Pollen flow out of greenhouses for wind-pollinated species, in the context of current GM containment regulations in the Netherlands

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DISCLAIMER

All suggestions in this report reflect the personal scientific view of the authors, which may become subject to (scientific) debate. We do not claim to obtain completeness in terms of studies and data cited. Furthermore, all quantitative values are measured and valuable only for this specific greenhouse in the summer of 2008 for those two cultivars. Our results are presumed to be indicative for the relative differences on pollen escape with and without insect mesh elsewhere under similar conditions, but they do not predict quantitative values of escape elsewhere.

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INTRODUCTION

Incidental formation of hybrids between cultivated plants and their wild relatives is well-documented for areas where they co-occur (Ellstrand 2003, Hails & Morley 2005, Chapman & Burke 2006). While crop-wild hybridization has most likely been going on for ages (Dale 1999), the effect of gene escape from cultivated plants into wild plant populations has become cause for public concern, connected to the construction of transgenic crops. Transgenes built into the crop through genetic modification might become introgressed into the genomes of nearby wild relatives. If these newly introduced genes confer a competitive advantage of hybrids over wild relatives, the gene may spread and in the extreme case this might result in invasiveness that can not be easily reversed (Pilson & Prendeville 2004, Chapman & Burke 2006). This has resulted in increased attention for methods and policies that would circumvent the initial formation of hybrids (den Nijs et al. 2004, Snow et al. 2005), and legislation to regulate the use of transgenic plants.

One potential route of gene flow from crops to wild relatives is by outcrossing after pollen escape from a greenhouse. In order to prevent this from happening, the Dutch government has implemented the "inhullingsplicht" for transgenic wind pollinated species with compatible relatives (crops or wild species) in the Netherlands: such plants may only be grown if inflorescences are covered by a fine, pollen-proof mesh, a time-consuming and expensive measure. The use of insect-proof screens is a common practice in greenhouses in many countries. These screens act as barriers that prevent insects entering the greenhouse, thus avoiding crop damage by herbivory and/or pathogen transfer (Teitel 2007). Potentially, these screens could also serve as a barrier restraining pollen escape from the greenhouse, although the mesh size used is often much larger than pollen size. Reduced wind speeds near the netting could provide a barrier for at least part of the pollen (Teitel 2007), and also air flows and turbulence in the greenhouse might be affected. As yet the empirical data on the actual pollen flow from greenhouses is very sparse. Exceptions are two related studies measuring the escape of Maize pollen from an open pipe-frame greenhouse covered with 1mm mesh on all sides (Watanabe et al. 2006b, 2006c). Watanabe et al. 2006b estimated the outcrossing rates in 6000 trap plants (white Maize variety) directly surrounding a pollen source of 200 yellow Maize plants in a duplex 1 mm mesh covered pipe frame, adjacent to 200 uncovered black Maize plants in the open. Most outcrossing events on the trap plants occurred within 5m of the central pollen donor area, and the mesh reduced the number of outcrossed kernels from 594 for black to 139 for yellow pollen donors, indicating that the mesh indeed reduced gene flow. However, it is difficult to translate these results to the situation in the Netherlands, where this type of mesh covered pipe frames is uncommon and predominantly the "Venlo" type greenhouses are used. This instigated the COGEM to commission a study aiming to gather relevant data on pollen escape from such greenhouses.

Our research specifically asks how the presence of insect netting affects pollen escaping from greenhouses. We tested this using two monocots, *Lolium multiflorum* and *Zea mays*. We experimented with and without insect mesh in the roof windows under a normal practice of opening the windows under warm conditions. We also artificially increased pollen concentrations inside the greenhouse, by generating extra upward air flow with large fans, to simulate a high pollen concentration in the greenhouse. Pollen was trapped both inside and directly outside the windows of the greenhouse using Rotorod® samplers. Finally, we investigated the pollen concentration at different heights inside the greenhouse.

MATERIAL AND METHODS

Greenhouse – The experiments were performed in and outside a 38 m^2 double-span greenhouse compartment. The compartment is located in the North-Eastern corner of the greenhouse complex of the University of Amsterdam at Science Park Amsterdam (N52° 21.29'; E4° 57.52'). The greenhouse is of the Venlo-type with a gutter height of 4.50 m. The eight windows have an angle of 30° from horizontal and are situated at 4.80 – 5.30 meters height (Fig. 1). Total greenhouse volume is around 175 m^3. Individual window surface area is 2.5 m^2, for eight windows per compartment this leads to 20 m^2. The windows are normally covered by insect-proof netting with a mesh size of 400 x 450 µm (0.4 x 0.45 mm), providing a 30% reduction in ventilation relative to open windows, according to the manufacturer's specifications.

Model species – Model species were the wind pollinated species *Lolium multiflorum* (Westerwolds Ryegrass/Raaigras) and *Zea mays* (Maize), further refered to as *"Lolium*" and "Maize". The *Lolium* was collected as sod cuts from cultivated fields from Barenbrug Holland BV at 08-07-2008 in Leens, Groningen (N53° 21'; E6° 24'). The greenhouse flowering was the second flowering period after a seed and biomass field harvest in mid-June. The sweet Maize was commercially obtained from Gebr. Eveleens, Aalsmeer (catalogue nr 522). Two subsequent series of plants were used resulting in two distinct flowering periods (Appendix 1).

The two species differ in pollen size and therefore also in weight. Maize has relatively large pollen grains (mean Ø 89 µm; Beug 2004), whereas *Lolium* has relatively small pollen (mean Ø 32 µm; Beug 2004). Pollen size is an order of magnitude smaller than the mesh (see above). The *Lolium* sods were grown on 90 cm high tables, the surface totaling 12 m^2 (Fig. 1), and they flowered continuously during a nine-week period. The resulting pollen release height was 1.20-1.50 m. Surrounding the tables, four rows totaling 120 Maize plants were placed on the floor (Fig. 1) resulting to a pollen release height of 2.50-3.00 m. Maize plant flowering peaked around mid July.

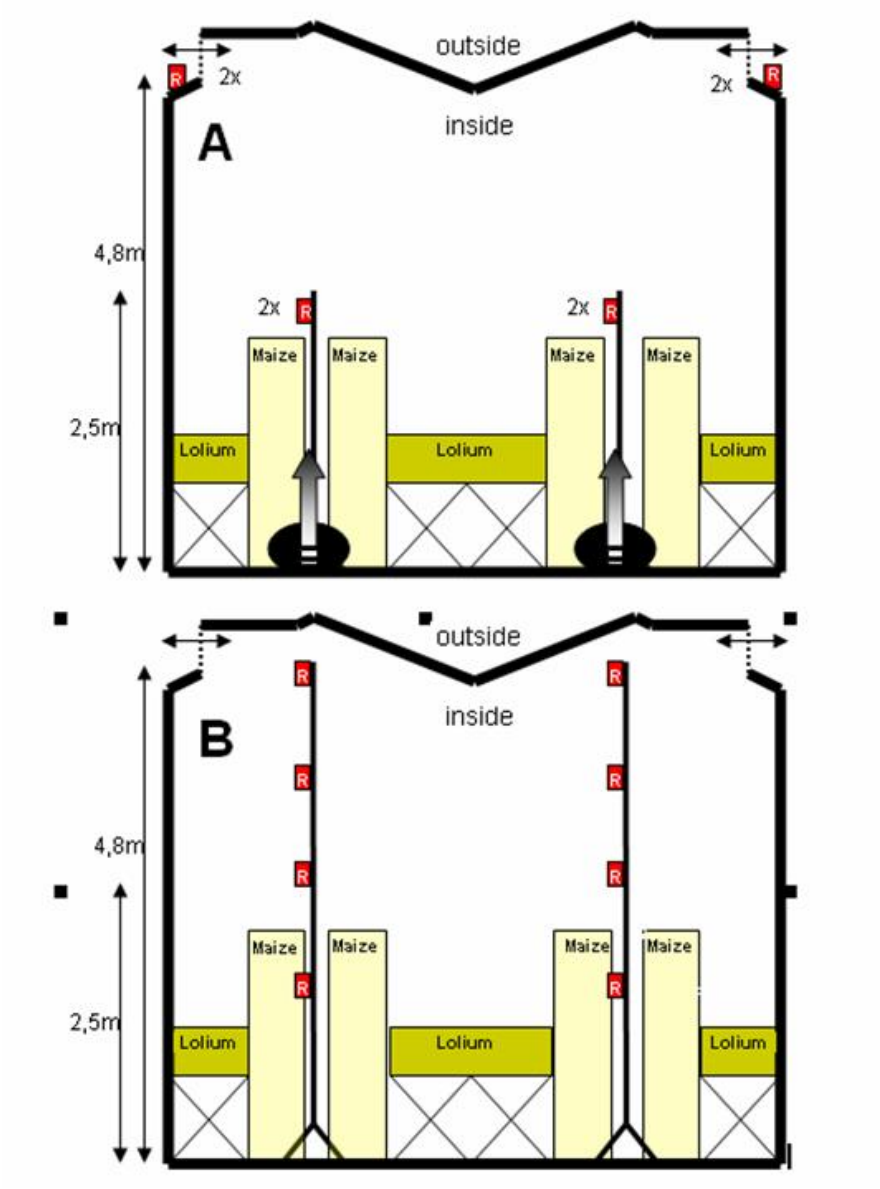

Figure 1. Side view of the greenhouse compartment. Setup (A) was used to measure pollen escape; setup (B) to measure vertical pollen dynamics inside the greenhouse. Rotorod samplers indicated by "R". The large shaded arrows indicate the direction of the artificial airflow.

Pollen traps – In order to measure the pollen concentration, Rotorod® samplers Model 20 (Multidata LLC) were used. Rotorods are a reliable and commonly used trapping system for airborne particles such as pollen. A rotating arm traps particles onto two rapidly spinning polystyrene rods per sampler. These samplers have a wide range of applications, from healthcare related studies (Hugg et al. 2007, Hugg & Rantio-Lehtimaki 2007) to studies on the dispersal dynamics of plant species (Spijkerboer et al. 2002, Aylor 2005, Aylor et al. 2006. Van Hout et al. 2008). Four Rotorods were placed inside the greenhouse at four different positions, just above the Maize inflorescences at approx. 2.5 meters height (Fig. 1A). Outside the greenhouse, four Rotorods were attached to the roof just in front of the windows (Fig. 1A). The Rotorod plastic collector rods are treated with a thin layer of silicone grease, catching all small airborne particles they encounter. For practical reasons the rods were running parallel to the airflow both inside (upwards)

and outside (facing the window) the greenhouse. Each collector rod has an effective area of 1.52 x 22 mm and runs at exactly 2400 RPM. Consequently, a total volume of 1.3 m^3 is sampled per hour per collector rod according to the Rotorod manual (Multidata 2002).

Sampling scheme – Samples were taken during the whole flowering period of Maize and *Lolium*, which was approximately from the July 23 to September 17. Sampling was restricted to non-rainy days, since windows are closed during the rain and the electrical equipment could not be used outside. Several runs were taken between 9 a.m. and 5 p.m. on sampling days, with a maximum of five runs per day. A single sampling run consisted of one hour running all eight Rotorods simultaneously inside and outside the greenhouse. Daily runs included three types of measurements:

- Non-disturbed ('normal") conditions with natural air movements within the greenhouse and warm air vented through the roof windows.
- A "worst-case scenario" with artificial airflow. Four vertically placed living room fans (Proline Ø 40 cm, 1800 Watt) blowing upwards towards the windows, were used to increase vertical airflow and turbulence.
- Outside background sampling. Daily background samples were taken (with exception of the first week for logistic reasons) to estimate the background pollen concentration of *Poaceae* that can not be distinguished from *Lolium*. Greenhouse windows were closed during these measurements.

The presence of netting was changed every 5-6 days (Appendix 1) to obtain data on the impact of insect netting on pollen escape from the greenhouse.

Sample treatment and counting – Collector rods were analyzed with light microscopy (Leica; enlargement 400x) after being placed in a stage adapter and stained with Calberla's stain (Multidata 2002; Benton Franklin Health District 2009). All putative *Lolium* and Maize pollen grains were counted on the full collector rod area of 1.52 x 22 mm, corresponding to 1.3 m^3 of air sampled per hour. Concentrations in this report are expressed as pollen capture per m^3 per hour.

Pollen grain distribution in the greenhouse – To assess the change in concentration with increasing distance above the floor two poles were placed on opposite sides of the greenhouse compartment, each equipped with four Rotorods at 2, 3, 4 and 5 meters above the floor (Fig. 1B). Samples were taken both with windows open and closed and repeated this six times between August 27 and September 4, using all eight Rotorods. Note that therefore no escape data are available in this period (period 2: Appendix 1).

Statistical analysis – The data was analyses using a generalized linear model approach, to test how presence of insect netting had altered the amount of pollen escaping from the greenhouse. The statistical model fitted the pollen count outside (PCO) in each of the four outside Rotorods, depending on the average pollen counts inside (PCI) at 2.5 m

height and the presence of insect nets, while correcting for the background pollen count (BPC) outside measured on the same day. The generalized model used was:

$PCO_i \leftarrow \alpha\ PCI + \beta_i\ [Rotorod_i * PCI] + \gamma\ [Insect\ Net * PCI] + \delta\ BPC + error$

Since the number of pollen captured for Lolium was high the standard ANOVA method could be employed, i.e. use the identity link (so "="for "←") and minimize the least-squares error to fit the statistical model to the data. All statistics were done with untransformed counts. Therefore, α can be directly interpreted as the average ratio (slope) of the outside to inside pollen count, the β_i parameters allow for differences in this ratio among the four outside Rotorods at different positions, the γ parameter estimates the difference in the ratio due to the insect mesh, and δ corrects for potential effects of background pollen from other sources and determines the intercept of the model. Type III Sums of Squares were used, i.e. statistical significance of effects was evaluated after correcting for all other effects. Count data and predicted values were plotted on $^{10}\log(x+1)$ axes for visual reasons (but note that predicted values not necessarily form a linear trend on a log-log scale for the model employed). This approach has the advantages that there is no need to calculate any ratios or averages (except for PCI) prior or after the statistical analysis: parameter estimates and their standard errors are directly given by the statistical program.

The Maize pollen was much rarer, and a Poisson-log link function for "←" was used, and minimizing errors as derived the difference between observed and predicted counts given the Poisson distribution. The latter model is appropriate for counts containing many zero values. For these statistical tests procedures UNIANOVA and GENLIN of SPSS v17.0 were used (SPSS Inc., Chicago, IL).

RESULTS

Descriptive statistics are provided in Appendix 2 and are not discussed here; the number of rods counted, untransformed average pollen counts and standard deviations are reported.

BACKGROUND POLLEN COUNTS

The average background pollen capture of Poaceae pollen was 2.2 per m^3 per hour, measured outside the greenhouse with windows closed. No background Maize pollen grains were found during these measurements, not surprisingly as there were no Maize fields in the vicinity of Science Park Amsterdam within 2 km distance. Pollen grains from the *Lolium* species that we used (Westerwolds Raaigras) are not visually distinguishable from pollen grains from most other Poaceae species. The average daily background

pollen count (BPC) was used in the model, thus correcting for the presence of *Lolium* and other Poaceae in outside measurements.

LOLIUM POLLEN ESCAPE

Normal conditions – Fig. 2A shows the outside pollen counts, plotted against the average pollen count inside the greenhouse at 2.5m height. The graph shows a lot of scatter, both for the counts in- and outside the greenhouse. It is clear that there was a relationship between in- and outside pollen count, which was confirmed by the statistical analysis (Table 1). The total statistical model explained 71.2 of the variation in outside counts. Effect of inside pollen count (PCI) was highly significant, the effect of the covariate background Poaceae pollen was according to the expectation (estimated parameter δ close to one, 0.957), and there also was a consistent difference between the four Rotorods. The most important factor, the presence of insect netting, was also statistically significant (Table 1). Surprisingly, relatively *more* pollen was found outside the greenhouse in the *presence* of het insect nets. The estimated ratio of outside to inside pollen concentration was 3.0 ± 0.4 (mean ± S.E.) per cent without insect nets and 13.8 ± 1.5 per cent with insect nets in place. It is not clear what caused this difference; note that the low ratio without netting is mostly due to the only two runs with an inside pollen count over 1000, coupled to - relatively - low outside counts. Possibly a chance combination of a high flowering incidence and relatively cool weather conditions (for the entire series with/without nets: 16.8–24.8°C, average 20.6°C, observations without nets with inside counts >1000: 17.2 and 18.4°C) may have played a role here. It is clear, however, that there is no statistical support for a *reduction* of the escape due to the insect nets, as the observed effect is in the opposite direction (Fig. 3A).

Table 1. Analysis of *Lolium* pollen escape using a Type III General Linear Model ANOVA. Focus is on the interaction between Insect mesh presence (yes/no) * inside pollen concentration (PCI). Two conditions are separately tested: normal airflow and with artificial airflow added (fans on).

Source of variation	Normal Conditions			Artificial airflow added		
	df	*MS*	*F*	df	*MS*	*F*
Pollen concentration inside the greenhouse (PCI)	1	57082	191.53 ***	1	482799	42.26 ***
Background pollen concentration (BPC)	1	2019	6.77 *	1	1962	0.17
Rotorod position * PCI	3	9268	31.10 ***	3	26956	2.36 ***
Insect mesh presence * PCI	1	24605	82.56 ***	1	8791	0.77
Residual error	144	298		104	11425	

* P < 0.05; ** P < 0.01; *** P<0.001

Increased airflow – Fig. 2B shows the outside pollen counts when fans were on and airflow was increased in the greenhouse. Indeed both the inside and outside counts were higher than under the normal conditions. The total statistical model explained 62.2 of the variation in outside counts. Effects of average inside pollen count (PCI) was again highly significant, whereas the effect of the covariate background Poaceae pollen was not significant (estimated parameter δ again close to one, 1.259), and there also was a consistent difference in counts for the four outside Rotorods. The presence of insect netting had no statistically significant effect (Table 1). The estimated ratio of outside to inside pollen concentration was of 13.0 ± 4.3 per cent without nets and 16.9 ± 1.4 per cent with nets (marginal means over the four outside Rotorods). However, as already mentioned, the difference due to the mesh nets was not statistically significant, which can also be seen from the graph of the predicted values (Fig. 3B).

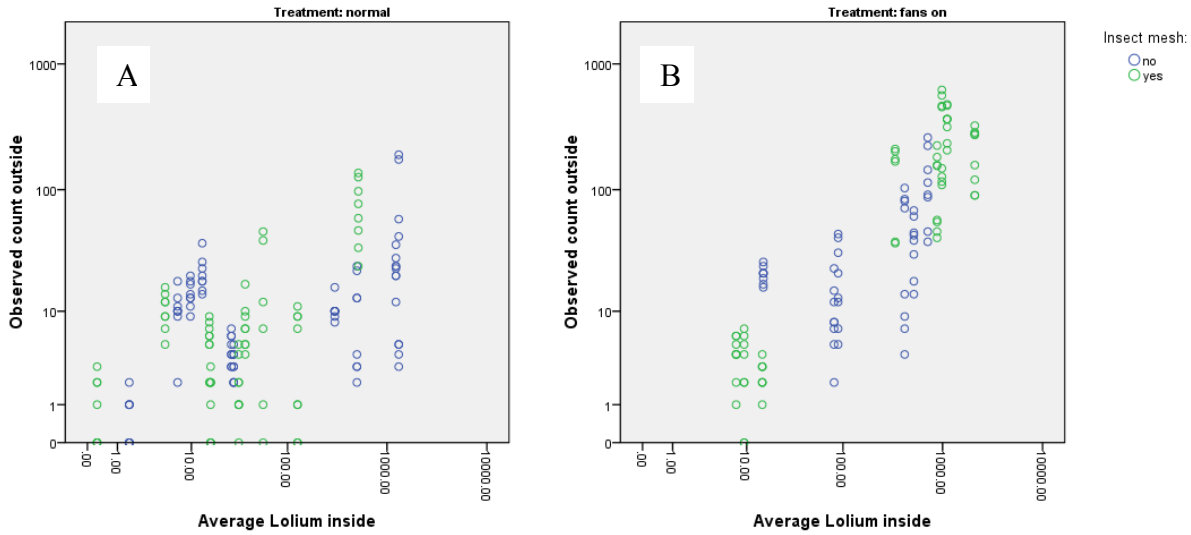

Figure 2. Average Lolium pollen capture outside the greenhouse, with and without insect netting in front of the windows, plotted against the average pollen count within the greenhouse, at 2.5m height. Each point represents the pollen count of one of the four outside Rotorods, plotted against the average inside count measured at the same time. Normal (A) and artificial airflow conditions (B). Note the data are plotted on a logarithmic scale for visual reasons.

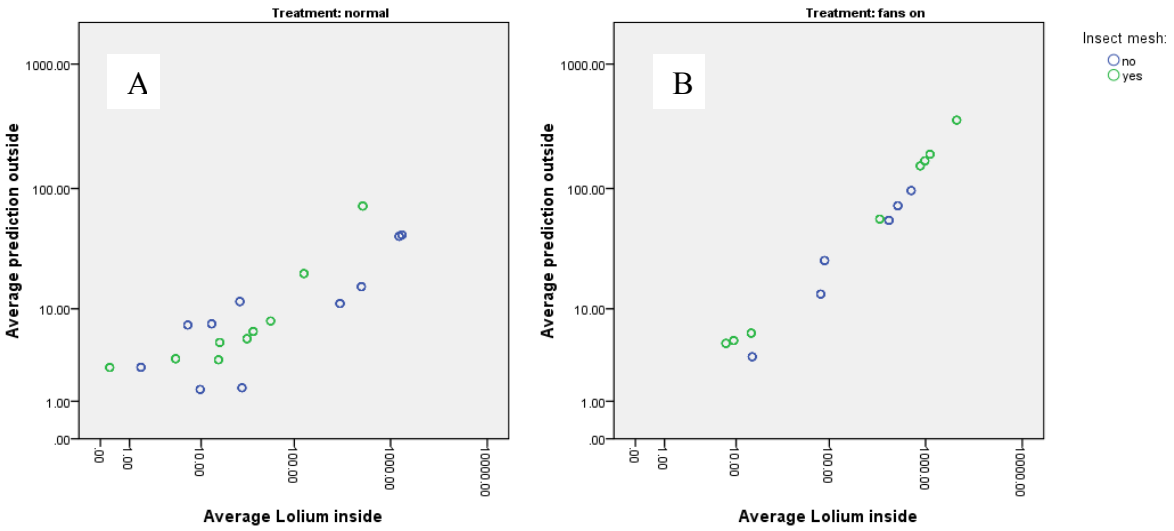

Figure 3. Predictions of the Lolium pollen capture outside the greenhouse (averaged over the four outside Rotorods), with and without insect netting in front of the windows, depending on the average pollen count within the greenhouse, at 2.5m height. Normal (A) and artificial airflow conditions (B). Note the data are plotted on a logarithmic scale for visual reasons.

MAIZE POLLEN ESCAPE

Escape under normal conditions – Under normal conditions the amount of airborne pollen at 2.5m height was extremely low, and Maize pollen was only sporadically found outside (Fig. 4A). The reasons for the low numbers are the high weight of Maize pollen, but also the production of Maize pollen was low during part of the experimental periods (see below). No statistical significant effects were found (Table 2), but obviously the power of the analysis was low, as in total only 10 pollen grains were found outside and 55 inside (n=72 rods), resulting in average outside to inside ratio of 21.5 ± 8.0 per cent without nets and 14.1 ± 6.3 per cent with nets.

Table 2. Analysis of Maize pollen escape, using a Generalized Linear Model with Poisson-log link and Poisson errors. Focus is on the interaction between Insect mesh presence (yes/no) * inside pollen concentration (PCI). Two conditions are separately tested: normal airflow and with artificial airflow added (fans on).

Source of variation	Normal conditions		Artificial airflow added	
	df	*Wald Chi-square*	df	*Wald Chi-square*
Pollen concentration inside greenhouse (PCI)	1	2.18	1	9.16**
Rotorod position * PCI	3	1.19	3	17.8***
Insect mesh presence * PCI	1	0.65	1	2.62
Residual (deviance)	66	38.0	42	82.1

* P < 0.05; ** P < 0.01; *** P<0.001

Escape under artificial airflow conditions – Artificial airflow was effective in increasing the number of airborne Maize pollen (Fig. 4B). The statistical analyses revealed that the outside count increased significantly with inside pollen count at 2.5m, with estimates of 6.3 ± 2.5 and 4.1 ± 1.0 per cent for the ratio outside to inside without and with nets, respectively. Also a significant effect of Rotorod position was detected, but no statistically significant effect of the presence or absence of the insect nets, as can be seen from the standard errors.

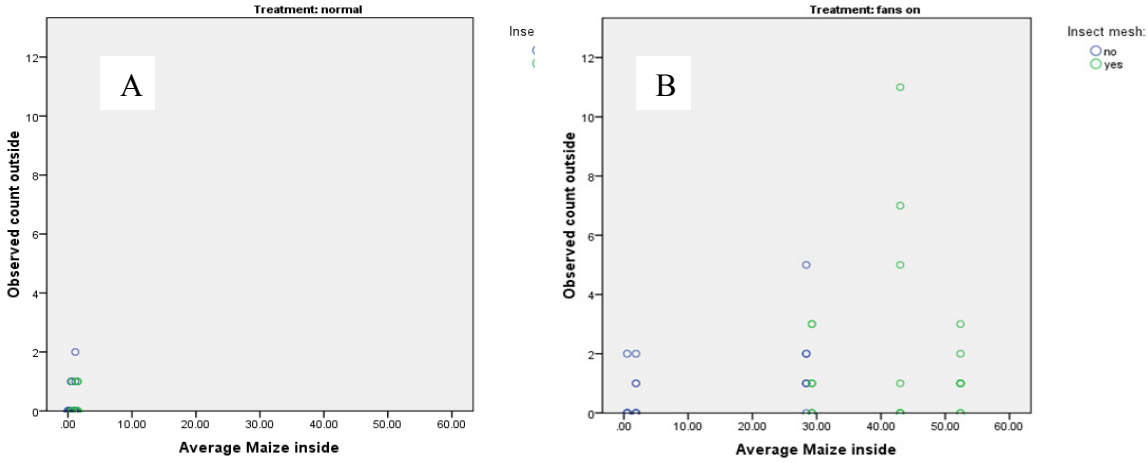

Figure 4. Average Maize pollen capture outside the greenhouse, with and without insect netting in front of the windows, plotted against the average pollen count within the greenhouse, at 2.5m height. Each point represents the pollen count of one of the four outside Rotorods, plotted against the average inside count measured at the same time. Normal (A) and artificial airflow conditions (B). Note the axes are linear.

POLLEN DYNAMICS INSIDE THE GREENHOUSE

In order to assess the dynamics inside the greenhouse we did a series of measurements focusing on the difference in airborne pollen concentration with closed vs. open windows. Both Maize and *Lolium* pollen concentrations were much higher when the windows were open (Fig. 5). Furthermore, the heavier Maize pollen grains remained closer to the ground, with a pronounced decrease in pollen concentration from 2 to 3 meters high. The concentrations of the lighter *Lolium* pollen grains show a much more gradual, though still substantial decrease with height. The steep decrease in pollen concentration in Maize is in agreement with the low escape rates observed. This also indicates that turbulence is very important, presumably especially so for the heavier pollen grains of Maize.

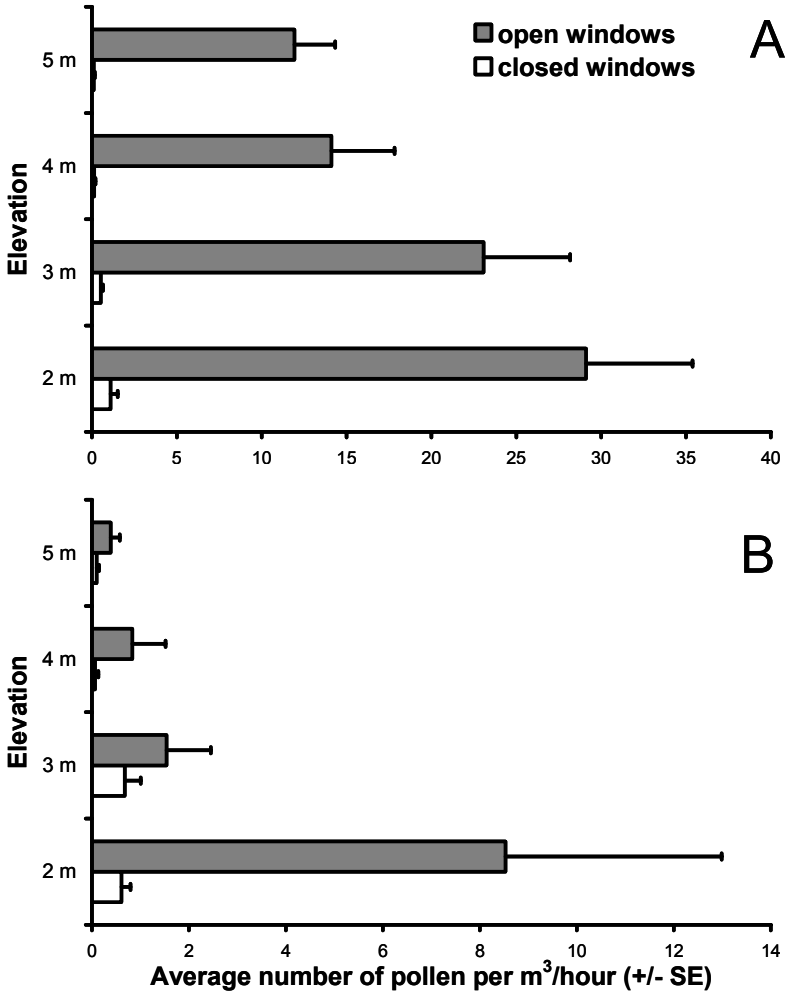

Figure 5. Pollen counts inside the greenhouse. Pollen capture at four different heights in the greenhouse, with the roof windows either opened (with insect nets in place) or closed. A) Lolium; B) Maize

DISCUSSION

The estimated pollen concentration inside the greenhouse at 2.5m height as well as outside varied considerably from day to day for both *Lolium multiflorum* and *Zea mays*.. Also, the range of values found for the ratio of *Lolium* pollen outside relative to that inside varied considerably. Values up to 13 percent under normal conditions, and 17 percent under enhanced airflow indicated that there is a considerable escape of the small-sized airborne *Lolium* pollen through the roof windows. We found no indication that pollen escape was in any way reduced by insect netting, for either *Lolium multiflorum* or *Zea mays*. If anything, under artificial air movement more pollen grains of *Lolium* escaped with insect netting in place than without. No effect of insect nets was found with the fans on, it seems also possible that it was due to unknown differences, e.g. in altered air flows within the greenhouse. Differences in average wind speed or outside air temperature could not explain the different results (data not shown). The general conclusion is that if there is pollen in the greenhouse a considerable fraction of that

pollen will find its way out of the greenhouse when the roof windows are open, irrespective of the presence of insect netting. Below we will discuss these results in a wider context.

Wind dynamics in the greenhouse – Screening the windows with insect netting affects airflows inside a greenhouse in complex ways (Teitel 2007, Shilo et al. 2004, Majdoubi et al. 2007). Greenhouse screening by insect netting can cause a significant decrease of turbulence (Katsoulas et al. 2006, Kittas et al. 2008) depending on the type of netting. Bartzanas et al. (2004), Dayan et al. (2004) and Kittas & Bartzanas (2007) simulated airflow and temperature patterns under different regimes. Shilo et al. (2004) found that air flows in the lower part of the greenhouse were mostly towards the ground and to the sides, and then upward along the walls; they did not look at pollen flows, but this suggest that a substantial fraction of pollen would end up adhered to plants, soil, pots, and the floor, especially so for the heavy pollen. Indeed, the artificial upward airflow conditions under screened conditions increased the concentration of pollen in our experiment, possibly also older pollen of the previous days. Optimal airflow within greenhouses aims to create homogenous temperature and growth conditions for plants (reviewed in e.g. Critten & Bailey 2002, Teitel 2007). It is possible that smaller mesh sizes would be more efficient in blocking pollen; however, this would also reduces the venting of the greenhouse, and would soon make forced and filtered air management needed (i.e. different and more expensive types of greenhouses).

Differences in the fate of pollen – We found large differences in the in vertical distribution of Maize and *Lolium* pollen, as predicted from the difference in pollen size between the species (Beug 2004). The concentration of Maize pollen within the greenhouse decreases steeply with height, so that the escape rate is reduced. In fact, the bigger Maize pollen grains rarely reached the level of the roof windows, unless turbulence was (artificially) high. The lighter *Lolium* pollen comes much higher and can more readily escape through the roof windows. For a quantitative risk assessment it is therefore important to take pollen grain size into account. Of course, size also affects the fate of pollen after escape (see e.g. Watanabe et al. 2006a, Kuparinnen et al. 2007). In this report we do not assess whether the pollen escape might causes increase the frequency of crop-wild hybridisation. The bulk of the escaping pollen may not reach a compatible wild relative at all, or the pollen may have died before pollination occurs. The absence of Maize pollen in the background measurements indicates that there were not a lot of flowering Maize plants in the neighbourhood at the time of the experiments. Indeed, the phenology of the wild (or crop) plants may or may not overlap with the phenology of the plants in the greenhouse. Species with light pollen and a locally common recipient population like *Lolium* would have a much higher likelihood of hybridizing than e.g. Maize, given that Maize pollen are heavy, hardly escape from greenhouses and are carried less far in the air. For Maize, data on outcrossing rates related to distance between the crop fields and recipient wild populations are available (e.g. reviewed in Hooftman & den

Nijs 2007), as well as on pollen movement through space (Aylor 2005, Aylor et al. 2006). A difference between pollen escaping from greenhouses and from crops and that is that the release height of pollen is higher, which might cause pollen to travel further. Whether this causes a significant alteration of the pollination-distance relationship is unknown.

Methodological limitations – For practical reasons the treatments (mesh/no-mesh) could not be done simultaneously and were performed during several periods of several days. This led to a difference in weather conditions during observations with and without netting, and presumably also to variation in flowering intensity of plants. However, in both treatments there was a considerable range of observed values, and the design allowed us to compare inside and outside counts that were obtained simultaneously (i.e. paired observations). The consistent relationship between in- and outside measurements (as plotted in Fig. 3) confirms that this was a sensible approach, and we do not consider the variation in pollen presence in time as problematic.

As seen in Appendix 1, especially Maize shows a clear peak flowering period in which it releases all of its pollen within a few days. Therefore it was only possible to sample Maize pollen up to ten days after the initiation of the flowering period, limiting the power of the analysis. An alternative approach would have been to use maize plants of different ages, to spread the release of pollen more evenly in time. On the other hand this might reduce the pollen density to low levels, and in our setup there were at least some periods with a high pollen production.

Wind also affected the Rotorod sampler collection efficiency. Although the Rotorod sampler performs better under influence of varying wind speeds than other pollen traps, the maximum change in collection efficiency is still reported at 39% (Frenz 2000), increasing sample error and decreasing statistical power. We captured pollen inside the greenhouse above the canopy and did not measure the total pollen production or release. Therefore, it is not possible from the present data to estimate the proportion of pollen produced that escapes through the roof windows. It is clear that this proportion will be much lower than the relative ratio of pollen concentrations outside to inside at 2.5m height.

RECOMMENDATIONS & CONCLUSIONS

1. **The flow of pollen from greenhouses is relatively high.** The high ratios of the concentration outside to concentration within greenhouse at 2.5m height indicate that a significant proportion of airborne pollen leaves a greenhouse during periods that the greenhouse is vented through opening the roof windows.

2. **Insect netting is not effective in reducing the pollen flow out of a greenhouse.** The addition of insect netting in our experiments did not lower the pollen concentrations measured outside of the greenhouse. Further research would be needed to see if alternative (cheap) pollen barriers are more effective.

3. **The amount of pollen escape from a greenhouse is not relevant during periods where there are no flowering, compatible recipients present in the vicinity of the greenhouse.** Although this issue is not specifically addressed here, quantitative risk assessment of the escape phase is only relevant if the other steps in the formation of crop-wild (or crop-crop) hybrids are feasible. If there are no wild relatives flowering in nature crop-to-wild gene flow is not possible.

4. **The amount of turbulence in the greenhouse is a decisive factor for pollen escape.**
   More (artificial) turbulence increased the concentration of airborne pollen inside the greenhouse and hence also outside. Variation in airflows among greenhouses with different ventilation regimes are expected to lead to different rates of pollen escaping, also depending on pollen sizes. The escape of particles from greenhouses could be further studied using artificial pollen grains, like *Lycopodium* spores (Ø 30 μm), allowing better standardized releases and independence of plant flowering behaviour.

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APPENDICES

Appendix 1

Overview of average daily inside measured pollen concentrations of *Lolium multiflorum* and *Zea mays*, daily temperature, and daily wind speed.

Appendix 2

Overview of experimental data.

Appendix 3

"Inperkingsmaatregelen bij activiteiten met genetische gemodificeerde planten": Copy of manual provided by VROM/Buro GGO (in Dutch)

APPENDIX 1

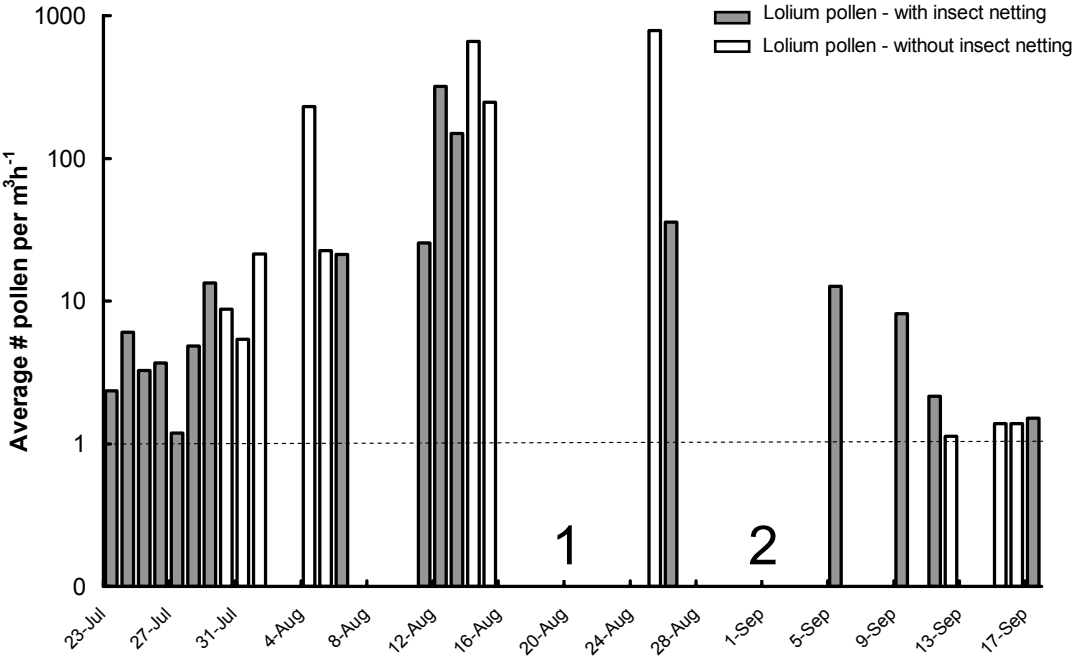

Fig. A1.1. Overview of average measured pollen concentrations inside the greenhouse (# per m² per hour) of *Lolium multiflorum* indicating the intensity of flowering over time. Bars represent days with escape measurements (coupled inside and outside measurements). Different bar colors indicate insect mesh being present in front of 20-m² of top windows in the greenhouse (yes/no). Days with no escape measurements are because of bad weather (closed windows) and (*1*) unavailability of researcher (TH) and (*2*) a week of measurements identifying within greenhouse stratification using all available Rotorods. The detection threshold is indicated by a dashed line.

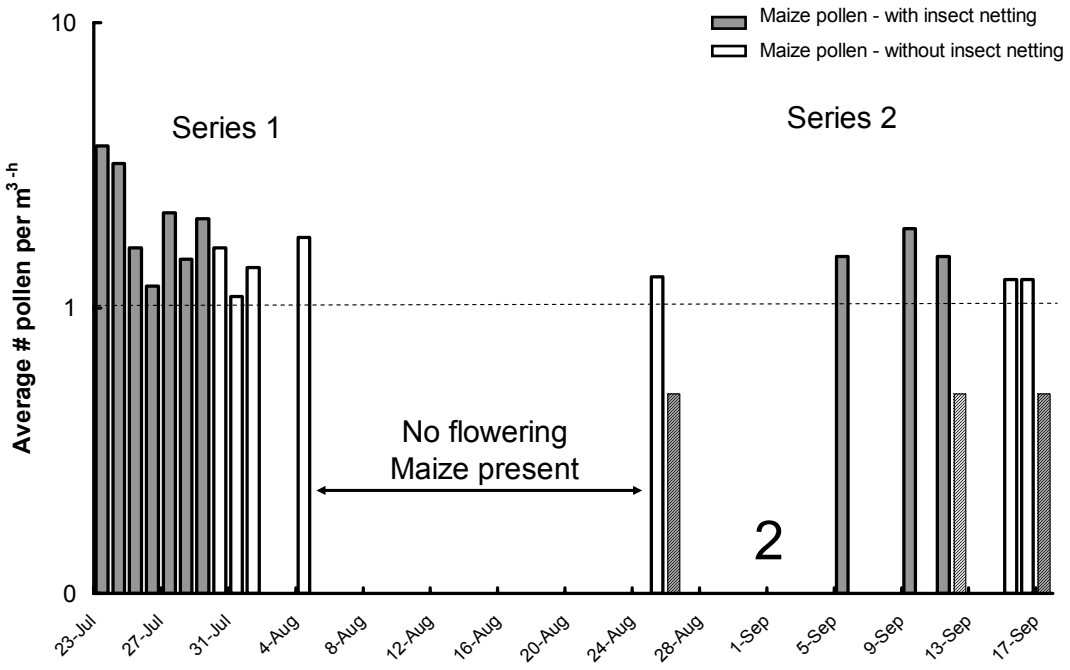

Fig. A1.2. Overview of average measured pollen concentrations inside the greenhouse (# per m² per hour) of *Zea mays* indicating the intensity of flowering over time. Bars represent days with escape measurements (coupled inside and outside measurements). Different bar colors indicate insect mesh being present in front of 20-m² of top windows in the greenhouse (yes/no). Dashed bars present 0-values below the indicated detection threshold (dashed line). Two subsequent series of flowering plants are included. Days with no escape measurements are because of bad weather (closed windows), pre-flowering growth of second series (indicated)) and (*2*) a week of measurements identifying within greenhouse stratification using all available Rotorods.

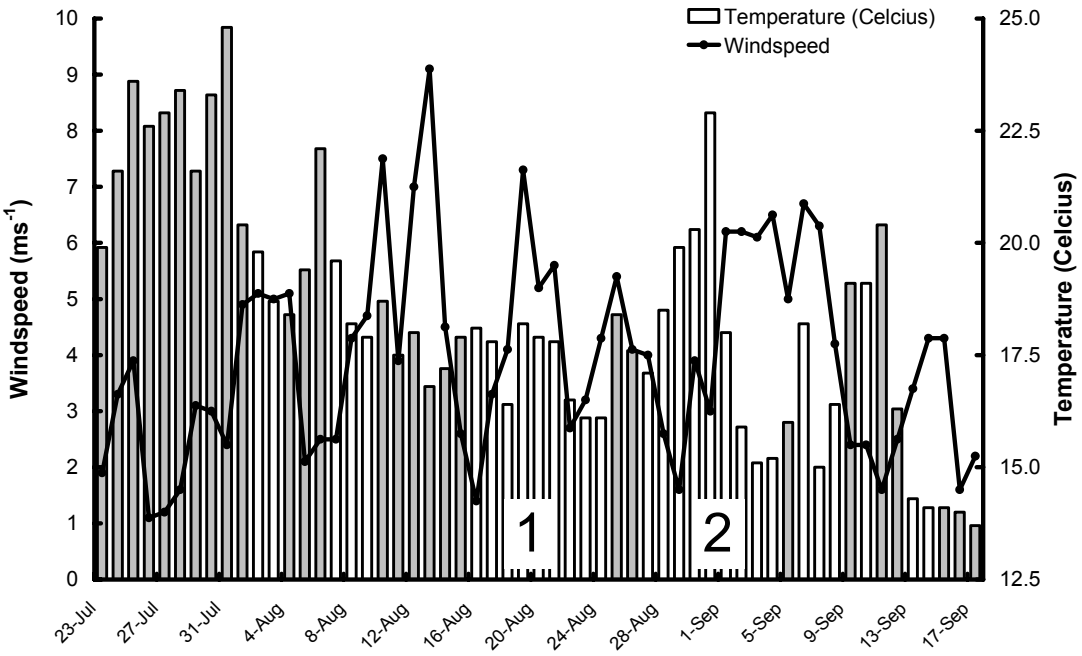

Fig. A1.3. Overview of average daily temperature (bars) and wind speed outside the greenhouse. Filled bars present days with escape measurements (coupled inside and outside measurements). Days with no escape measurements are because of bad weather (closed windows), (*1*) unavailability of researcher (TH) and (*2*) a week of measurements identifying within greenhouse stratification using all available Rotorods.

APPENDIX 2

Table A2.1. Descriptive statistics of experimental data for measurements of pollen escape (coupled inside and outside measurements). Included is the number of individual rods on which pollen were counted (two per Rotorod per run).

Lolium

Insect mesh	Treatment	location	# Rods	Mean	Std. Error	Minimum	Maximum
No Mesh	Background pollen	outside	78	3.10	0.43	0.00	14.6
No Mesh	Artificial airflow	inside	36	172	32.4	6.15	696
No Mesh	Artificial airflow	outside	48	33.9	5.90	1.54	200
No Mesh	Neutral conditions	inside	68	172	44.5	0.00	1850
No Mesh	Neutral conditions	outside	80	12.2	2.48	0.00	146
With Mesh	Background pollen	outside	38	0.73	0.12	0.00	2.31
With Mesh	Atrificial airflow	inside	52	513	89.7	3.08	2219
With Mesh	Atrificial airflow	outside	62	110	16.1	0.00	478
With Mesh	Neutral conditions	inside	60	46.6	13.1	0.00	518
With Mesh	Neutral conditions	outside	70	10.7	2.47	0.00	105

Maize

Insect mesh	Treatment	location	# Rods	Mean	Std. Error	Minimum	Maximum
No Mesh	Background pollen	outside	48	0.06	0.03	0.00	0.77
No Mesh	Artificial airflow	inside	24	7.88	3.08	0.00	48.5
No Mesh	Artificial airflow	outside	24	0.64	0.19	0.00	3.85
No Mesh	Neutral conditions	inside	48	0.43	0.10	0.00	3.08
No Mesh	Neutral conditions	outside	48	0.10	0.04	0.00	1.54
With Mesh	Background pollen	outside	38	0.00	0.00	0.00	0.00
With Mesh	Atrificial airflow	inside	24	32.0	5.00	0.77	73.8
With Mesh	Atrificial airflow	outside	24	1.35	0.41	0.00	8.46
With Mesh	neutral	inside	24	0.90	0.20	0.00	3.08
With Mesh	neutral	outside	24	0.13	0.06	0.00	0.77

Table A2.2. Descriptive statistics of experimental data for the pollen dynamics inside the greenhouse, stratified in 4 layers. Included is the number of individual rods on which pollen were counted (two per Rotorod per run).

Lolium				
Height	**Treatment**	**# Rods**	**Mean**	**Std. Error**
2 m	Closed windows	24	1.09	0.43
3 m	Closed windows	24	0.51	0.14
4 m	Closed windows	12	0.13	0.08
5 m	Closed windows	24	0.10	0.07
2 m	Open windows	24	29.1	6.29
3 m	Open windows	24	23.1	5.09
4 m	Open windows	12	14.1	3.72
5 m	Open windows	24	11.9	2.41
Maize				
Height	**Treatment**	**# Rods**	**Mean**	**Std. Error**
2 m	Closed windows	24	0.61	0.19
3 m	Closed windows	24	0.67	0.33
4 m	Closed windows	12	0.06	0.06
5 m	Closed windows	24	0.10	0.05
2 m	Open windows	24	8.53	4.46
3 m	Open windows	24	1.54	0.91
4 m	Open windows	12	0.83	0.68
5 m	Open windows	24	0.38	0.19

APPENDIX 3

"Inperkingsmaatregelen bij activiteiten met genetische gemodificeerde planten". Copy of manual provided by VROM (in Dutch)

24 maart 2005

INPERKINGSMAATREGELEN BIJ ACTIVITEITEN MET GENETISCH GEMODIFICEERDE PLANTEN

Onderstaande tabel is gebaseerd op appendix C behorend bij de Regeling genetisch gemodificeerde organismen en Richtlijnen van de COGEM bij deze regeling van juni 1998. Aan de hand van een herzien COGEM advies (met kenmerk CGM/030214-01 d.d. 28 februari 2003) is deze tabel bijgesteld en uitgebreid. Sindsdien zijn er nieuwe gewassen door de COGEM beoordeeld die in deze tabel opgenomen zijn. Ook nieuw beoordeelde gewassen worden in onderstaande tabel opgenomen, dit betekent dat deze tabel regelmatig wordt ge-update.

Van de onderstaande gewassen zijn de maatregelen met betrekking tot de fysische inperking vastgesteld. Indien u een gewas wilt modificeren dat niet in deze tabel vermeld wordt, dan dient u nadere gegevens aan te leveren die betrekking hebben op de voortplantingswijze, de bestuivingswijze, het bloeiseizoen, het voorkomen van kruisbare verwanten in de Nederlandse flora, de zaad-karakteristieken (grootte, vorm, vastzadigheid e.d.) en vegetatieve voortplanting.

Verklaringen behorend bij de tabel:

Type gewas:

A	Apomict
I	Insectenbestuiver, kruisbevruchter
W	Windbestuiver, kruisbevruchter
V	Vogelbestuiver, kruisbevruchter
Z	Zelfbevruchter, I en W geven respectievelijk aan dat ook insecten- of windbestuiving plaats kan vinden
*	Onder Nederlandse teeltomstandigheden vindt geen vruchtzetting plaats.

#1 *Dieffenbachia* spp. en *Schefflera* spp. vormen onder nederlandse kascondities nauwelijks of geen bloeiwijzen. Voor *Ficus* spp. komen de insecten die voor bestuiving kunnen zorgen niet in Nederland voor. Op grond van COGEM advies CGM/041201-01 mogen deze genetisch gemodificeerde planten op PK-I vegetatief vermeerderd worden waarbij evt. gevormde bloeiwijzen verwijderd worden.

Gegevens m.b.t. fysische inperking

Pollendichte inhulling:

+	Maatregelen voor inperking van pollen noodzakelijk
–	Geen maatregelen voor inperking van pollen noodzakelijk

Plant			*Gegevens m.b.t. fysische inperking*		
			Pollendichte inhulling		*Bijzondere maatregelen voor zaden en grond*
Familie	*Soort*	*Type gewas*	*PK-I/II kas insectendicht*	*PK-I kas, niet insectendicht*	
Amaryllidaceae	*Narcissus spp.*	*I*	-	+	*Ja*
Alstroemeriaceae	*Alstroemeria*	*I*	-	+	*Ja*
Apiaceae	*Carum spp.*	*I*	-	+	*Ja*
	Daucus carota	*I*	-	+	*Ja*
Apocynaceae	*Catharanthus roseus*	*	-	-	*nee*
	Tabernaemontana pandaqui	*	-	-	*nee*
Asteraceae	*Artemisia spp.*	*W*	+	+	*Ja*
	Brachyscome multiflora	*I*	-	+	*Ja*
	Brachyscome melanophora	*I*	-	+	*Ja*
	Bidens ferulifolium	*I*	-	+	*Ja*
	Cichorium spp.	*I*	-	+	*Ja*
	Dendranthema grandiflora	*	-	-	*Ja*
	Gerbera jamesonii	*	-	-	*Ja*
	Helianthus annuus (cultuurplant)	*Z/I*	-	-	*nee*
	Lactuca spp.	*Z/I*	-	+	*Ja*
	Sanvitalia speciosa	*I*	-	+	*Ja*

Plant			Gegevens m.b.t. fysische inperking		
			Pollendichte inhulling		Bijzondere maatregelen voor zaden en grond
Familie	Soort	Type gewas	PK-I/II kas insectendicht	PK-I kas, niet insectendicht	
Araceae	Anthurium andreanum	I	-	+	Ja
	Dieffenbachia spp.	I, #1	-	-	Nee
	Zantedeschia	I	-	+	Ja
Araliaceae	Schefflera spp.	I, #1	-	-	Nee
Balsaminaceae	Impatiens spp.	I	-	+	Ja
Begoniaceae	Begonia spp.	I	-	+	Ja
Brassicaceae	Arabidopsis thaliana	Z/I	-	-	Ja
	Arabis holboellii	A/Z/I	-	+	Ja
	Arabis drummondii	A/Z/I	-	+	Ja
	Brassica spp	Z/I	-	+	Ja
	Raphanus sativus	Z/I	-	+	Ja
	Sinapis spp.	Z/I	-	+	Ja
	Thlaspi caerulescens	Z/I	-	+	Ja
	Thellungiella halophila	Z/I	-	+	Ja
Canabinaceae	Cannabis sativa	W/I	+	+	Ja
Caryophyllaceae	Dianthus spp.	*	-	-	Ja
	Gypsophila paniculata	*	-	-	Ja
Chenopodiaceae	Beta vulgaris	W	+	+	Nee
	Spinacia oleracea	W	+	+	Nee
Cucurbitaceae (inhullingsplicht geldt alleen voor de mannelijke bloeiwijzen)	Citrullus lanatus	I	-	+	Nee
	Citrullus vulgaris	I	-	+	Nee
	Cucumis spp.	I	-	+	Nee
	Cucurbita spp.	I	-	+	Nee
Droseraceae	Drosera spp.	I	-	+	Ja
Euphorbiaceae	Manihot esculentum	*	-	-	Nee
Fabaceae	Glycine max	Z	-	-	Ja
	Lotus corniculatus	I	-	+	Ja
	Lotus japonicus	I	-	+	Ja
	Mediacago truncatula	Z	-	-	Ja
	Medicago varia	I	-	+	Ja
	Phaseolus spp.	Z/I	-	+	Nee
	Pisum sativum	Z/I	-	-	Nee
	Trifolium repens	I	-	+	Ja
	Vicia faba	Z/I	-	+	Nee
	Vicia hirsuta	I	-	+	Nee
	Vigna unguiculata	Z/I	-	+	Nee
Fagaceae	Quercus robur	W	+	+	Nee
Geraniaceae	Pelargonium spp.	I	-	+	Ja
Gesneraceae	Saintpaulia ionantha	*	-	-	Ja
Goodeniaceae	Scaevola aemula	I/W	+	+	Ja
Hydrangeaceae	Hydrangea macrophylla	*	-	-	Ja
Hyperaceae	Hypericum	I/W	+	+	Ja
Iridaceae	Freesia	*	-	-	Ja
	Gladiolus	*	-	-	Ja
	Iris spp.	I	-	+	Ja
Lamiaceae	Mentha	I	-	+	Ja
	Lavandula spp.	I	-	+	Ja
Liliaceae	Allium cepa	I	-	+	Ja
	Allium porrum	I	-	+	Ja
	Hyacinthus spp.	I	-	+	Ja
	Lilium spp.	*	-	-	Nee
	Tulipa spp.	I	-	+	Nee
Linaceae	Linum usitatissimum	Z/I	-	+	Ja
Malvaceae	Gossypium hirsutum	I	-	+	Nee
Marantaceae	Calathea roseopicta	I	-	+	Ja
Moraceae	Ficus spp.	I, #1	-	-	Nee
Musaceae	Musa spp.	*	-	-	Nee
Nyctaginaceae	Bougainvillea vera	I	-	+	Ja
Onagraceae	Fuchsia hybrida	V*	-	-	Nee

Plant			**Gegevens m.b.t. fysische inperking**		
			Pollendichte inhulling		Bijzondere maatregelen voor zaden en grond
Familie	Soort	Type gewas	PK-I/II kas insectendicht	PK-I kas, niet insectendicht	
Poaceae	Agrostis stolinifera	W	+	+	Ja
	Agrostis tenuis	W	+	+	Ja
	Dactylis glomerada	W	+	+	Ja
	Festuca spp.	W	+	+	Ja
	Hordeum vulgare	Z	-	-	Nee
	Lolium spp.	W	+	+	Ja
	Oryza sativa	Z	-	-	Nee
	Phleum pratense	W	+	+	Ja
	Poa pratensis	Z/A/W	+	+	Ja
	Poa trivialis	W	+	+	Ja
	Triticum aestivum	Z	-	-	Nee
	Zea mays	W/Z	-	-	Nee
Polemoniaceae	Phlox paniculata	I	-	+	Ja
Polygonaceae	Rumex palustris	W	+	+	Nee
Primulaceae	Cyclamen persicum	I	-	+	Ja
Ranunulaceae	Delphinum belladonna	I	-	+	Ja
	Delphinum elatum	I	-	+	Ja
Rosaceae	Fragaria spp.	Z/I	-	+	Nee
	Malus spp.	I	-	+	Nee
	Pyrus spp.	I	-	+	Nee
	Rosa cultivargroepen: Floribunda group Climbing floribunda group Grandiflora group Climbing Grandiflora group Hybrid Kordesii group Hybrid Moyesii group Hybrid Musk group Hybrid Rugosa group Hybrid Wichurana group Hybrid Tea group Climbing Hybrid Tea group Large Flowered Climber group Miniature group Climbing Miniature group Mini-Flora group Polyantha group Climbing Polyantha group	*	-	-	Nee
	Rosa hybride	I	-	+	Ja
Rubiaceae	Bouvardia spp.	*	-	-	Nee
Rutaceae	Citrus spp.	I	-	+	Ja
Salicaceae	Salix alba	W	+	+	Ja
	Salix matsudana	*	-	-	Nee
Scrophulariaceae	Craterostigma plantagineum	Z	-	-	ja
	Sutera diffusa	I	-	+	ja
Solanaceae	Capsicum annuum	I	-	+	nee
	Lycopersicon spp.	Z	-	-	nee
	Nicotiana spp.	Z	-	-	ja
	Petunia hybrida	Z	-	-	nee
	Salpiglossis sinuata	Z	-	-	nee
	Solanum melongena	I	-	+	nee
	Solanum tuberosum	Z	-	-	nee
	Solanum verrucosum	Z	-	-	nee
Sterculiaceae	Theobroma cacao	Z/I	-	-	nee