

## Article

# Pollen Flow of Winter Triticale (*x Triticosecale* Wittmack) Investigated with Transgenic Line Expressing $\beta$ -Glucuronidase Gene

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**Abstract:** A transgenic winter triticale line expressing the *uidA* gene, encoding  $\beta$ -glucuronidase, was used to assess the pollen flow in field experiments over two consecutive vegetation seasons in central Poland. The experimental design included two variants of mixed transgenic and non-transgenic lines. Pollen grains were collected using passive traps located at 0, 10, 30, 60 and 85 m from the transgenic line. GM pollen grains were detected histochemically by staining with x-Gluc. A positive effect of temperature increase, as well as the strength and direction of the wind on the number and spread of pollen grains was observed. Regardless of the experiment year and variant, only few pollen grains were observed at a distance of 85 m. In the first year of the study the amount of pollen grains at 85 m was 300-fold lower than at the source and 140-fold lower in the second year. The number of transgenic pollen grains was two times lower when the field with the transgenic triticale was surrounded by a non-transgenic line, compared to an empty field. On the basis of the obtained results, we suggest 100 m as the distance for triticale pollen migration, although longer flight incidents are possible in extreme atmospheric conditions.

**Keywords:** aerobiology; GM plants; risk assessment; pollen transfer; transgenic winter triticale



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## 1. Introduction

Pollen from cultivated plants can be transmitted over long distances depending on the plant reproductive biology and atmospheric conditions. Predicting pollen flow is an important factor when planning deliberate release of genetically modified (GM) plants into the environment. The presence of pollen from unauthorized GM plants in honeybee food products might limit their trade. Pollen from genetically modified fields can impact the adjacent organic production. Pollen flow studies can be an important part of the research required for conducting risk assessment before introducing GM plants to the environment. The impact of GM pollen on non-target insects that feed on *Bt* crops, has been reported by many authors [1,2]. Moreover, pollen flow studies deliver data that are useful for appropriate field separation in the production of seed material, especially in the case of hybrid triticale varieties. Aerobiological methods offer valuable tools that help in studies of pollen and spore air transport and are useful in many aspects of agricultural and breeding applications. They enable studies of plant phenology and pathogen epidemiology and their changes driven by weather [3], monitoring of local and global distribution of plant pathogens [4,5], and studies of health hazards caused by allergens dispersed by air currents [6]. Mathematical modeling based on aerobiological methods is possible, with

exceptions [7]. Atmospheric conditions alter the efficiency of pollen grain release from anthers and affect the pollen flow distance among wind-pollinated crop plants. Crop species represent different rates of autogamy (self-pollination) and allogamy, which lead to various rates of outcrossing [8]. In addition some crops can hybridize with wild relatives so there is a risk of gene flow from GM plants to the natural ecosystems [9]. On the other hand such risks are not present in the case of the cultivation of crops, which are uncommon in the particular ecosystem or do not have any wild relatives [10].

Triticale is a man-made hybrid of wheat (*Triticum aestivum*, self-pollinating) and rye (*Secale cereale* open-pollinating, anemophilous) [11,12]. As such, triticale is an amphiploid species consisting of the genomes of wheat and rye [11]. Generally it exhibits cleistogamy similarly to wheat as well as chasmogamy similarly to rye [13], although, some propensity for open pollination (chasmogamy) is expressed in different genotypes of triticale, due to the ability of florets to outcross with other florets within the same inflorescence, or with other plants [13–15]. Outcrossing of triticale with other cultivars has been observed in nature however interspecific outcrossing with wheat and rye could only be obtained by manual pollination [16–20].

The degree of allogamy of triticale depends on the genetic origin, as well as, weather conditions during anthesis [15]. The spread of triticale pollen can vary depending on air humidity and temperature, wind force and direction, time of sowing, pollen production, and mechanical barriers [21]. In addition, the dispersal of triticale pollen results from its vitality, as well as its concentration in the air [22]. Good weather conditions, i.e., warm weather and low relative humidity, as well as air movements caused by earth heating and the formation of ascending air currents are particularly important for free pollen movement. Atmospheric disturbances may cause pollen movement over longer distances. High air humidity, as well as rainy days limit the duration of flower opening, anther extrusion and pollen dispersal. All mentioned factors interfere with each other causing spatiotemporal changes in flowering conditions [16,21,22].

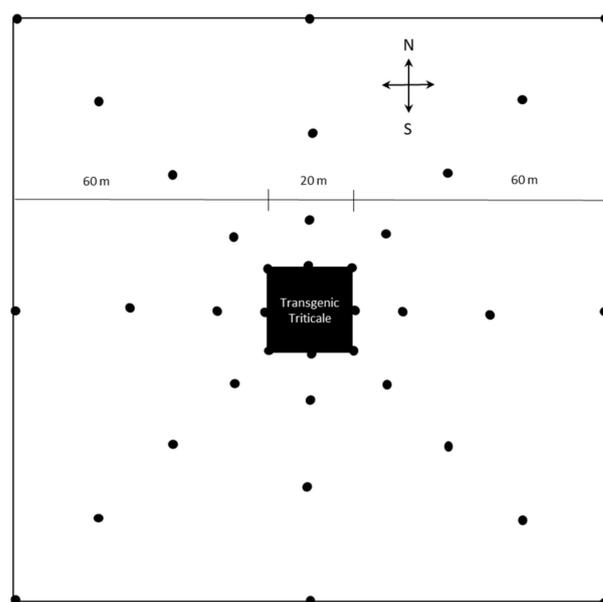
The first genetically modified triticale plants were obtained in 1993 [23]. Since that time, despite several other successful transformations, studies on the evaluation of risks associated with the release of triticale to the environment and the impact of transgenic products on living organisms have been published only occasionally [24–26]. The problem of allogamy among cultivated species, including triticale is particularly important in relation to the appearance of GM plants. Future, deliberate release of transgenic triticale will generate a need for risk assessment in many countries, therefore, we conducted studies to investigate potential pollen grain release in climatic conditions of Central Europe. The main goal of this study was to investigate the spread of triticale pollen grains by examining the extent of pollen transmission from genetically modified triticale. The GM triticale was used as a model because GM pollen grains could be easily distinguished (blue color after staining) from any other pollen including non GM triticale pollen potentially present in this environment. The additional aim was to compare two isolation methods which represent two systems used for minimizing outcrossing when GM plants are cultivated in a coexistence regime (border rows and spatial isolation) or during seed production (spatial isolation).

## 2. Materials and Methods

The field experiment was located in Radzików, central Poland (N 52°13', E 20°39') in the years 2010 and 2011. The monitoring of pollen flow was conducted using a transgenic winter triticale line as a model plant [23]. Transgenic triticale contains the *bar* gene conferring resistance to phosphinothricin, an active ingredient of herbicide, and the *uidA* gene [27]. The product of the *uidA* gene-β-glucuronidase-catalyzes the hydrolysis of β-glucuronide substrates, fluorometrically and histologically assayable, and hence the activity of this enzyme (blue colour) is easily detectable in plant organs including pollen grains. For this experiment double haploid line obtained by androgenesis was used [28] as all pollen grains produced by such a homozygous line contain the *uidA* gene. The GM line

does not differ morphologically from the isogenic variety Bogo (Plant Breeding Strzelce Ltd., Strzelce, Poland). This also applies to the size, weight, shape and surface of pollen grains. The average size of pollen grains for the transgenic line was  $59.85 \mu\text{m}$  and for the isogenic line  $59.17 \mu\text{m}$ .

The field experiment consisted of two experimental variants, each of  $19,600 \text{ m}^2$  ( $140 \text{ m} \times 140 \text{ m}$ ). In the first variant called “border rows” field with the GM triticale of  $400 \text{ m}^2$  ( $20 \text{ m} \times 20 \text{ m}$ ) sown in the middle, was surrounded by non-transgenic winter triticale cv. Bogo. The pollen traps were fixed in the non-transgenic triticale field. In the second variant called “spatial isolation” only the field with the GM triticale ( $20 \text{ m} \times 20 \text{ m}$ ) was planted and pollen traps were fixed around the empty field (Figure 1).



**Figure 1.** Schematic representation of experimental fields. Black dots represent the location of passive pollen traps. In ‘border rows’ variant the transgenic triticale was sown in the middle (black square,  $20 \text{ m} \times 20 \text{ m}$ ) and it was surrounded by non-transgenic winter triticale cv. Bogo. In ‘spatial isolation’ variant the transgenic triticale was sown in the middle but it was surrounded by an empty field.

Pollen flow of the transgenic line was monitored using 36 passive traps. The passive pollen traps composed of two glass slides ( $76 \times 26 \text{ mm}$ ) were fixed with clamps on a wood base ( $10 \times 7 \text{ cm}$ ) and mounted at a  $10^\circ$  vertical angle at the crop height. Slides were coated with an adhesive substance consisting of petroleum jelly dissolved in hexane according to Lacey and West [29]. Traps were set in eight geographical directions at distance of 0, 10, 30, 60 and 85 m diagonally from the field with the transgenic winter triticale line.

Collection of samples started on the day when the first anthers appeared on the main shoot spikes (beginning of the anthesis phase, DC 61, according to the decimal growth scale published by Zadoks et al. [30]). During the anthesis phase (from DC 61 to DC 69) slides were changed every 24 h. After application of a few drops of the X-Gluc solution (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide, Sigma-Aldrich, Merckgroup, Warsaw, Poland) the slides were covered with coverslips ( $24 \times 60 \text{ mm}$ ). The slides were scanned using an Epson Perfection 4990 Photo scanner (Seiko Epson Corporation, Suwa, Japan via Epson Polska). Blue stained pollen grains originated from transgenic plants. The number of such pollen grains on the total surface of each slide was marked on the image of a slide enlarged to the size of a computer screen, using the dedicated software—ImageJ <http://imagej.nih.gov/ij/> (ImageJ ver 153 Win Java-8, National Institute of Health, Bethesda, MD, USA). Meteorological data were recorded during anthesis by the weather station Vaisala Oyj (Vantaa, Finland) located 500 m from the experiment site in the first year

of the study and 1000 m from the experiment site in the second year of the study. On the basis of the number of pollen grains on each slide, the following calculations were made:

1. The total number of pollen grains per day for a given distance (0, 10, 30, 60, 85 m).
2. The number of pollen grains for a particular geographical direction: N, E, S, W (4 replicates per distance) and for NE, SE, NW, SW (5 replicates).

In order to approximate functions describing the number of pollen grains on slides as a function of the distance from the field with transgenic triticale plants analysis of linearized nonlinear regression functions was carried out [31–33]. After selection of the power function  $y = a \cdot x^b$  (where  $y$  is the number of pollen grains,  $x$  is the distance in meters and  $a$  and  $b$  are model parameters) as the best match for the phenomenon under study, a linearization transformation was conducted. The transformed function  $\log_{10} y = \log_{10} a + b \cdot \log_{10} x$  was then classically analyzed as a linear regression model—the values of the model parameters ( $a$  and  $b$ ) were estimated using the least squares method and the coefficient of determination ( $R^2$ ) was used as a measure matching the regression model to the original data. All statistical calculations were made using Statistica version 10 (StatSoft Inc., Tulsa, OK, USA) and Microsoft Excel version 2010 software. Inference about the significance of differences was conducted using one way ANOVA and was determined using Tukey's test ( $\alpha = 0.05$ ). Dependence between the number of pollen grains on the surface of the slide and the distance from the micro field with transgenic plants was calculated using two parameters:

(a) The average number of pollen grains for a given distance from the field with transgenic plants and geographical direction; the data were calculated for all subsequent observation days from both experimental variants, for each year of the study;

(b) The maximum and minimum numbers of pollen grains for a given distance from the field with GM plants (regardless of geographical direction), for each year of the study.

### 3. Results

#### 3.1. Weather during the Anthesis Phase

The beginning of anthesis, when anthers were extruded on the central part of the main stem spikes (DC 61) started on 7 June 2010 and 1 June 2011, in the first and in the second year of the study, respectively. The anthesis complete phase (DC 69), when anthers were practically white and devoid of pollen grains, was observed on 14 June 2010 and 8 June 2011, respectively. The duration of anthesis was identical in both years of this study, but the weather conditions during the anthesis phase were different each year (Table S1).

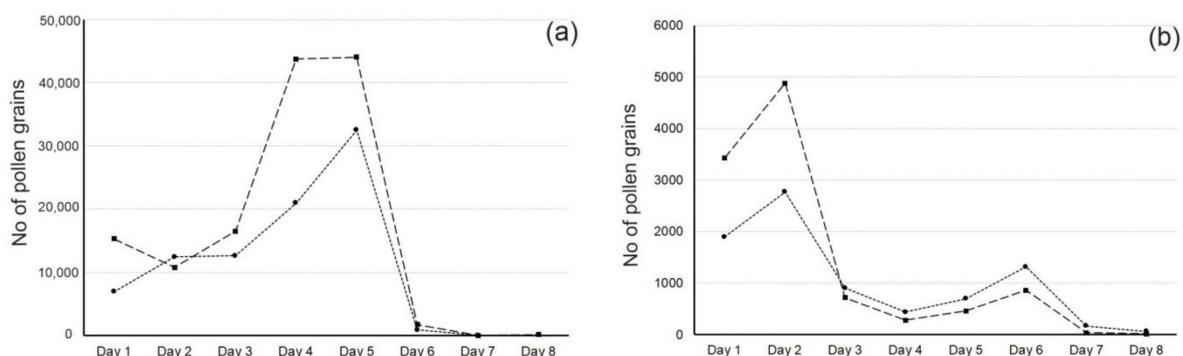
In the first year of the experiment, at the time of anthesis most of the weather was sunny and dry (Table S1A). The thermal conditions, sunshine, and lack of rain (except day 3) were favorable for anthesis. At the beginning of the anthesis phase, when anthers on the main shoot spikes began to release pollen grains, the average daily air temperature was 19.4 °C. With the advancement of plant anthesis to days 4 and 5, the average daily air temperature increased to 26.2 °C. In the following, final days of the anthesis phase, the daily air temperature decreased to 11.8 °C (on the eighth day). Longer periods of insolation, for more than fourteen hours and without rain were observed on the first two days and on the fifth day of anthesis. Shorter periods of insolation and the low rainfall were recorded only on days 3 and 7. During the eight days of anthesis south-west wind directions prevailed during days 1, 2, 5 and 7, with a moderate wind force (range 1.1–2.0 m/s, mean 1.39 m/s) (Table S1A). The relative air humidity during the most intense anthesis ranged from 54 to 95%. More intense rains, lack of insolation and higher relative humidity were recorded on the final days of the experiment, especially on day 8, when the anthesis phase was coming to an end.

In the second year of the experiment the weather course during the anthesis phase greatly differed (Table S1B). Average daily air temperatures ranged from 10.1 °C to 19.5 °C and they were lower by 7.0 °C (day 1) and 12.8 °C (day 4) in comparison to the first year of study. Insolation level was lower (from 1.3 to 8.7 h) than in the first year of experiment (from 8.5 to 14.1 h). During the plant anthesis rainfalls with different intensity took place—

from 0.2 mm (day 2) to 4.8 mm (day 4). Air humidity was higher than in the previous year, and relative humidity rates were variable. Moreover, at this time the wind was mostly blowing towards the south (days 1, 6 and 7) and north east (days 2 and 3). The mean wind strength was greater (1.9 m/s) than in the previous year (1.4 m/s). In the period of the most intense pollen grains release (day 2) the relative air humidity was nearly the same (about 63%) as in the previous year (62%, day 5).

### 3.2. Pollen Flow

During each of the two years of this study, the spread of pollen grains was monitored within eight days of anthesis. The number of pollen grains released from anthers of transgenic plants changed day to day during this period, regardless of experimental variant (full or empty space around the source of GM pollen). We observed big differences in pollen numbers between the first year (higher values) and the second year of this study, irrespectively on a day of observation (Figure 2a). Moreover, a greater dynamics of changes in the number of pollen grains between particular days of monitoring was observed in the second year compared to the first year of this study.



**Figure 2.** Fluctuations of the number of pollen grains in subsequent days of anthesis depending on the variant of the experiment and year of study; (a): first year, (b): second year of the study. In both graphs: dashed line—spatial isolation, dotted line - border rows.

In the first year, the number of transgenic pollen grains on slides increased gradually from day 1 (7000 and 15,000) to day 3 (12,600 and 16,500). Then, it rapidly increased in day 4: 21,000 and 44,000 pollen grains in the first and the second variant, respectively (Figure 2a). A further increase in the number of pollen grains up to day 5 was noted, when the maximum numbers of pollen grains on a slide were 32,500 and 44,000 in the first and in the second variant, respectively. While from day 6 to the end of observation (day 8) a sharp decrease in the number of transgenic pollen grains was noted. On day 6 the number of transgenic pollen grains was reduced to about 900 and 1800; while on day 8 the number of pollen grains was the lowest and equal to about 100 and 150, in the first and in the second variant, respectively (Figure 2a). The number of pollen grains was almost twice as high on day 4 and one third higher on day 5 in the spatial variant than in the variant with the border rows (Figure 2a). In the remaining days the number of pollen grains was similar in both variants (Figure 2a).

In the second year of experiment, the number of transgenic pollen grains recorded on the first day was low; in the border rows variant it was 1900 and in the spatial isolation variant it was 3400. On the second day the number of pollen grains increased to 2700 (border rows) and 4800 (spatial isolation). The number of pollen grains observed on this day was the highest during the second year of this study. On the third day we noted a reduction in the number of pollen grains to 700 and 900 (in the spatial isolation and in the border rows variants, respectively). On the following days a further reduction in the number of pollen grains was observed (Figure 2b). Between day 3 and day 8 fluctuations in the number of pollen grains were recorded. On day 8 only single pollen grains were

detected on a single trap, in total 60 pollen grains in the border rows and 20 in the spatial isolation variant (Figure 2b).

The number of pollen grains noted in the second year was four times lower than in previous year, regardless of the variant. The maximum number of transgenic pollen grains was 9-fold lower in the second year than in the first year. The maximum intensity of releasing of pollen grains from anthers in anthesis period was observed on day 2 in the second year versus day 5 in the first year, which indicated strong influence of weather conditions on the releasing course of triticale pollen and also on intensity of its dispersal.

### 3.3. Number of Pollen Grains and Distance from the Source

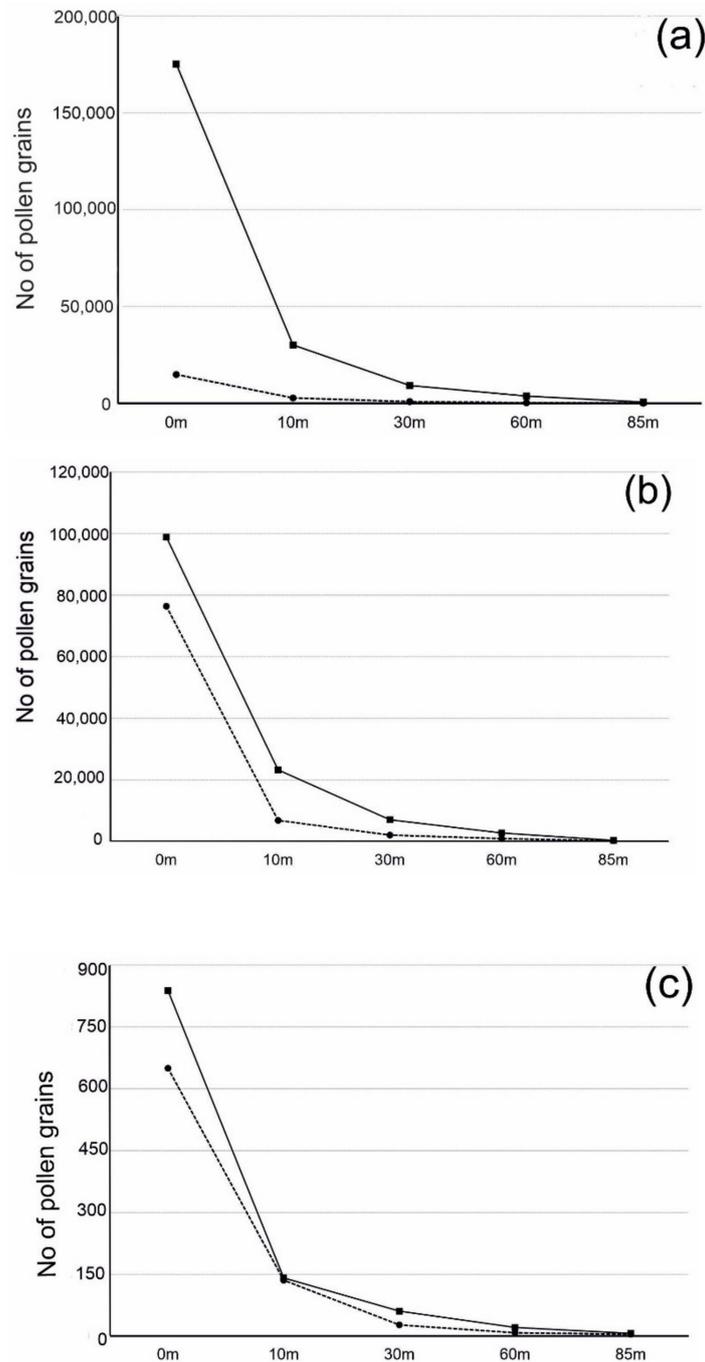
The total number of pollen grains on slide recorded over eight days of anthesis varied depending on distance between the field with transgenic plants and the trap location in the field. The highest number of pollen grains was noted in the direct vicinity of transgenic plants, and decreased sharply with the increase of the distance of the trap from the field, regardless of the year of study (Figure 3a). The total number of transgenic pollen grains recorded on traps localized in the closest vicinity of the field with plants (0 m) was 175,000 and 15,000 in the first and in the second year, respectively (Figure 3a). In both years at the same time, the number of transgenic pollen grains was 5 times lower at the distance of 10 m from the field with transgenic plants than at the source. The number of pollen grains decreased successively from the field with transgenic plants to the distance of 85 m. At distances of 60 and 85 m from the source the total number of pollen grains was very low; it was 3700 and 300 pollen grains in the first year and 600 and 100 in the second year, respectively (Figure 3a). Higher values of transgenic pollen grains were obtained in the spatial isolation variant as compared to the border rows variant, at all distances and in both years. At a distance of 85 m from the field with transgenic plants the total number of pollen grains was very low. In the first year it was 253 and 374 in border rows and spatial isolation, respectively and in the second year it was 46 and 67 pollen grains (Figure 3b,c).

The relationship between the number of pollen grains and the distance from the field with transgenic winter triticale plants is presented in Figure 4. The values of average, maximum and minimum of the number of pollen grains were the greatest in the immediate vicinity of the transgenic triticale, irrespective of the year and experiment design. As the distance from the field of transgenic plants increased, the number of pollen grains was getting smaller, independently of the year of study. The differences were higher in first year (Figure 4a) than in the second year (Figure 4b). Regression equation indicates that the number of pollen grains depended on the distance from transgenic triticale, regardless of the year of study. Values of the coefficients of determination ( $R^2$ ) were high (0.9). The regression function obtained fitted well to the actual data in each year of study.

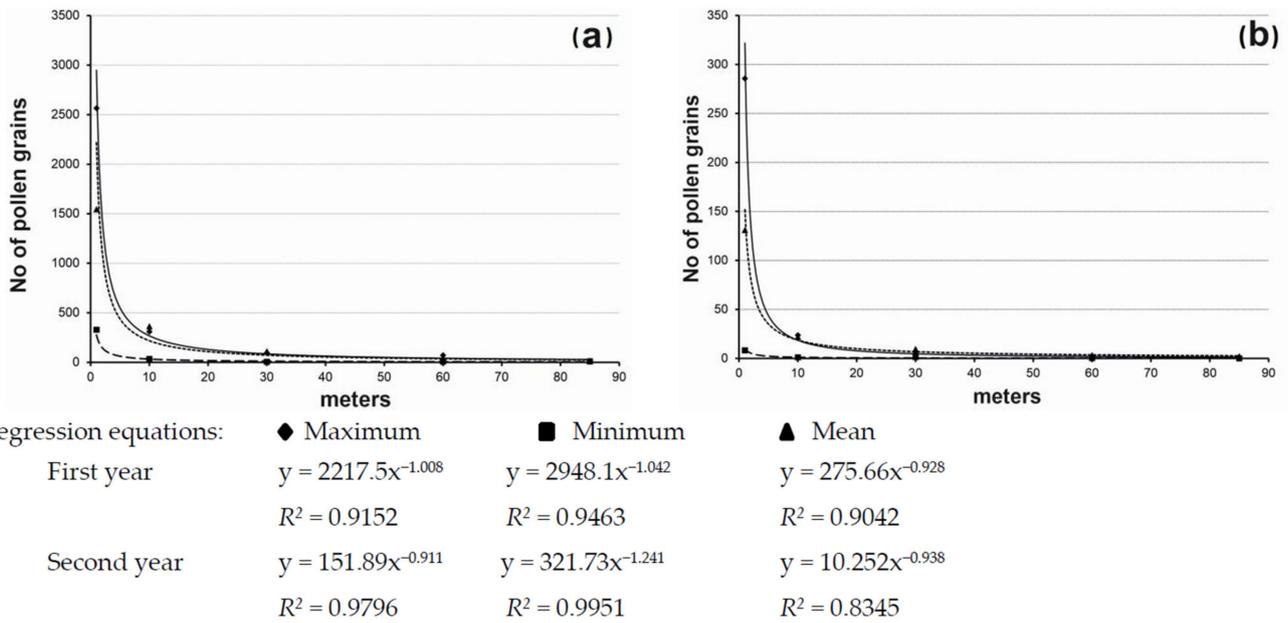
### 3.4. Number of Pollen Grains and Geographical Directions

The dispersal of transgenic pollen grains in the air highly depended on the weather conditions and on the geographical direction of the wind (Figure 5a,b). In the first year of the study, regardless of the experimental design, more pollen grains were moving towards the north (N), north-east (NE) and east (E) than to the west (W) and south (S) (Figure 5a). In the spatial isolation variant the number of detected transgenic pollen grains (total number counted over the eight days) was higher by 36% to 43% from the north direction to the east direction and by 73% on south east direction. However, the number of pollen grains was low in both variants. In other geographical directions differences in the number of pollen grains between the both variants were very small (Figure 5a). In the period of maximal pollen grain release from anthers (days 4 and 5) the amount of pollen noted in the N, NE, and E was higher by about 40% in the variant with spatial isolation than in border rows variant (Figure 5a). In the second year of the study most of pollen grains were transferred by wind to the south-west (SW) and south (S) and the lowest amount was moved to the north-east (NE) and north (N), in both experiment variants (Figure 5b). In the spatial isolation variant the number of detected transgenic pollen grains (total number counted

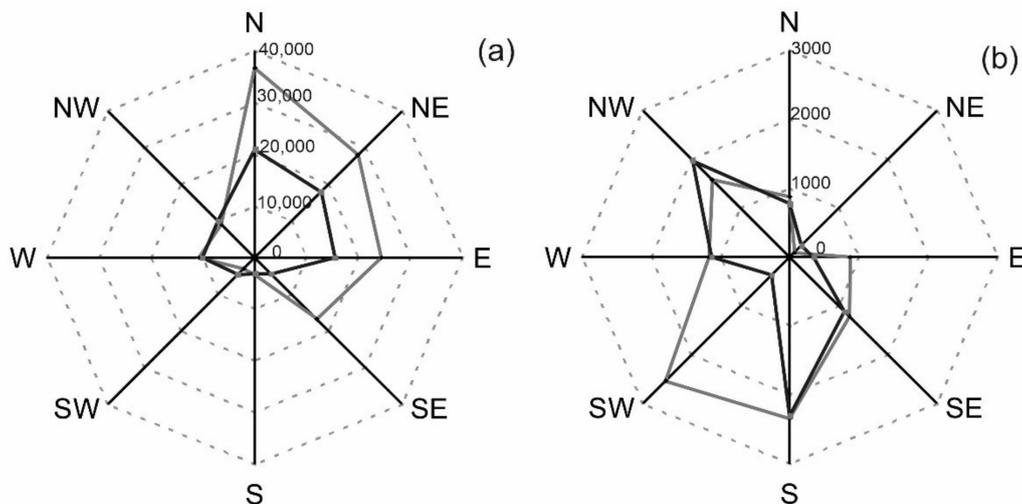
from eight days), compared with border rows variant, was higher by about 85% and 60% in south west (SW) direction and in the east (E), respectively. In the remaining geographical direction differences in the number of pollen grains between both variants were very small (Figure 5b). In the period of maximal pollen grain release from anthers (day 2) higher amount of pollen in the SW (by about 10 times) was noted in variant with spatial isolation than in the variant with border rows (Figure 5b).



**Figure 3.** Relationship between the number of transgenic triticale pollen grains and the distance in the first year (solid line) and second year of study (dotted line)—(a), and the relationship between the number of transgenic triticale pollen grains and the experiment design in the first (b) and the second year (c). In both graphs: solid line—spatial isolation variant, dotted line - border rows variant. For experiment design description see Figure 1.



**Figure 4.** The number of pollen grains on slides as a function of distance from the field with transgenic winter triticale plants in the first (a) and the second (b) year of the experiment; for both graphs: maximal values—solid line, minimal values—dashed line, mean values—dotted line.



**Figure 5.** Number of pollen grains of triticale plants captured at different geographical directions in first (a) and second year (b), in border rows (black line) and spatial isolation (grey line). Please note the different scale of (a,b) graphs.

The ANOVA analysis taking into account the wind directions showed significant differences in pollen spreading in both years. In the first year the biggest amount of pollen grains was recorded in the north, east and north-east, and was significantly different from other directions ( $F = 7.393, P = 0.000$ ). In the second year the significantly higher amount of pollen grains was found in the south ( $F = 3.122, P = 0.002$ ).

#### 4. Discussion

The results presented in this paper are discussed with literature data in the following aspects: the course of anthesis, dispersal rate and dispersal distances of transgenic winter triticale pollen grains. In this experiment the anthesis in the second year started seven days earlier than in the first year, regardless on experiment design. The anthesis phases of transgenic line and non-transgenic winter triticale cv. Bogo lasted for eight days and their

length was identical in both years of the study. During this anthesis phase, extrusion of anthers from spikelets and pollen grain release from anthers occurred. In field conditions, the duration of anthesis in triticale plants was caused by biological traits of flowering—firstly anthers of basal florets in central spikelets and in following spikelets towards the top and the base of main spikes were extruded. In next days of anthesis, the extrusion of anthers from other, younger spikes were observed. Although weather conditions in two consecutive years of the experiment were different, warm and dry in the first year versus cold and wet in the second year, it did not affect the course and duration of anthesis among tested transgenic, as well as of non-transgenic winter triticale plants. In another experiment conducted in Canada, Kavanagh with coworkers [12] showed that transgenic spring triticale plants (containing a dominant blue aleurone trait) were flowering earlier than non-transgenic spring triticale. Other data show, that the beginning of anthesis of transgenic plants was one to two days ahead of the non-transgenic plants of wheat and barley [34]. The duration of anthesis phase depended on properties of the cultivars, as well as on weather conditions and the anthesis time in wheat was 2–6 days (Percival 1921, Leighty and Sando 1924 cited by Waines and Hedge [18]), 8 days [34], 8.3 days in cold, wet conditions and 2.7 days in hotter, drier conditions [35].

In our study the number of transgenic pollen grains of winter triticale depended on the variant of the experiment as well as on the year of the study. In the first year, during few days, the number of pollen grains was higher compared with the second year. Furthermore, the number of transgenic pollen grains was higher in the variant with spatial isolation compared with border rows variant, regardless on the year of the study. The maximal number of transgenic pollen grains was observed on the fifth day in the first year and on the second day in the second year. The course and duration of anthesis, the intensity of pollen release from fully developed florets within matured spikes among tested transgenic plants in the field and the intensity of pollen dispersal into the atmosphere were affected by different weather conditions in both years.

Literature data show that the pollen dispersal is related to the number of pollen grains produced in anther and to the length of anthers. Different triticale genotypes, as well as wheat and rye genotypes varied in length of anther what affected the number of pollen grains per anther [22,36–39], and both traits were genetically controlled [40]. Triticale generally had longer anthers and produced more pollen grains per anther than wheat. Its anthers were shorter and produced less pollen grains per anther in comparison to rye [36]. In our study, the low air temperature and high humidity caused lower pollen grain release rate and considerably lower numbers of transgenic pollen grains in the second year in comparison to warm period (higher air temperature and low humidity), during anthesis phase in the first year of the study. Weather conditions play an important role in releasing of pollen grains from anthers and in their dispersal into atmosphere. In this aspect our data support the previous findings [16,21,22].

Results presented in this paper showed, that the number of pollen grains varied considerably depending on the distance from the field with transgenic plants in both years, regardless on the experimental variants. We observed that the highest number of pollen grains was noted in the direct vicinity of transgenic plants. With the increase of the distance from the field with transgenic plants the number of transgenic pollen grains decreased sharply and the same phenomenon was found in both years. At the distance of 85 m only single pollen grains derived from these transgenic plants were found, especially in the seventh and eighth day of anthesis.

Weather conditions at anthesis affected the release of pollen grains from anthers and the dispersal rate of pollen in the air. In the first year, when strong spreading of pollen grains was noted, the air temperature was high, insolation was long, there was no rain and humidity was low. The day preceding anther extrusion was also very warm and dry, which favored the maturation of flowers. Most presumably it also affected the intensity of releasing and dispersal of pollen grains in the next days. The second year was cooler, cloudy and rainy, and the florets (anthers) matured under unfavorable conditions. Anthesis started

under unfavorable conditions, it was cold and rainy, which shortened anther extrusion and release of pollen from anthers to two days and later a rapid decrease of intensity of pollen dispersal was observed. The number of transgenic pollen grains of winter triticale decreased in leptokurtic pattern with increasing distance from the field with transgenic triticale plants.

Literature data show that environmental factors such as humidity and temperature play significant role in pollen dispersal [11]. According to Aylor [41] the settling speed of corn pollen is fundamental for determining the distance of corn pollen transport in the atmosphere. Values of settling speed of corn pollen ranged from  $0.31 \text{ m s}^{-1}$ ,  $0.21 \text{ m s}^{-1}$  and  $0.17 \text{ m s}^{-1}$  for fully hydrated, mostly dehydrated and completely dehydrated pollen, respectively. These values depended on water content, shape, size and density of corn pollen [41]. Aylor concluded that dry corn pollen grains settle slower than fresh pollen [41]. Environmental factors such as rainfall, temperature, light intensity, wind and relative humidity as well as various stress conditions can directly affect floret opening, stigma receptivity, amount of pollen released, pollen viability and pollen movement [42]. The impact of various environmental factors on pollen flow and pollen mediated gene flow (PMGF) has been described for many species, such as wheat [42–45], rice [46], barley [47] maize, oilseed rape, sugar beet, and potatoes [45]. Many authors noted that the range of pollen flow is much greater than the distance at which the cross-fertilization can appear [10,43]. Pollen flow data can be used as the basis for establishing an appropriate isolation distance to avoid outcrossing in seed production. However data on PMGF should be collected to establish the isolation distance in the case of coexistence between conventional and GM crops [48]. Both pollen flow and PMGF (crop to crop and crop to wild relatives) are part of GMO risk assessment [48]. According to Loureiro et al. [49] isolation distance is the main barrier for crop-to-crop gene flow. The most effective means to reduce outcrossing and PMGF in GM triticale may be developing cultivars that exhibit a lower propensity for floret opening [12]. Additionally, barriers at field borders, such as tall, dense plants or nets are highly desirable [50].

We have demonstrated that the dispersal of transgenic pollen grains of winter triticale in the air depends on wind direction and depends on the year. In the first year of our study, during releasing of pollen grains from anthers (anthesis), more pollen grains were moving to the north (N), north-east (NE), east (E), while in the second year pollen grains were mostly moving toward the south west (SW) and south (S). In the experiments of Kavanagh et al. [12] the highest average PMGF, based on the number of triticale blue seeds occurred in the easterly direction at 0.2 m (5.07%) and at 50 m (0.14%). The lowest PMGF frequency at 0.2 m (1.92%) was recorded in the west and the lowest at 50 m (0.04%) was in the north [12]. The results depend on wind directions during the anthesis phase and other local conditions. In both years, in the experimental variant with spatial isolation, the higher number of pollen grains was measured in comparison to those in the experimental variant with border rows.

Summarizing, observed differences in the number of pollen grains between both variants studied in this experiment can be explained by the absence of plants, and more dynamic air circulation over the fallow in the spatial isolation variant, as compared to the fields full of plants (border rows variant). There are wind gusts and convection currents that take pollen and carry it over greater distances than the field surroundings only, but they are rare and highly dispersed. Weather conditions including such parameters as daily air temperature, air humidity, rain, insolation, wind force and wind direction influenced the beginning of anthesis phase, duration of this phase, and rate of pollen flow of tested transgenic winter triticale plants. The results were also consistent with the literature data [22].

The results obtained in this study are very important not only for the future cultivation of transgenic plants, but also for conventional and organic agriculture, especially for heterosis breeding and for the coexistence of transgenic, organic and conventional crops.

## 5. Conclusions

We have monitored pollen flow of triticale using the transgenic line and we have found that the number of pollen grains dramatically dropped down with the distance from the source. Only single pollen grains were reaching 85 m, which would not be enough for outcrossing, even in days with gusts of wind of  $3.1 \text{ m s}^{-1}$ . This is in line with the OECD Seed Schemes for self-pollinating varieties of triticale which shall be isolated from all other crops of triticale by 50 m for basic seed production data [51]. The conventional, ecological and transgenic triticale may be cultivated in the proximity provided the abovementioned distance is maintained. Natural barriers, such as high and densely growing plants at field borders can additionally secure the safety of crop coexistence and enrich the biodiversity of agricultural environment.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2073-4395/11/3/431/s1>, Table S1: Atmospheric conditions during the anthesis phase in first year (A) and in second year of study (B).

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**Data Availability Statement:** The data presented in this study are available on request from the first authors.

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