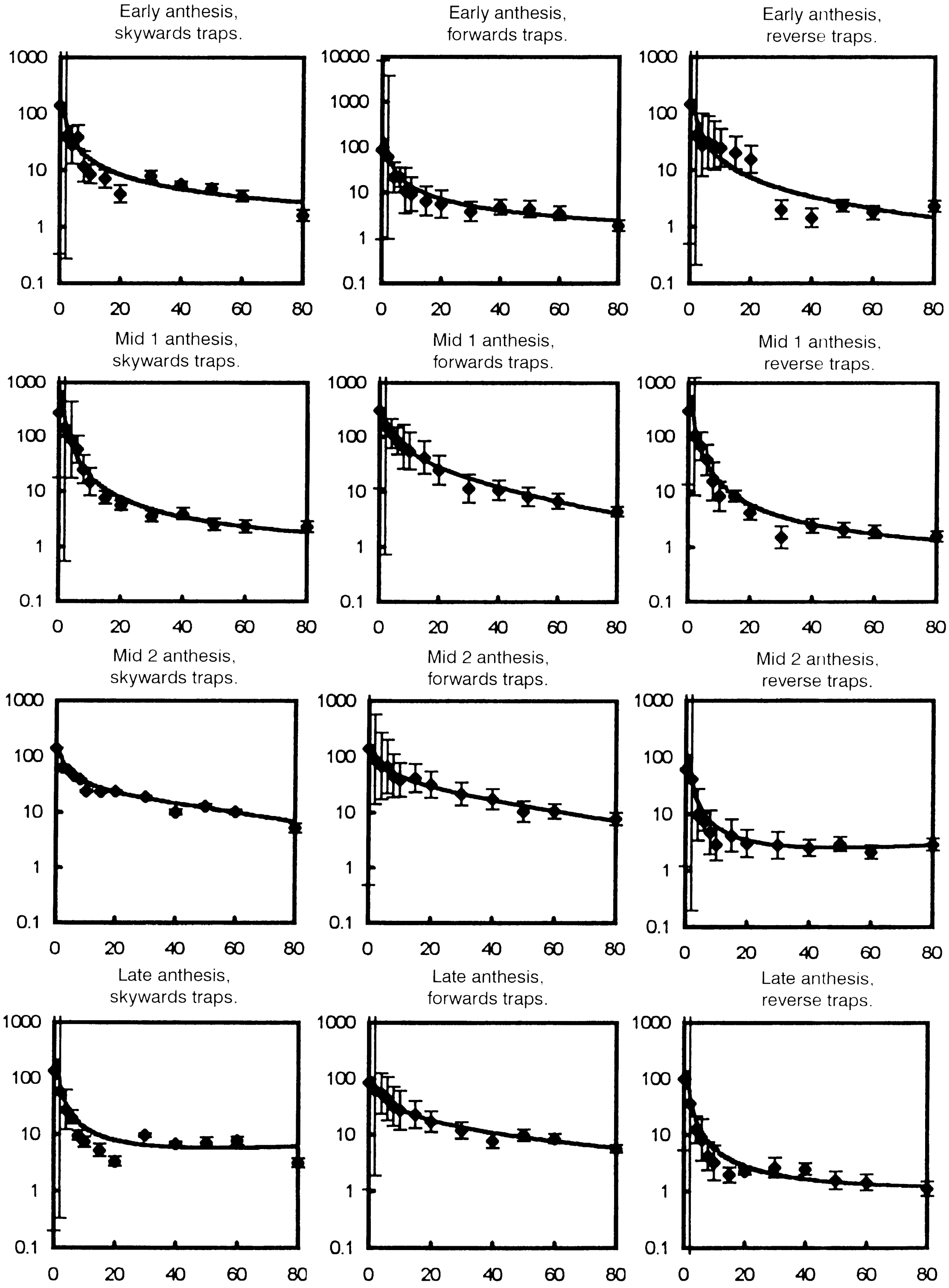
Early anthesis, skywards traps.
Early anthesis, forwards traps.
Early anthesis, reverse traps.
Mid 1 anthesis, skywards traps.
Mid 1 anthesis, forwards traps.
Mid 1 anthesis, reverse traps.
Mid 2 anthesis, skywards traps.
Mid 2 anthesis, forwards traps.
Mid 2 anthesis, reverse traps.
Late anthesis, skywards traps.
Late anthesis, forwards traps.
Late anthesis, reverse traps.

Fig. 4 Pollen count (*y axis*, log scale) versus distance from source (metres). Equation 3 fits five datasets (*solid lines*) but not the others (*dashed lines*). *Error bars* show 95% confidence limits of the observed means

second mid anthesis, where the estimated turning point of 56 m lies within the experimental range of distances, the evidence for a real turning point is weak with no significant increase in pollen deposition between 56 m and 80 m (Fig. 4).

It is more likely that the turning point is not real. Instead, it appears that the initial decline in pollen deposition is steeper than can be modelled by Eq. 1, and that the best fit to such a steep initial slope is obtained by allowing a spurious turning point. The

Table 4 Summary of curve fits for the five datasets for which Eq. 2 gives a statistically acceptable fit

Datasets	Parameter estimates			Turning point (m)	Percentage of variance accounted for
	a	*b*	*m*		
Forward, early	94.21	0.00011	– 0.15		52.7
Forward, mid 1	317.5	0.005	– 0.27		84.5
Forward, mid 2	159.60	0.001	– 0.47		29.4
Forward, late	111.70	0.00083	– 0.41		43.7
Reverse, mid 2	64.28	– 0.207	0.392	52.09	35.2

Table 5 Summary of curve fits for the five datasets for which Eq. 3 gives a statistically acceptable fit

Datasets	Parameter estimates				Turning point (m)	Percentage of variance accounted for
	a	*b*	*c*	*d*		
Forward, early	92.87	– 0.455	1.227	0.305	15906	52.9
Forward, mid 1	317.9	0.052	0.6319	0.784		84.6
Forward, mid 2	157.6	0.002	0.5207	1.387		29.2
Forward, late	105.8	0.0028	0.5033	0.732		43.7
Reverse, mid 2	67.39	– 0.456	1.683	0.508	49.81	35.2

regression equations account for only about a third to a half of the variation (Table 3), and the error variation is high (Figs. 3 and 4). It is concluded that the fits appear statistically acceptable only because the high error variance generates a weak test and not because the fit is good.

Dilution and deposition with turbulence

Allowing for turbulence provided a statistically acceptable fit to 3 more datasets besides those 2 for which the simpler equation was statistically acceptable (Table 2). For all the datasets from forward-facing traps *b* is positive and there is no ‘turning point’ (Table 4). There is a negative *b* for the data from reverse-facing traps exposed during second mid anthesis. The turning point is similar to that found for Eq. 1 as discussed above. For the fits to data from forward-facing traps *m* is negative. This is contrary to Sutton-s predictions that *m* lies between 0.5 and 1 and brings into doubt the biological interpretation of the parameter values.

Line plus exponential model

The line plus exponential model (Eq. 3) fitted no further datasets (Table 2). In 2 cases a fitted equation 3 is biologically implausible since it predicts an eventual increase in pollen deposition with distance (Table 5). The results of the ‘blank run’ suggest that this is not due to a background pollen cloud. It is possibly due to the effects of wind gusts and turbulence during the experiment, and to the fact no account is taken of variation in dispersal and deposition with wind direction.

Figure 4 shows that there is considerable variation in pollen counts between traps at the same distance. This is particularly so for forward-facing traps and may account for the statistically acceptable fit of the line plus exponential (Eq. 3) to these datasets.

A feature of data from skywards-facing traps is of a rapid fall in pollen deposition over a short distance (about 15–20 m) followed by erratic deposition over greater distances, a pattern not well-fitted by the line plus exponential equation (Fig. 4). This pattern is possibly due to deposition from pollen clouds that are taken high into the atmosphere and settle out when the wind drops.

Discussion

The most striking feature recurring throughout this analysis is the high variability of pollen deposition. When regarded as a Poisson process pollen deposition is highly overdispersed (*sensu* Ross 1987) even after allowing for variation in mean deposition with time, trap orientation and distance (Fig. 3). The variance of pollen deposition varies with time, orientation and distance. The pattern of pollen deposition with distance shows highly significant variation with time and trap orientation. Particularly notable is the failure to provide a satisfactory description of pollen dispersal based on classical theory. Even the most generalized form of equation failed to provide a statistically acceptable fit to 7 of the 12 datasets. When the fit was statistically acceptable, statistical acceptability was attributable more to high error variances providing weak significance tests rather than to genuinely good fits, and parameter values obtained from these fits were generally biologically implausible.

We therefore conclude first that classical pollen dispersal theory, and hence the equations presented here, can not provide a satisfactory basis for prediction of pollen flow in relation to risk assessment for the release of transgenic crops; and second that pollen dispersal must be strongly influenced by other factors that have not been considered here. In a subsequent paper we consider the influence of wind direction on pollen dispersal.

Acknowledgements The technical assistance of Mr. J. Buckley and Mr. M. Caine is gratefully acknowledged. This work was initiated under a MAFF Open Contract (RG0203).

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