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Rationalizing the isolation distance needed for field trials involving genetically modified rapeseed (*Brassica napus* L.) in China

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The isolation distance required for field trials of genetically modified (GM) rapeseed varies widely worldwide, with a 50–400 m distance in most nations contrasting with a minimum 1000-m isolation distance in China. The goal of this study was to evaluate the relevance of current regulations in China regarding the isolation distance needed for GM rapeseed trials. A pollen flow experiment was conducted based on the design of concentric circles, with the GM plants in a 20-m diameter circle at the centre, surrounded by non-GM plants to a distance 60 m from the perimeter of the circle containing GM plants. The rate of pollen flow was the highest at the isolation distance of 0.5 m, where it ranged from 2.3091% to 2.6711%. The general pattern of the pollen flow rate (y) with distance (x) was well described by the equation $y = 1.3936x–0.9136$ ($R^2 = 0.9950$). The long-distance pollen flow tested at the isolation distance of 800 m was 0.0018%, which agrees with the theoretical prediction. The results suggested that 300 m, rather than 1000 m, is a reasonable distance to ensure a tolerable threshold of pollen flow (less than 0.01%) under conditions of winter rapeseed production in China.

regulated isolation distance, genetically modified winter rapeseed, pollen flow rate, tolerance threshold

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The isolation distance required for trials involving genetically modified (GM) rapeseed (*Brassica napus*) varies widely, although for most nations, it ranges from 50–400 m. A 50-m isolation distance is recommended for GM trials in the United Kingdom (UK) [1,2], with a 200-m isolation distance recommended for organic crops. In the UK, an isolation distance of 50 m is also required for growing crops with high levels of erucic acid [2,3]. Canadian regulations stipulate a 200-m isolation distance for GM canola trials, or a 10-m wide border of synchronously flowering non-GM plants around the entire trial area [4]. An isolation distance of 400 m is required for GM trials in France, Belgium and Sweden. Australian GM trial requirements include a 400-m isolation distance and a 15-m non-GM buffer [2]. However, in China, more than 1000 m is required for isolation from other *Brassica* plants in confined field trials of GM rapeseed according to the Implementation Regulations on Safety Assessment of Agricultural GMOs of China [5]. The 1000-m isolation distance is the longest of all of the minimum isolation distances defined by governments throughout the world for trials involving GM rapeseed.

Longer isolation distances increase both costs and the difficulty of implementing regulations, which together substantially restrict GM-crop development. In contrast, insufficient isolation distances have potential health and environmental risks, and reduce the market acceptability of the product [6–9]. With the increasing number of field trials of GM rapeseed in China, more and more debate has centered around whether the isolation distance of 1000 m can be justified in scientific terms, or whether the regulation should be

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revised to better address the needs for the development of GM rapeseed varieties [10,11].

The regulated isolation distance is usually established based on the tolerance threshold of the exogenous gene flow rate (outcrossing rate) [2,7,9,12,13]. Numerous studies on rapeseed's pollen flow distance and pollen flow rate have been conducted in Canada, Australia, and some European countries [4,6,7,14–17], but few has been conducted in China [18].

Studies have shown that pollen flow varies with various factors, such as cropping systems, the availability of pollinating insects, wind direction, temperature, topography and other natural environmental conditions [2,6,12,17–21]. Given that different countries have different environmental conditions, their requirements for isolation distances may vary [2]. Unlike other rapeseed production countries, such as Canada and Australia, where spring-type rapeseed is grown, China is characterized by predominant growth of winter-type rapeseed, with the seed sown in Autumn and harvested the following May [22]. China is also a biodiversity centre of many *Brassica* species, such as *Brassica rapa* (*B. rapa*) and *Brassica juncea* (*B. juncea*), which are indigenous throughout China and cross readily with *B. napus* [22–24]. In particular, wild *B. juncea* is a common weed in fields used for rapeseed cultivation [25]. Therefore, a sound isolation distance is required to guarantee the food and environmental biosafety of GM crops, and the market acceptability of the products.

The objectives of this study were to assess the efficacy of isolation distances in minimizing pollen flow from external pollen sources under the condition of Chinese winter rapeseed production and to find a scientifically rational isolation distance to keep the risk of pollen flow within an acceptable threshold for GM rapeseed field trials. The result of this paper provides a scientific basis for possible revision of the current Chinese regulation regarding the mandatory isolation distance for GM rapeseed field trials.

1 Materials and methods

1.1 Materials

Pollen donor Z7B10, a genetically modified glufosinate-resistant strain of *B. napus*, was developed by the Oil Crops Research Institute (OCRI) of the Chinese Academy of Agricultural Sciences (CAAS) through *Agrobacterium*-mediated transformation. It is a winter-type strain containing one copy of the bar (phosphinothricin acetyltransferase) gene [26], which is a dominant gene conferring resistance to the herbicide glufosinate ammonium (tradename BastaR, Hoechst). This strain was approved for environmental release in China in 2009 [27].

Pollen recipient Zhongshuang No.10, a winter-type cultivar of *B. napus* registered in 2005, was developed by the same institute, flowers at the same time as Z7B10, and has a high hybrid affinity for Z7B10.

The seed purity and germination rates of Z7B10 and Zhongshuang No. 10 were examined under laboratory conditions before the seeds were sown in the field.

1.2 Experimental design of pollen flow

The experiment was carried out at Huai'an, Jiangsu, China, from September 30, 2009 to December 20, 2011. With the longitude of E118°12′00″–119°36′30″ and latitude of N32°43′00″–34°06′00″, it is on the climatic demarcation line of northern and southern China, and falls within the area of China where most winter rapeseed is grown.

The pollen flow experiment was conducted based on a concentric circle design (Figure 1), with GM Z7B10 planted within a circle with a 20-m diameter, and surrounded by non-GM Zhongshuang No.10 plants planted within a

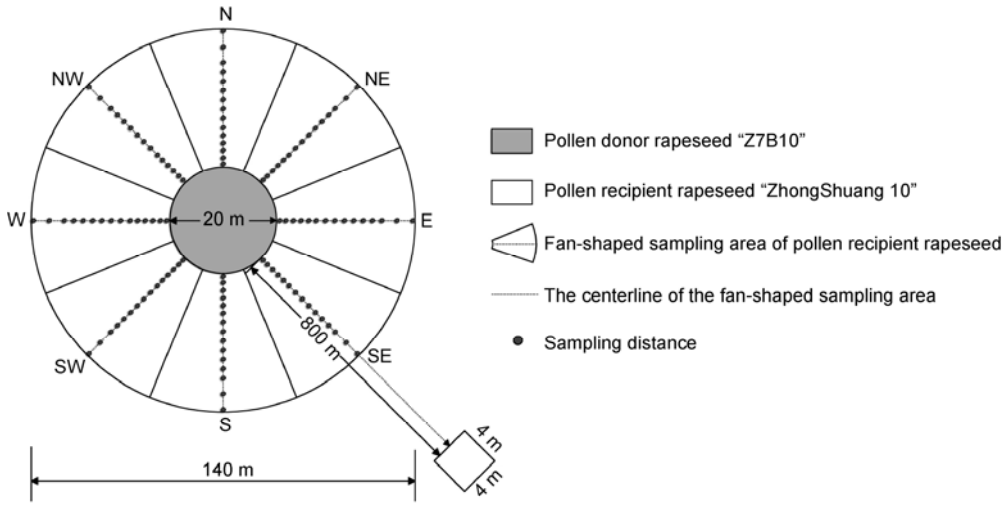

Figure 1 Design of the concentric circle experiment (the figure is not drawn to scale). The abbreviations E, W, S, N, SE, NE, SW and NW represent the east, west, south, north, southeast, northeast, southwest and northwest directions, respectively (the same hereinafter).

surrounding circle that extended 60 m from the outer boundary of the circle containing Z7B10 plants. The total area of the concentric circle experiment was 2 ha. Both donor and recipient cultivars were sown on 30 September, 2009 at a rate of 7.5 kg of seed ha^{-1}. The density of plants was adjusted to 18 plants per m^2 when seedlings were at the 5-leaf stage.

In order to estimate the pollen mediated long-distance gene flow rate, an additional 4 m × 4 m square plot of Zhongshuang No. 10 was grown to the southeast of the concentric circles, 800 m from the boundary of the GM Z7B10 (inner) circle. Between the concentric circles and the 4 m × 4 m square plot there was a lawn where Tifdwarf Bermudagrass was grown. Within the experimental area, growth of other oilseed rape and *Brassica* vegetables was prohibited.

The plants in the pollen flow experiment were allowed to pollinate naturally during the flowering period, without any measures to assist pollination, such as provision of artificial beehives.

1.3 Sampling

In order to estimate the pollen flow rate, samples of mature plants were taken from 8 points (east, west, south, north, southeast, northeast, southwest, and northwest) of the Zhongshuang No.10 (outer) circle. For each direction, the samples were collected at 15 distances (0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 50 and 60 m) from the outside boundary of the circle containing GM rapeseed Z7B10. For each sample, 10–20 plants were selected and their seeds were threshed and pooled for further analysis. Consequently, a total of 120 samples at eight directions were harvested, with 15 samples for each direction.

For the 4 m × 4 m square plot 800 m away from the edge of the pollen donor circle, seeds from all of the plants were harvested together.

All samples were air-dried and threshed, starting with the samples from the 800-m square plot and working toward samples taken from closest to the centre of the concentric circles. This approach prevented contamination of the outer samples by those nearer the center.

1.4 Environment survey of the experimental field

During the flowering period, the major meteorological data (including wind direction, wind speed, temperature, humidity, light intensity, and rainfall) were recorded at 1-h intervals every day from 8:00 am to 17:00 pm by an automatic weather station (FSR-4, Beijing Yude Company) installed at the center of the circular experiment area. The average wind speed in each direction and the frequency of wind gusts were calculated based on the above record.

When the rapeseed plants were in full bloom, the number of bees was counted by 9 workers by visual inspection for 30 min at noon on each sunny day. Numbers of bees were counted in the pollen donor area and recipient areas between 0.5–5 m, 20–30 m, and 50–60 m away from the edge of the pollen donor circle in all of the same eight directions used for sampling.

1.5 Seed screening for presence of the transgene

In this study, the rate of pollen flow was estimated as the proportion of the progeny harvested from Zhongshuang No.10 plants, which contained the bar gene and were resistant to glufosinate, per number of progeny plants tested.

Seed samples collected from the eight directions (15 sampling sites used for each direction) in the concentric circle field experiment were sown on October 2, 2010 and arranged according to a triplicate randomized block design with 30 m^2 for each plot and at a seeding rate of 350–430 plants m^{-2}. Seeds collected from the plot 800 m away from the edge of the pollen donor circle were directly sown on July 20, 2011 in three randomly arranged plots of 90 m^2 at a density of 410–430 plants m^{-2}. Zhongshuang No. 10 and GM rapeseed Z7B10 were used as negative and positive controls, respectively.

Final plant populations were estimated for each plot at the two-leaf stage by counting the number of plants in five 1 m × 1 m wooden frames based on a 5-point sampling method. The total number of seedlings per plot was calculated as follows:

Number of seedlings per plot = Number of seedlings m^{-2} × Plot area (m^2).

At the two- or three-leaf stage, the seedlings were sprayed with the herbicide BastaR (Bayer Cropscience Co.), containing 13.5% active ingredient phosphinothricin (PPT) ammonium salt, at the optimal concentration recommended by Xiao [27]. The same concentration of herbicide was sprayed both 10 and 20 d after the first spraying to prevent the survival of plants sensitive to glufosinate. The survivors in each plot were counted 10 d after the third spray.

The pollen flow rate was calculated as follows:

Pollen flow rate = number of glufosinate resistant seedlings per plot/number of total seedlings per plot × 100%.

1.6 Confirmation of positive plants by PCR detection

The transgenic nature of plants that survived repeated spraying with the herbicide glufosinate was confirmed by PCR-mediated screening for the presence of the bar gene. For the samples taken from 20, 25, 30, 35, 40, 50, 60 and 800 m, all of the glufosinate-resistant seedlings were analyzed for the presence of the bar resistance gene. For those from other distances, a total of 500 glufosinate-resistant seedlings were randomly selected and examined for the presence of the bar resistance gene.

Total DNA extracts were prepared from all plant samples using the SDS method with an instrument for rapid nucleic

acid extraction (FastPrep®-24, MP). The DNA samples were used for amplification of the bar gene using the primers 35SF (5′-CCTTCGCAAGACCCTTCCTC-3′) and BarR (5′-AAACCCACGTCATGCCAGTT-3′) in a 20-μL reaction system containing 1 U *Taq* DNA polymerase, 1× buffer, 2.2 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.15 mmol L^{-1} primers, and 30 ng template DNA. The amplification conditions included a 3-min denaturation at 94°C, followed by 35 cycles that each comprised 30 s denaturation at 94°C, 30 s annealing at 60°C, and 1 min extension at 72°C, followed by a final 7 min extension at 72°C. The PCR products were analyzed using 1% agarose gel electrophoresis.

1.7 Data analysis

For a specific distance and direction, the mean and average deviation of the pollen flow rate were calculated from three replicates of the seed screening trial results.

A two-way analysis of variance was performed on the replicated data set collected for all directions and distances, followed by multiple comparisons performed using Duncan's method.

The statistical relationship between sampling distances and pollen flow rate was studied using regression of pollen flow rate on distance from the edge of the pollen donor plot. The model that best fits the data was an exponential decay model $y = bx^{-a}$, where y is the pollen flow rate (%), x is the distance from the edge of the pollen donor circle (m), and a is the nonlinear coefficient of determination of the curve. The model was fitted to these data using Microsoft Excel 2003 and SPSS19.0. The coefficient of determination, R^2, was calculated as previously reported [28].

The regression equations and the regression curves for each direction were obtained through the above regression analyses. A general regression equation was derived from the distance as x and the mean pollen flow rate of 8 directions as y. The theoretical estimates were checked with the data from the pollen flow experiment.

2 Results

2.1 Pollen flow rate with distance

Seed samples collected from 8 directions and 15 sampling sites of each direction in the concentric circle field experiment were sown separately in a plot according to a triplicate randomized block design. A total of 4241904 seedlings from 360 plots were investigated. The number of seedlings per plot ranged from 7818 to 12798, with a mean of 11783. After spraying the seedlings three times with the herbicide glufosinate, a total of 18150 glufosinate-resistant seedlings survived from the 360 plots, while in the control field, all Zhongshua No. 10 seedlings died and all Z7B10 seedlings survived. The pollen flow rate of GM rapeseed varied with both the distances and directions (Table 1). Analysis of variance (Table 2) showed that the difference of pollen flow rate was highly significant among both isolation distances and directions, but not significant among replications. The results indicated that the isolation distance was the predominant factor accounting for about 99% of the total variance of pollen flow rate. The interaction between direction and isolation distance was not significant, indicating that the tendency of pollen flow rate with distance was similar in all of the eight directions tested.

As shown in Table 1, the pollen flow rate (y) declined rapidly as isolation distance (x) increased in all of the eight directions. The highest pollen flow rate was 2.3091% to 2.6711% found at the isolation distance of 0.5 m. Between an isolation distance of 0.5 m and an isolation distance of 20 m, the gene-flow rate dropped sharply from 2.3091%–2.6711% to about 0.0625%–0.1035%, and then declined very slightly with further increases in the isolation distance. The lowest pollen flow rate, 0.0296%–0.0416%, found at the isolation distance of 60 m.

The highest accumulated pollen flow rate was found in a northwesterly direction (6.9713%), followed by 6.7029% in a northerly direction and 6.6919% in a westerly direction. The lowest accumulated pollen flow rate was 6.1113%, found in a southeasterly direction (Table 1). Duncan's multiple comparison between directions indicated that differences in the rate of pollen flow were only significant between the rates observed in the northwesterly and southeasterly directions.

2.2 Modeling pollen flow rate

Regression analysis of the experimental data showed that the pollen flow rates in all eight directions fitted an exponential decay model $y = bx^{-a}$, with $R^2 \geqslant 0.98$ (Figure 2). The parameters a and b varied between the directions, but all 8 equations showed the same tendency with a sharp decrease in the gene-flow rate within 20 m of the isolation distance, and a slight decline with further increases in the distance (Figure 2). This general tendency was described by the general equation $y = 1.3936x^{-0.9136}$ ($R^2 = 0.9950$), which was calculated by the average data determined from samples collected in all eight directions. Given that the rates of pollen flow were highest in the northwesterly direction and lowest in the southeasterly direction, the upper and lower limits of pollen flow rates were estimated using the equations $y = 1.4310x^{-0.8788}$ (for the northwesterly direction) and $y = 1.3850x^{-0.9689}$ (for the southeasterly direction). Based on the general equation, and the equations for the southeasterly and northwesterly directions, the theoretical pollen flow rate with its range can be calculated for any given isolation distance (Table 3). This enabled us to estimate the pollen flow rate beyond 60 m from the perimeter of the inner circle of GM Z7B10 plants.

As shown in Table 3, at an isolation distance of 100 m,

Table 1 Pollen flow rates (%) at different distances in different directions

Distance (m)	Direction									Significance of difference	
	E	N	S	W	NE	NW	SE	SW	MEAN	5%	1%
0.5	2.5030±0.1431	2.5645±0.1569	2.4929±0.1121	2.4740±0.1566	2.4788±0.1421	2.6711±0.2133	2.3091±0.1865	2.5289±0.1265	2.5028±0.1632	a	A
1	1.2211±0.1874	1.3210±0.1396	1.2816±0.1001	1.2744±0.1395	1.2387±0.1655	1.4046±0.1522	1.1942±0.1440	1.2840±0.1192	1.2775±0.1365	b	B
2	0.7657±0.0564	0.7750±0.1026	0.7032±0.0706	0.7900±0.0531	0.6526±0.0583	0.8349±0.0664	0.6887±0.0836	0.7301±0.0829	0.7425±0.0838	c	C
3	0.5179±0.0366	0.5692±0.0586	0.5019±0.0360	0.6235±0.0511	0.4945±0.0408	0.5361±0.0449	0.5192±0.0389	0.5595±0.0339	0.5402±0.0544	d	D
4	0.4172±0.0270	0.4333±0.0301	0.4066±0.0326	0.4929±0.0367	0.3740±0.0291	0.4030±0.0313	0.4205±0.0254	0.4484±0.0341	0.4245±0.0425	e	E
5	0.3423±0.0247	0.3283±0.0383	0.3225±0.0147	0.3897±0.0294	0.3106±0.0197	0.3071±0.0248	0.3397±0.0305	0.3673±0.0174	0.3384±0.0346	f	F
10	0.1972±0.0392	0.2250±0.0332	0.1975±0.0336	0.1825±0.0227	0.1911±0.0246	0.2141±0.0237	0.2194±0.0375	0.1902±0.0326	0.2021±0.0301	g	G
15	0.1307±0.0132	0.1414±0.0221	0.1242±0.0162	0.1132±0.0124	0.1284±0.0201	0.1418±0.0251	0.1301±0.0210	0.1090±0.0237	0.1274±0.0199	h	H
20	0.0889±0.0145	0.0784±0.0198	0.0890±0.0093	0.0739±0.0093	0.0848±0.0051	0.1035±0.0100	0.0625±0.0145	0.0725±0.0072	0.0817±0.0157	i	HI
25	0.0690±0.0019	0.0582±0.0139	0.0693±0.0071	0.0605±0.0065	0.0616±0.0194	0.0843±0.0130	0.0527±0.0109	0.0631±0.0050	0.0648±0.0130	ij	I
30	0.0576±0.0062	0.0490±0.0154	0.0599±0.0040	0.0582±0.0074	0.0535±0.0052	0.0717±0.0119	0.0421±0.0022	0.0518±0.0144	0.0555±0.0115	ij	I
35	0.0526±0.0058	0.0421±0.0097	0.0520±0.0089	0.0438±0.0084	0.0534±0.0112	0.0598±0.0162	0.0365±0.0126	0.0497±0.0018	0.0487±0.0111	ij	I
40	0.0505±0.0023	0.0417±0.0156	0.0445±0.0102	0.0417±0.0152	0.0506±0.0051	0.0511±0.0131	0.0344±0.0064	0.0489±0.0059	0.0454±0.0103	ij	I
50	0.0468±0.0175	0.0384±0.0118	0.0417±0.0076	0.0386±0.0098	0.0496±0.0143	0.0466±0.0067	0.0324±0.0094	0.0420±0.0105	0.0420±0.0109	ij	I
60	0.0416±0.0085	0.0377±0.0052	0.0390±0.0044	0.0350±0.0106	0.0384±0.0035	0.0416±0.0080	0.0296±0.0038	0.0347±0.0113	0.0372±0.0073	j	I
Sum	6.5022	6.7029	6.4257	6.6919	6.2607	6.9713	6.1113	6.5802	6.5308		

the average pollen flow rate is 0.0207%, with a range between 0.0160% and 0.0250%. At an isolation distance of 1000 m, the average pollen flow rate is 0.0025%, with a range from 0.0017% to 0.0033%. In this way, the isolation distance can be estimated if a threshold rate of pollen flow is determined (Table 3). For example, if the threshold rate of pollen flow is set as 0.1%, the isolation distance should be 25 m, and if the threshold is set as 0.01%, the isolation distance should be 300 m.

2.3 Verification experiment for the long-distance pollen flow rate

To verify the reliability of the theoretical model, we conducted an experiment in a rapeseed field 800 m southeast of the concentric circle field. Two positive plants were detected from 111510 seedlings (Table 4). This indicates a pollen flow rate of 0.0018%, which is very close to the theoretical estimation of pollen flow rate at 800 m, which ranged between 0.0021% to 0.0040%, with a mean of 0.0031% (Table 3).

2.4 Detection of glufosinate-resistant bar gene using PCR

All of the 1075 herbicide-resistant seedlings obtained at isolation distances of 20, 25, 30, 35, 40, 50, 60 and 800 m, and 500 other randomly selected herbicide-resistant seedlings were collected and used for PCR-mediated detection of the glufosinate-resistant bar gene. The results (Figure 3) showed that a 595-bp specific band of the bar gene was amplified from all the herbicide-resistant seedlings and the positive control Z7B10, but not from the negative control Zhongshuang No. 10. This indicated that all of the surviving seedlings in the field possessed the bar gene. These results confirmed that the pollen flow rates obtained in the field trial are reliable.

2.5 Analysis of natural environmental conditions and pollination media of the experimental fields during rapeseed flowering

In this study, the flowering period of pollen donor GM rapeseed Z7B10 was synchronous with that of the pollen recipient Zhongshuang No. 10. Both varieties began flowering around April 1, were in full bloom between April 10 and April 21, and finished flowering around May 4.

The major climate data of the rapeseed fields during the flowering season, which were obtained from the automatic weather station, were similar to the local climate data in recent years provided by the Huai'an Meteorology Bureau. The area had an average ambient temperature of 15.7°C, ambient relative humidity of 59.1%, and a light intensity of 140.3 Lx. The total rainfall during the entire flowering period was only 0.6 mm. The fastest average velocity was 1.89 m s^{-1} of northwest wind, followed by the 1.40 m s^{-1} and 1.28 m s^{-1} of northeast and southeast winds, respectively. There were a total of 30 occurrences of southerly wind, followed by the 29 occurrences each of southeasterly wind and northeasterly wind. Northwesterly wind was recorded only nine times, the fewest of all of the incidences of wind directions (Figure 4). This is consistent with the differences of pollen flow rate observed between the different directions.

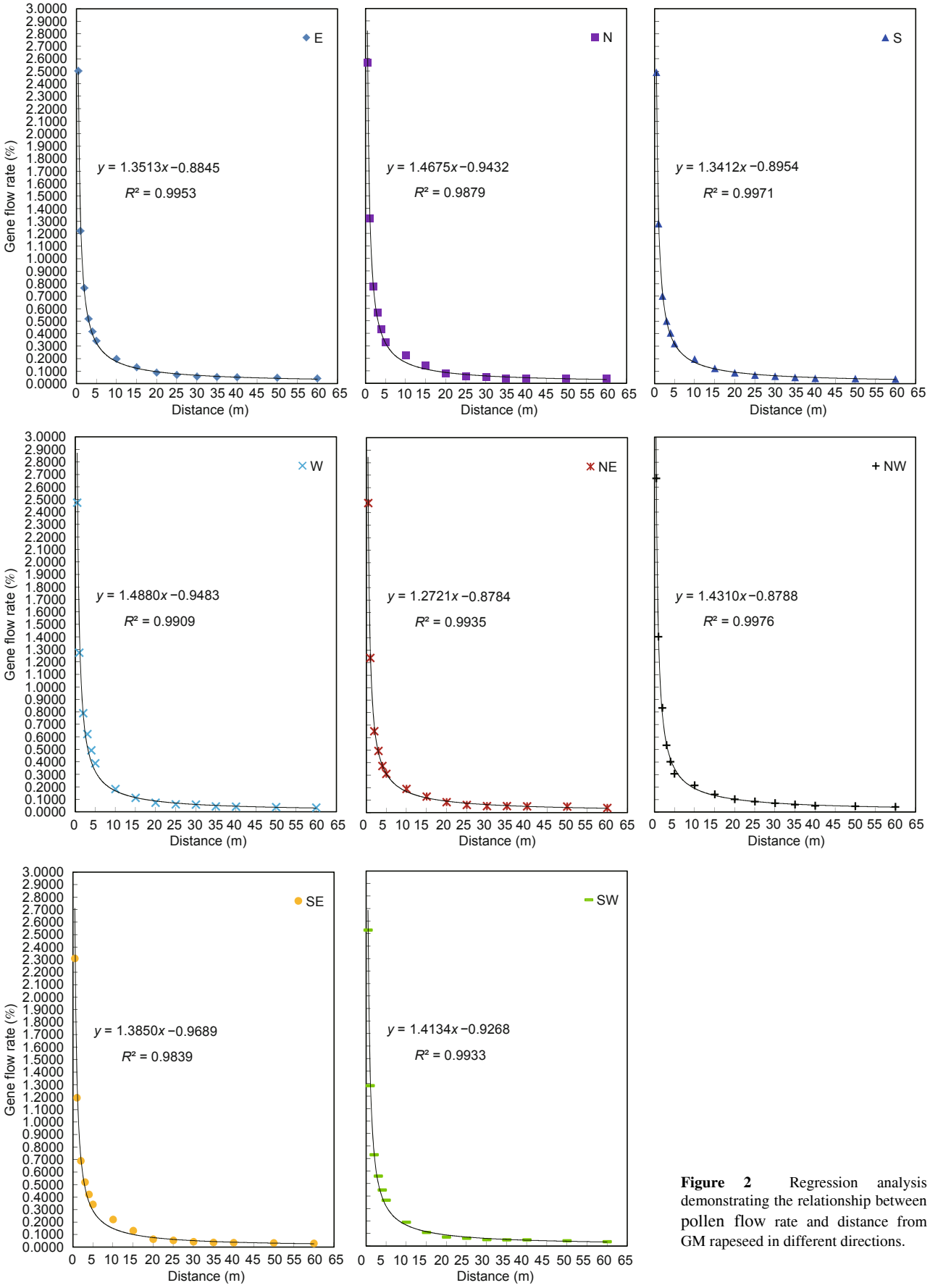

Figure 2 Regression analysis demonstrating the relationship between pollen flow rate and distance from GM rapeseed in different directions.

Table 2 Analysis of variance of data describing pollen flow rates

S.O.V	d*f*	*SS*	*MS*	*F*	*P*
Blocks	2	0.002142	0.001071	0.27987	0.75613
Distance	14	150.4552	10.7468	2824.753	0
Direction	7	0.10256	0.014652	3.8512	0.000544
Interaction	98	0.37588	0.003836	1.0081	0.47148
Error	240	0.91308	0.003805		
Total	359	151.8467			

Table 3 Theoretical pollen flow rate based on the exponential decay model $y = bx^{-a}$

Isolation distance (x) (m)	Pollen flow rate (y) (%)		
	Southeast (y=1.3850$x^{-0.9689}$)	Northwest (y=1.4310$x^{-0.8788}$)	Average (y=1.3936$x^{-0.9136}$)
0.5	2.7109	2.6314	2.6252
1	1.3850	1.4310	1.3936
2	0.7076	0.7782	0.7398
3	0.4777	0.5449	0.5108
4	0.3615	0.4232	0.3927
5	0.2912	0.3478	0.3203
10	0.1488	0.1892	0.1700
15	0.1004	0.1325	0.1174
20	0.0760	0.1029	0.0903
25	0.0612	0.0846	0.0736
30	0.0513	0.0720	0.0623
35	0.0442	0.0629	0.0541
40	0.0388	0.0559	0.0479
50	0.0313	0.0460	0.0391
60	0.0262	0.0392	0.0331
100	0.0160	0.0250	0.0207
150	0.0108	0.0175	0.0143
200	0.0082	0.0136	0.0110
250	0.0066	0.0112	0.0090
300	0.0055	0.0095	0.0076
350	0.0047	0.0083	0.0066
400	0.0042	0.0074	0.0058
450	0.0037	0.0067	0.0053
500	0.0034	0.0061	0.0048
550	0.0031	0.0056	0.0044
600	0.0028	0.0052	0.0040
650	0.0026	0.0048	0.0038
700	0.0024	0.0045	0.0035
750	0.0023	0.0043	0.0033
800	0.0021	0.0040	0.0031
850	0.0020	0.0038	0.0029
900	0.0019	0.0036	0.0028
950	0.0018	0.0035	0.0027
1000	0.0017	0.0033	0.0025
1500	0.0012	0.0023	0.0017
2000	0.0009	0.0018	0.0013

Table 4 Estimation of pollen flow rate at a distance 800 m away from the edge of the GM rapeseed circle

Plot	Total seedling number/plot	Number of glufosinate-resistant seedlings	Pollen flow frequency (%)
I	37242	0	0.0000
II	38034	1	0.0026
III	36234	1	0.0028
Total	111510	2	0.0018

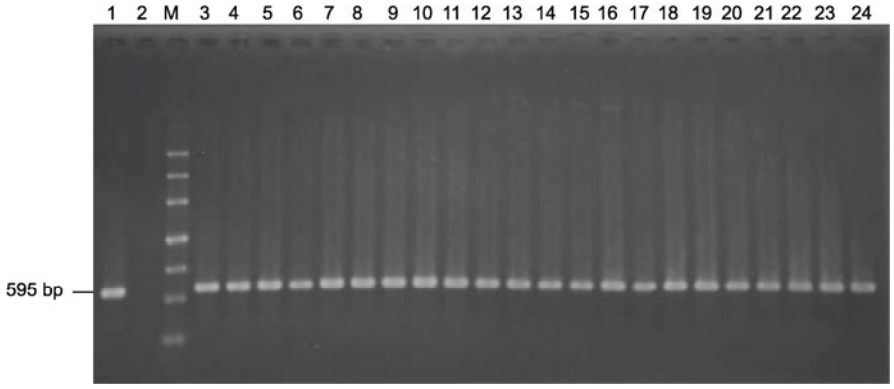

Figure 3 Detection of the bar gene, which confers glufosinate resistance, using PCR. M, Marker III; 1, positive control of GM rapeseed "Z7B10"; 2, negative control of non-GM rapeseed "Zhongshang 10"; 3–24, the surviving "Zhongshuang 10" rapeseed seedlings after three separate sprays with the herbicide glufosinate.

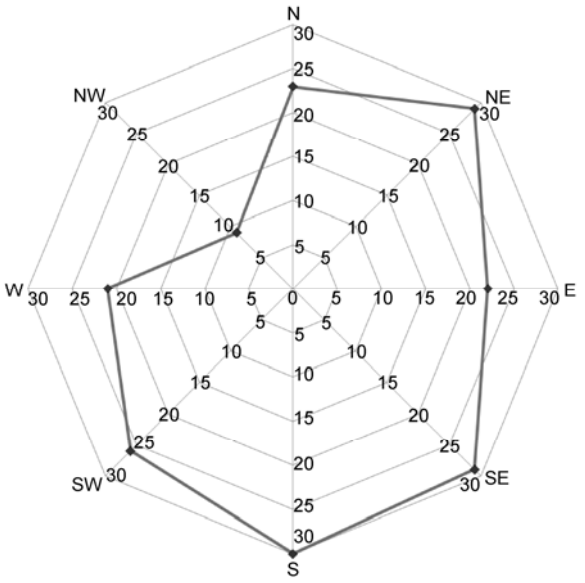

Figure 4 The number of wind occurrences per hour at different directions in the trial field of rapeseed during the flowering period.

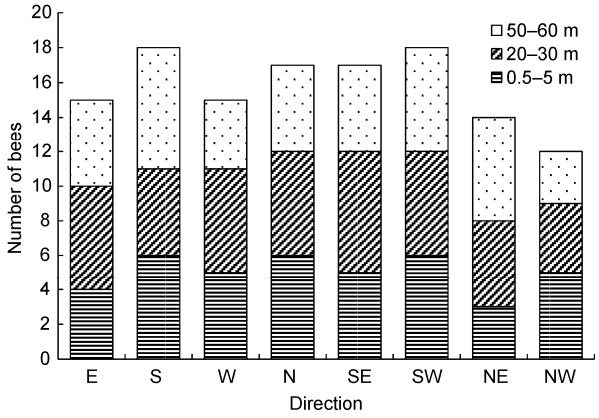

Figure 5 Bee distributions at different distances in different directions in the trial field of rapeseed during the flowering period.

The survey of bees (*Apis cerana cerana Fabricius*) during full bloom of rapeseed plants showed that only 3 bees were found within the GM circle, and 12–18 bees were found within the non-GM planting area in every direction (Figure 5). The distribution of bees at different isolation distances in different directions was not significantly different (P>0.05), suggesting that bees were not a major factor that accounts for the differences in rates of pollen flow in this study.

3 Discussion

Only one study, which involved the herbicide-resistant spring rapeseed variety Ms8Rf3, has investigated pollen flow from GM rapeseed under the conditions of winter rapeseed production in China [18]. In contrast, many broadly comparable studies have done in Canada, Australia and European counties [4,6,7,14–17]. The isolation distance required for field trials involving GM rapeseed varies widely between different countries. This is probably because different countries have considerably different cropping systems, the availability of pollinating insects and other natural environmental conditions [2,6,12,17–21]. In Canada, early maturing varieties are sown in spring, and develop rapidly in the long days to be harvested before the onset of winter, with less than a 4-month growing season. In Australia, rapeseed is usually sown in autumn, but with spring type varieties that do not need vernalization (winter chilling) to flower. Crops ripen in late spring or early summer, after a 5- to 7-month growing season. In Europe, most of the crop is of winter varieties which require vernalization, are sown in early autumn, and harvested late in the following summer, nearly 12 months after sowing. However, in China winter-type rapeseed (*Brassica napus*) occupies 90% of the rapeseed production area, where the crop is sown in Autumn and harvested the following May [22]. It is therefore important to estimate the pollen flow rate under the local condition and with the proper experimental materials.

The pollen donor used in this study, Z7B10, is a winter-type *B. napus* variety developed in China and approved for environmental release [27]. The pollen receptor Zhongshuang 10 is also a winter-type rapeseed variety that is widely cultivated in China. To our knowledge, this is the first large-scale study of pollen flow rate involving a winter-type rapeseed pollen donor and a winter-type rapeseed recipient under conditions typical of those used for winter rapeseed production in China.

The results showed that the highest pollen flow rate of GM rapeseed in all directions was 2.3091%–2.6711% at adjacent sites (a 0.5-m isolation distance) (Table 1). Staniland et al. [4] conducted a two-year experiment in two areas of Canada and found that the average pollen flow rate was 0.7% at adjacent sites. Beckie et al. [29] performed an experiment at 11 sites using glyphosate-resistant and glufosinate-resistant rapeseeds as pollen donors, and found that their average pollen flow rates were 1.1% and 1.4% at adjacent sites, respectively. Rieger et al. [6] conducted an experiment in multiple locations in Australia, and found that the highest pollen flow rates of rapeseed were 0.225% in West Australia, 0.197% in South Australia, 0.151% in Victoria, and 0.155% in New South Wales, respectively. Morris et al. [30] found that the pollen flow rate at adjacent sites was 2%–3.5% in the United States. Dietz-Pfeilstetter and Zwerger [31] conducted a two-year pilot experiment in Germany and found that the pollen flow rates of rapeseed at adjacent sites were 0.94% and 0.67%, respectively. Götz and Ammer [32] reported that the pollen flow rate was 0.43%–2.32% at a distance 0.5 m from the source of pollen. Scheffler et al. [14] found that the rate was 4.8% at adjacent sites in the UK. Therefore, the maximum pollen flow rate of GM *B. napus* at adjacent sites observed in this experiment is not very different from those obtained in other countries.

Our results indicated that the pollen flow rate declined sharply within 20 m, and then changed only slightly at distances beyond 20 m. This finding is also consistent with the results of many previous studies [4,14,18,31]. When the isolation distance was 40–60 m, the pollen flow rate decreased to 0.022% [33], 0–0.00034% [14], 0.05%–0.33% [34], 0.05%–0.11% [35], 0.15%–0.22% [29], and 0.04%–0.09% [31]. In this study, the pollen flow rate at distances 40–60 m from the source of pollen is 0.0372%–0.0454%, which is slightly less than for most broadly comparable experiments. The reduced rate of pollen flow beyond 20 m can be explained by the scarcity of pollinators observed in this experiment. Whereas wind-borne pollen appears to make no or only a negligible contribution to long-distance pollination of oilseed rape [36,37], pollinating insects—in particular honeybees (*Apis mellifera*) and bumblebees (*Bombus* sp.)—play a major role in *B. napus* pollination and are believed to be involved in the transfer of pollen over long distances. A report on the global bee status quo of the United Nations Environment Programme, which was issued in Geneva, indicated that the number of bees across the globe is being adversely affected by the habitat degradation, air pollution, widespread use of pesticides, and other factors [38]. Low rates of pollen flow at long distance may thus be closely related to the reduced number of pollinating insects [39].

It is difficult to measure the pollen flow rate of GM *B. napus* at isolation distances larger than 400 m, which are believed to be rare [2,6,15,18,29]. Scheffler et al. found that the pollen flow rate was 0.0156% and 0.0038% at isolation distances of 200 and 400 m, respectively [15]. Beckie et al. [29] showed that the pollen flow rates were 0.04%–0.05% at an isolation distance of 400 m, and that no gene transfer was apparent at 600 m. Cai et al. [18] performed an experiment in Wuhan, China using glufosinate-resistant rapeseed Ms8Rf3 (spring type) as pollen donor, and found that the pollen flow rate was 0.0031% at the isolation distance of 1000 m. Even in a large-scale field trial after commercial cultivation, the pollen flow rate of GM rapeseed was only no more than 0.11% at an isolation distance of 400–1000 m [6]. This study shows that the relation between pollen flow rate and isolation distance followed the equation $y = 1.3936x–0.9136$ (R^2=0.9950). According to this equation, an isolation distance of 300 m is sufficient to prevent pollen flow rates to exceed the tolerance threshold of 0.01% (Table 3). If a 0.1% tolerance threshold of pollen flow rate is considered to be acceptable, then an even shorter isolation distance (e.g., 25 m) is acceptable. The pollen flow rate in the field 800 m away in the southeast from the GM rapeseed was only 0.0018%, which is very close to the predicted 0.0021%–0.0040% (Table 3). This indicates that it is feasible to predict the long-distance pollen flow rate by using the model established in this study. Combined with all of the available data collected from all over the world, we recommend that 300 m, rather than 1000 m, should be adopted as the mandatory isolation distance for field trials of conventional GM rapeseed under conditions typical for winter-rapeseed production in China. The tolerance threshold of a 0.01% gene-flow rate is chosen because it is below a critical level of PCR detection, which is 0.1% in general, and below 0.9%, which is the lowest threshold value for labeling of GM in food and feed in the world [40,41]. Therefore, keeping the pollen flow rate below 0.01% should ensure both seed purity and the market acceptability of products.

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