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**Genetically modified maize:
Factors affecting cross-pollination and coexistence**

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II. List of abbreviations

ASI: Anthesis-silking-interval

Bt: *Bacillus thuringiensis*

CCM: corn-cob-mix

CPR: cross-pollination rate

Ct: cycle threshold

DNA: deoxyribonucleic acid

E: early maturity group

EC: European Commission

EU: European Union

FAO: world food and agriculture organisation

GM: genetically modified

GMO: genetically modified organism

GV: gentechnisch verändert

GVP: gentechnisch veränderte Pflanzen

HT: herbicide tolerant

IR: insect resistant

IRMM: Institute for Reference Materials and Measurements

JRC: Joint Research Center

L: late maturity group

qPCR: quantitative polymerase chain reaction

SA: seed admixture

SL: seed lot

UV: ultra violet

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V. Summary

Maize is worldwide one of the most important crops in terms of area grown and yield. Cross-pollination is a natural process occurring in maize. It was studied by plant breeders in former years in order to guarantee seed purity and to preserve identity of hybrids. With the introduction of commercial cultivation of genetically modified (GM) crops in the year 1996, there have been concerns about the possible cross-pollination between GM and non-GM maize. According to maize biology, with about 95% cross-pollination, adventitious presence can arise through many pathways. These include cross-pollination between adjacent fields, seed dispersal from volunteers and mechanical transfer at sowing, harvesting, transport and storage operations. A central point of the European agricultural policy is that farmers should be able to use the agricultural system of their choice. To achieve this policy, recommendations and guidelines have been released. This includes field management strategies to reduce the level of cross-pollination. Worldwide field trials have been conducted using colour makers or qPCR for the detection and quantification of gene flow. To date no field trials have been conducted with the aim to compare the effects of different gap crops separating the donor and the recipient fields on the cross-pollination rate. Moreover no field trials have been conducted to investigate the effects of GM seed admixture on the non-GM harvest product.

Three chapters (Chapter 2 to 4) describe the conducted field experiments to investigate the possible coexistence of farming systems with and without the use of GM crops.

Field trials described in the second chapter were to investigate the consequences of GM seed admixture on the non-GM harvest product. We determined the effect of maturity group on the level of cross-pollination and we compared the visual detection with the detection method using qPCR. The results of the field trials showed that the GM output is equal to the GM input. The standard deviations observed depend on the location, the flowering time which determine the pollen concurrence and weather conditions which determine the viability of pollen. By the admixture of seeds with different maturity groups, the GM output was lower than the GM input. In addition, we showed that the visual detection differed from the detection using qPCR. Certified Reference Material is used to calibrate housekeeping gene and transgene in unknown samples during the qPCR run. The seed DNA content per mass unit can be different among maize cultivars. Thus, the use of plasmid DNA as a standard for quantification can be recommended.

The objective of the third chapter was to verify appropriate separation distances for ensuring coexistence between GM and non-GM maize production fields and to analyse the extent of cross-pollination into donor-facing field edges of maize recipient plots. Moreover, the effect of different gap crops (wet vs. dry microclimatic conditions) on the cross-pollination rate between two adjacent maize fields has been investigated. Although the cross-pollination rates varied from year to year, it is shown that the cross-pollination rate is highest at border rows facing the donor (edge effect) and decreases rapidly with increasing distance from the donor field.

Chapter four was undertaken to investigate whether the level of cross-pollination is correlated with the flowering time on the one hand and with climatic conditions on the other hand using data from two cross-pollination field experiments over two years. No clearly defined correlation was found between the flowering time, weather conditions and the cross-pollination rate.

The following recommendations can be made to ensure the better coexistence of farming systems with and without GM in the case of good agricultural practice:

- Our recommendation is to permit a maximum contamination of 0.3 or 0.5% GM seed in non-GM seed lots, to be sure that the contamination of the harvest crop remains below the critical limit of 0.9%.
- Our qPCR results were more variable due to uncertainties because of the use of maize flour as Reference Materials. We recommend the use of plasmid DNA to avoid uncertainties by the calculation of the copy number of reference materials.
- There was no significant difference between the cross-pollination rates using clover-grass or barley stubble as gap crops. As mentioned by many authors, isogenic maize field adjacent to the donor field may better reduce the cross-pollination in a recipient field due to pollen dilution.
- The cross-pollination rate at border rows facing the donor in the case of adjacent maize fields in the main wind direction depends more on wind speed than on the isolation distance between the donor and the recipient field. Our recommendation for good agricultural practise is to harvest the first 12 recipient rows separately from the rest of the field.

VI. Zusammenfassung

Mais ist weltweit eine der wichtigsten Kulturarten und die wichtigste Getreideart bezogen auf die Anbaufläche und den Ertrag. Auskreuzung ist ein natürlicher Prozess beim Mais. Auskreuzung wurde in den vergangenen Jahren von Pflanzenzüchtern untersucht, um Saatgutreinheit zu gewährleisten und die Identität der Hybride zu sichern. Mit der Einführung und dem kommerziellen Anbau von gentechnisch veränderten Pflanzen (GVP) im Jahr 1996 entstand die Möglichkeit eines Genaustausches zwischen GV Mais und Nicht-GV Mais. Ungewollte Vermischungen können auf mehreren Wegen geschehen. Zu diesen Wegen gehören Auskreuzung zwischen zwei benachbarten Feldern, Durchwuchsmais und mechanische Übertragung von Maiskörnern während der Ernte, Transport und Lagerung. Ein Zentralpunkt der Europäischen Landwirtschaftspolitik ist, dass Landwirte die Möglichkeit haben müssen, Landwirtschaftssysteme ihrer Wahl durchzuführen. Um diese Politik zu ermöglichen, sind Empfehlungen erlassen worden. Diese beinhalten Feldmanagement-Strategien, um die Auskreuzungsrate zwischen zwei benachbarten Feldern zu reduzieren. Weltweit sind Feldversuche durchgeführt worden, um Auskreuzung zu detektieren und zu quantifizieren. Kein Feldversuch wurde bis heute durchgeführt, um den Einfluss von unterschiedlichen vegetativen Oberflächen zwischen einem Donorfeld und einem Rezipienten zu untersuchen. Zudem wurde nie ein Feldversuch durchgeführt, um die Auswirkung einer GV-Saatgutvermischung auf das Ernteprodukt zu untersuchen.

In Kapitel 2 bis 4 werden die hier durchgeführten Feldexperimente beschrieben, mit denen verschiedene Einflussfaktoren auf die Auskreuzung zwischen benachbarten Maisfeldern und die Koexistenz von Landwirtschaftssystemen mit und ohne Gentechnikanwendung untersucht wurden

Bei den im Kapitel 2 beschriebenen Feldversuchen wurde die Auswirkung einer GV-Saatgutvermischung auf das Nicht-GV Ernteprodukt untersucht. Wir haben ebenso die Auswirkung der Reifegruppe auf die Höhe der Auskreuzung untersucht sowie einen Vergleich zwischen der visuellen Detektionsmethode und der qPCR durchgeführt. Die Ergebnisse der Feldversuche haben gezeigt, dass der GV-Anteil im Saatgut ungefähr gleich dem GV-Anteil im Ernteprodukt ist. Bei der Saatgutvermischung mit unterschiedlichen Reifegruppen war der GV-Anteil im Ernteprodukt niedriger als im Ausgangssaatgut. Diese Unterschiede hängen von mehreren Faktoren ab, wie dem Standort, der Blühsynchronität zwischen Donor und Rezipient und den Wetterbedingungen. Wir haben auch gezeigt, dass es einen Unterschied gibt zwischen der visuellen Detektionsmethode und der qPCR. Referenz-Material wird benötigt, um Maisreferenzgene und Transgen in Proben zu kalibrieren. Der DNA-Anteil pro Masseneinheit variiert zwischen Maisvarietäten. Deshalb empfehlen wir die Benutzung von Plasmid-DNA zur quantitativen Kalibrierung.

Das Ziel der im 3. Kapitel beschriebenen Versuche war, Isolationsdistanzen zu verifizieren, um die Koexistenz zwischen GV und Nicht-GV Maisanbau zu gewährleisten und den Randeffekt am Rezipientenfeld zu untersuchen. Außerdem wurde der Einfluss von verschiedenen Zwischenkulturen (feuchte bzw. trockene mikroklimatische Bedingungen) auf die Auskreuzungsrate zwischen zwei benachbarten Feldern untersucht. Die Auskreuzungsrate variierte von Jahr zu Jahr. Es konnte gezeigt werden, dass die Auskreuzungsrate am Rand des Rezipienten schnell abnahm mit zunehmender Distanz vom Donor. Die Auskreuzung wurde mit unterschiedlichen Funktionen beschrieben. Der vertikale Pollentransport über die Distanz war mehr vom Windabhängig als von Unterschieden der Thermik, die durch feuchte oder trockene Bedingungen bei verschiedenen Zwischenkulturen entstehen.

Mit den im 4. Kapitel beschriebenen Versuchen sollte geklärt werden, ob die Auskreuzungsrate mit dem Blühbeginn einzelner Probestellen oder mit den klimatischen Bedingungen korreliert. Erfasste Wetterdaten während der Blühphase wurden verwendet, um die Korrelation zu berechnen. Keine klar definierte Korrelation wurde erhalten zwischen der Auskreuzungsrate und dem Blühbeginn sowie zwischen Auskreuzungsrate und klimatischen Bedingungen. Die Auskreuzungsrate hängt von vielen Faktoren ab, allerdings ist eine Vorhersage nicht immer möglich.

Von den hier erzielten Ergebnissen können folgende Schlussfolgerungen für die landwirtschaftliche Praxis gezogen werden:

Der GV-Anteil im Ernteprodukt entspricht ungefähr dem GV-Anteil im Saatgut. Bei der Koexistenz von Landwirtschaftssystemen in der EU könnte ein Schwellenwert von 0.3% im Saatgut eingeführt werden. Der Schwellenwert von 0.9% im Ernteprodukt wird dann nicht überschritten.

Ein Unterschied wurde festgestellt beim Vergleich zwischen visueller Detektion und qPCR. Grund für diesen Unterschied sind biologische Faktoren, wie Maiskörnerzusammensetzung. Eine Lösung wäre die Benutzung von Plasmid DNA bei der Kalibrierung der qPCR. Die Kopienzahl ist bei Plasmid DNA bekannt und dies kann die Umrechnung der DNA-Menge vereinfachen.

Es konnte kein Unterschied in der Auskreuzungsrate bei Verwendung von Klee-Gras und Gerste-Stoppel als Zwischenkultur festgestellt werden. Wie von einigen Autoren vorgeschlagen, ist Mais die einzige Pflanze, die wirkungsvoll als Barriere benutzt werden kann, um die Auskreuzungsrate zu reduzieren. Da spät blühende Pflanzen mehr zu hohen Auskreuzungsraten neigen, sollten für die Praxis homogenere Pflanzenbestände angestrebt werden.

Die Auskreuzungsrate nimmt mit zunehmender Distanz vom Donor ab. Aufgrund des Randeffektes ist es empfehlenswert, die Isolationsdistanz einzuhalten und die ersten 12

Rezipientenreihen separat zu ernten. Diese Maßnahmen reichen aus, um die Auskreuzungsrate unter dem Schwellenwert von 0.9% im Ernteprodukt zu gewährleisten.

1. General Introduction

1.1 Importance of maize crops

Maize (*Zea mays L.*) is one of the most important crops in the world in terms of growing area and utilisation (FAO, 2006) and the most widely distributed crop as well. Worldwide about 160 million hectares are used for grain maize cultivation (Deutsches Maiskomitee, 2009). Approximately half of this area belongs to developing countries. In the European Union (EU), the area grown with maize in 2008 was 8.9 million hectares for grain maize and 5.1 million hectares for silage. The main maize producers are the United States of America (USA) with more than 40% of the worldwide production. Other leading maize producers are China (21%), the EU (10%) and Brazil (8%) (Deutsches Maiskomitee, 2009). In 2008, the worldwide production of maize was around 800 million tonnes just slightly more than rice (~787 million tonnes) and nearly two-fold compared with wheat (~441 million tonnes). The EU production of maize was 61 million tonnes for grain maize and 208 million tonnes for silage maize. In the last decade the maize growing area has increased due to the increasing need for food and industrial raw material. Maize is capable of very high yields. According to UN's Food and Agriculture Organisation (FAO), maize yields currently reach on average 15 dt/ha in Africa, slightly more than 30 dt/ha in Latin America, and 17 dt/ha in India. The EU has the highest area productivity of 86 dt/ha followed by the USA (81 dt/ha). The worldwide diversity of maize use includes: maize grain, starch products, corn oil, baby food, popcorn, maize-based food items, maize flour, forage for animals, and maize stalks as soil mulch. The different use of maize depends on the requirements and the development status of the country. In the USA, Canada and other industrial countries (Europe) grain maize is mainly grown for livestock feed and in smaller proportions for sweeteners like maize syrup for industrial use as ethanol. Due to the growth in the consumption of meat and poultry the demand for maize increases steadily. In many developing countries of the world (Africa, South America) maize and maize meal constitute a staple food for human consumption. Depending on the growth of the population, the demand for maize grows in these countries as well (Ortiz, 1998; Pingali, 2001). In Germany, approximately 2.1 million ha were grown with maize in 2008. This is about 17% of the total growing area in Germany (Top Agrar, 2009), including 12.000 ha organic maize. This area is expected to increase in 2009 (Deutsches Maiskomitee, 2009). One third of the 2.1 million hectare maize growing area is used for grain maize production inclusive corn cob mix and two thirds for silage maize production. Silage is used for livestock feed or for biogas production and energy exploitation. Due to the rising need for energy production the maize acreage increased from 1.874.082 hectares in 2007 to 2.087.100 hectares in 2008. The total production volume was 5.1 million tonnes for grain maize and 70.1 million tonnes for silage maize in 2008. Germany belongs to countries with the highest area productivity. The area productivity in 2008 was 99.1 dt/ha for grain maize inclusive CCM (Corn-cobs-mix) and 451.4 dt/ha for silage maize. The use and cultivation of grain maize is restricted in Germany, due to climatic conditions. Germany belongs to the countries with temperate climatic conditions. This results in a short vegetation period. Hence the cultivation of maize is restricted to maize varieties with a maturity group under 350.

1.2 Maize biology

In plant kingdom maize belongs to the family poaceae and is a monoecious plant with a male (tassel) and a female (ear) inflorescence.

The male inflorescence, the tassel, arises from the shoot apical meristem. Pollen is produced in the anthers of the tassel at the tip of the maize plant. At the maturity stage anthers suspend on filaments that emerge from the tassel and from an opening on the top of the anthers, pollen is released. Pollen is the main carrier of genetic information. Maize pollen are very heavy and belong to the heaviest in the plant kingdom with a diameter of 90 to 125 μm (Raynor *et al.*, 1972) and a weight of 0,25 μg (Emberlin *et al.*, 1999; Jones and Newell, 1948; Miller, 1985). Pollen grain is made up of three layers: the outermost layer, the intine layer and the pollen coat. The outermost also multilayered exine is made up of a polymer called sporopollenin and is resistant to various chemicals such as sulphuric and phosphoric acid. The intine is made up of cellulose and the pollen coat contains proteins, lipids and pigments (Edlund *et al.*, 2004). For pollen release, even a slight breeze or vibration (e.g. as can be caused by a gently tapping of the stem) will release the pollen into the atmosphere, when it is mature. The low wind speed threshold for maize pollen release influences its dispersal into the environment. Eastham and Sweet (2002) and Urebelarrea *et al.* (2002) estimated that about 14 to 50 million pollen grains are produced per average-sized plant while some modern hybrids are producing 4 to 6 million pollen grain per plant during the flowering period. Maize ears need between 500 and 1000 pollen for the fertilization. Consequently, more pollen is produced than needed for the auto-pollination. Jones and Newell (1948) and Raynor *et al.* (1972) reported a production rate between 9.000 and 50.000 pollen grains per kernel set, depending on the genotype. Tassels may shed pollen for 2 to 14 days depending on genotype and environmental factors. A range of studies have focused on the pollen shed period. The main shed period varies between 5 to 8 days and begins about 3 days after the first anthers are visible. Cool and cloudy weather delays the period of shed by one to two hours per day (Burris, 2001). Jarosz *et al.* (2005) reported a pollen shed period between 5 to 8 days and from 7 am to 6 pm with the main shed time between 11 am and 12 am. Under favourable conditions the vast majority of pollen shed may occur within a period of 2 days. Under unfavourable weather conditions, this period expands up to 8 days (Treu and Emberlin, 2000; Wolt *et al.*, 2003). A flowering time between 10 to 15 days has been observed by tropical maize varieties (Paterniani and Stort, 1974). Maize releases pollen mainly during dry weather conditions. Jarosz *et al.* (2005) found a correlation between the dynamics of pollen release and the dynamics of air vapour pressure surrounding the tassel, suggesting that pollen release started once the vapour pressure deficit increased about 0.2 to 0.5 KPa.

The fundamental objective of pollen production in the plant kingdom is for species proliferation. Huge amounts of pollen are transported through the atmosphere to fertilize compatible plants. To fertilize neighbouring plants, pollen has to be viable when landing on the silk and this depends on environmental factors such as temperature, relative air humidity and air pressure. Many studies have been performed to determine the effect of environmental factors on pollen viability (Devos *et al.*, 2005). The simultaneous effect of several environmental factors on pollen viability is still unclear. Many published studies have focused only on one or two weather factors. Eastham and Sweet (2002), Glover (2002) and Schoper *et*

al. (1987) stated that maize pollen viability is enhanced by cool temperature and high relative humidity. According to Roy *et al.* (1995), maize viability decreases with increasing temperature above 50°C. This is in accordance with Luna *et al.* (2001) and Aylor, (2004) who found that pollen dehydration during hot and dry conditions should be the main factor of pollen mortality. By measuring the viability of pollen in the atmosphere after shedding, Luna *et al.* (2001) found a loss of pollen viability of 80% in 1 h and 100 % in 2 h. At pollen release, the moisture of the grain is about 55 – 60% and pollen viability is lost by moisture content of about 30%. Aylor (2004) measured the highest viability at the first 30 to 60 min after pollen release. When pollen is released it is subjected to sunlight and, therefore, UV-radiation. Aylor, (2004) investigated the effect of UV-C on pollen viability.

Compared to the sensitivity of fungi species and their development forms such as the conidia form of *Venturia inaequalis* and the urediniospores of *Uromyces appendiculatis* maize pollen features a higher resistance rate to UV-C radiation. Furthermore, the process of adhesion, germination and length of pollen tube decreases substantially during the dehydration process (Barnabas, 1985). The result of this decrease is the reduction of pollen fitness and the qualitative and quantitative pollen competition. Concluding, pollen vitality is more affected by the water status of the pollen, and high temperature and decreasing pollen viability affects the ability of pollen to cross-pollinate. When pollen takes up water, it is no more capable to germinate. During pollination processes, pollen is captured by trichomes, if it reaches the receptive area of the silk and if the pollen is viable and the silk receptive, the pollen fecundates the silk. A twofold fecundation occurs, the first one in the embryo sack with 2 sperm cells (Coe, 2001).

Silk is the style of the female part of the flower. Silk emerges from the ovary at the base of the ear. Depending on the genotype, silk undergoes a well defined sequence of emergence, elongation and senescence (Basseti and Westgate, 1993a), and this is done in a well coordinate time table. Deutsches Maiskomitee (2003) and OECD (2003) mention that usually one, sometimes two, female flowers are originated from the axillary shoot meristem despite an initiation in each axillary shoot meristem. A certain number of lines are arranged by pair in line on the ear. Bommert *et al.* (2005) stated that each spikelet pair meristem forms a short branch which bears two spikelet meristems and that only one meristem is developed into a fertile flower on the ears. There is a variation in the number of spikelet depending on the genotype and environment. Each spikelet is capable to be fertilized and develops into a kernel (Tollenaar *et al.*, 1997). According to Harris *et al.* (1976) the abortion or growth cessation of a second ear usually occurs during or just after silking of the uppermost ear. The dominance of the first formed organ (Bangerth, 1989) can explain the abortion or growth cessation of secondary ears. To date, plant breeders are focusing their effort to breed modern hybrids with only one ear. The silk receptivity begins at emergence and can remain for 10 days depending on environmental conditions and nutrient status of the plant. The silks themselves do not readily retain pollen, almost all of which is captured on the trichomes (hairs) on the silks (Aylor *et al.*, 2003). Every single silk directs the germ tube of germinating pollen grain to one ovule. Once fertilized the stop of silk elongation and a rapid desiccation have been reported. After entering the embryo sack, one sperm fertilizes the egg, forming the embryo; the other one fertilizes one of the polar nuclei before fusing with the second polar nuclei to form the

endosperm, which is triploid. The result is the development of a kernel. If the silk is not fertilized, it will continue the process of elongation until fertilization or the process of elongation is complete (Burriss, 2001). The silk can reach a length of 15 cm at the end of the elongation process (Aylor *et al.*, 2003; Burriss *et al.*, 2002). Bassetti and Westgate (1993) reported that the senescence of the silks begins 7 to 8 days after the emergence from the husk. At the stage of senescence, pollen tube would not be able to grow through the senescing area anymore. Herrero and Johnson (1981), Westgate and Boyer (1986) investigated that water deficit is the main limiting factor by silk viability during the day. By water deficit the senescence of silk exposed for several days is accelerated compared to the senescence of new emerging silk. Plants favour this way new emerging silk. Hence, only a restricted area of the silk is receptive and, therefore, a huge amount of pollen is needed for sufficient fertilization of the silk.

Maize, although self-fertile, is typically cross-pollinated by wind (95%) because of differences in floral synchrony between male and female flowers of a single plant (Burriss *et al.*, 2002). The time difference between male and female flowering is called anthesis-silking interval (ASI). In the case of cross-pollination, 2 ASI's are distinguished, the ASI1 and the ASI2. The ASI1 characterizes each variety and the ASI2 gives the difference in days between the start of male flowering of the donor variety and the start of female flowering of the recipient variety. Silk emergence occurs under favourable conditions 2 to 4 days after pollen shed (Deutsches Maiskomitee, 2009). The time between pollen shedding and silk emergence depends on the genotype and environmental factors. The ASI tends to increase by water deficit, nutrient, light and lack of thermal adaptation. Lafitte *et al.* (1997) denoted an increase of the ASI from 2 to 7 days when the normal environment of the plant changed.

1.3 Recent development in maize breeding

The centre of origin of maize is the Mesoamerican region, probably in the Mexican highlands, from where it spread rapidly. Archaeological records and phylogenetic analysis suggest that the domestication began at least 6000 years ago (Piperno and Flannery, 2001; Matsuoka *et al.*, 2002). Maize spread around the world after European discovery of the American continent in the 15th century, particularly in temperate zones (Paliwal, 2000; Farnham *et al.*, 2003). During the centuries of the domestication of maize, every growing region produced or selected its own maize cultivars or landraces. Farmers have maintained and improved these varieties and they are adapted to local requirements and characteristics (Paliwal, 2000). Maize can be grown in a number of environments (reviewed in Paliwal, 2000; Farnham *et al.*, 2003) from 58° North (e.g. Canada and the Russian Federation) to 40° South (e.g. Chile). Generally, tropical maize is grown between 30° North and 30° South, subtropical maize between 30° and 34° both North or South, and temperate maize beyond 34° latitudes. It can be grown in a range of altitudes from sea level up to 3800 m and with growing seasons between 42 and 400 days. This ability to grow in a wide range of environments is reflected in the high diversity of morphological and physiological traits.

Maize was domesticated from its wild ancestor teosinte (Matsuoka *et al.*, 2002) at a single domestication region in Central America. The north- and the southwards propagation began

from Central America, where maize was first discovered. The expansion of maize in the Northern and Southern hemisphere took place very late, because maize cropping was limited by day lengths, seasonality's and different climate (Diamond, 2002). With the introduction of hybrid maize to replace open pollinated varieties at the beginning of the 20th century, a great progress was made. From the low yielding open pollinated species, high yielding species were bred using modern maize breeding methods. Hence, maize became one of the most important crops in the world in terms of growing area and yield. Most of the maize grown in the USA in the middle of the 20th century was hybrid maize. Due to the intensive use of area for maize cultivation, breeders have to face new challenges, concerning crop protection caused by increasing pressure of diseases and pests.

Since 1970, with the discovery of techniques allowing the recombination of DNA, new ways were found to modify the genetic information in plants. Nowadays, three different traits are of great interest, the herbicide tolerance (HT), and insect resistance (IR) to the European corn borer (*Ostrinia nubilalis*) and to the Corn Rootworm (*Diabrotica virgifera*). In addition, combinations of herbicide tolerance and insect resistance traits (called stacked genes) are also available on the market (James, 2008). Many other traits like virus resistance, disease resistance, and altered composition are available but the use is still restricted to some countries or regions in the world. Transgenic maize crops for abiotic stress resistance (e.g. cold, drought and high salinity), pest resistance (nematodes, fungi) and yield improvement may be introduced in the near future (Kast, 2007).

Genetically modified (GM) maize is the second most important GM crop following soybean with about 23% of the total GM crop area worldwide (James, 2008). The first commercially grown GM maize was registered in the USA and Canada in 1997. The global area with GM maize is still (and steadily) increasing. Even between 2007 and 2008 it increased by 6 %. The number of countries growing GM maize has increased in 2008 as well, when GM maize has been cultivated in 17 countries worldwide. The biggest ones are the USA with about 28 million hectares followed by Argentina and Canada with more than 1 million hectares each (James, 2008). In 2008 one new country in Africa (Egypt) has introduced GM maize to commercial cultivation in addition to South Africa. The total area of GM maize in Africa aggregates to more than 1.6 million hectares in 2008 (James, 2008).

In the EU the growing area of GM maize has increased since the introduction of the commercial cultivation in 1998. In 2008, GM maize was cultivated in 7 EU countries and the area reached 107.717 hectares. Spain is the main producer of GM maize with more than 79.269 hectares. This is about 74% of the total area grown with GM maize in Europe (www.transgen.de). Only one GM maize event (MON810) is approved for commercial cultivation in Europe. A gene of the bacterium *Bacillus thuringiensis* (Bt) which expresses the lepidopteran-active Bt toxin Cry1Ab has been introduced into the maize genome. Once in the intestine milieu of insects, it will result in the perforation of the intestine wall and therefore the death of the insect (de Maagd *et al.*, 2001; Moeser, 2006).

In Germany, Bt-maize was cultivated and commercialized since 2005. The area used for Bt-maize cultivation has increased from 342 hectares in 2005 to 3171 hectares in 2008. This is about 3% of the total GM crop area in the EU. Since April 2009, a ban for cultivation of Bt-

maize MON810 has been enacted in Germany being already in force in the member states Austria, France, Greece, Hungary, and Luxembourg. Currently, the cultivation of genetically modified maize in Germany is restricted to field releases.

1.4 Worldwide Legislations for GMO

With the introduction of GM crops, concerns about the risk for the environment and human health have been raised. According to Brown (2001), Conner *et al.* (2003) and Dale *et al.* (2002), the advent of GM crops had led to considerable controversy concerning issues of agricultural and environmental impact, in part, because of the rapidly increasing GM acreage. Worldwide measures have been taken to ensure the transparency in the use of GM crops. Many countries have introduced thresholds to regulate the use and the commercialization of GM crops with the aim to enable the coexistence between farming systems with and without GM. According to Brookes *et al.* (2004), coexistence is an issue related to “the economic consequences of adventitious presence of material from one crop in another and the principle that farmers should be able to cultivate freely the agricultural crops they choose, be it GM crops, conventional or organic crops”. Labelling thresholds for the presence of genetically modified organism in food and feed are diverse: from 0% up to 5% in different countries and regions around the world (Table 1.1). For instance, GM labelling is voluntary in the USA and Canada and mandatory in Japan at a threshold level of 5%. This gives consumers the freedom of choice in the selection of their food.

Table 1.1: Regulation concerning the labelling of GM food and feed products in selected countries (Alexander *et al.*, 2007)

Country	Labelling status	Threshold (%)	Date of implementing threshold regulation
Australia and New Zealand	Mandatory	1	December 2001
Brazil	Mandatory	4	December 2001
Canada	Voluntary ^b	-	December 1994
China	Mandatory	0	July 2003
European Union	Mandatory ^c	0.9	July 2003
Hong Kong	Voluntary	5	February 2001
Israel	Mandatory ^d	1	
Japan	Mandatory for selected products	5	April 2001
Korea	Mandatory for selected products ^d	3	13 June 2001
Malaysia	Mandatory	3	Proposal
Russia	Mandatory for selected products	5	1 September 2002
Switzerland	Mandatory	2 or 3 (feed ^e), 0.5 (imported seeds)	
Taiwan	Mandatory	5	Proposal
Thailand	Mandatory for selected products	5	Proposal
United States	Voluntary	-	January 2001

Data have been converted to SI units.

^aData from Jia (2003) und ISAAA (2005).

^bLabelling required if safety concerns (allergenic, change in nutritional composition) exist.

^clabelling required for the adventitious presence at a 0.9% threshold for approved GM organisms or 0.5% for GM organisms given a favourable risk assessment but not yet approved. Includes both feed and food products.

^dLabelling required only if recombinant DNA or proteins are detected.

^eThreshold for feeds containing raw material of a single source is 3%. For mixed feeds, a threshold of 2% exists.

The European Commission (EC) has released guidelines for the development of strategies and managements to ensure the coexistence between all farming systems (EC, 2003). One of the most important management strategies is the implementation of separation distances. In the EU, separation distances for GM cultivation (proposed or implemented by Member States) range between 25 m and 800 m for grain maize in dependence on the neighbouring field being cultivated conventionally or organically (Devos *et al.*, 2009). In Germany, separation distances have been set at 150 m for conventional maize production and 300 m for organic maize production (www.biosicherheit.de).

1.5 Adventitious presence of GMOs in maize derived products

The term “adventitious presence” refers to the unintentional and incidental presence of trace amounts of foreign material in agricultural commodities. Adventitious presence is an unavoidable reality of plant biology, seed production and the distribution of commodity crops. While adventitious presence can be minimized, as a practical matter it cannot be eliminated entirely. Adventitious presence of GM maize in non-GM maize derived products may have several causes (Figure 1.2): Cross-pollination between non-GM crop and a neighbouring GM maize field, GM impurities in seed lots (cross-pollination during field production or admixture during post-harvest processes) and volunteers from previous maize crops (Devos *et al.*, 2005; Messéan *et al.*, 2007). Estimated average potential rates of adventitious presence within the 0.9% threshold set by the EU labelling legislation are 0.3% for seed impurity for cross-pollinating species like maize, 0.5 % for crop to crop cross-pollination and 0.1% for all other sources like volunteers, machinery etc. (Scientific Committee on Plants, 2001). Currently a zero tolerance for any GM material in maize seed lots is valid. The main source of GM presence in maize derived products is caused by pollen-mediated gene flow from neighbouring GM-maize fields. Pollen-mediated gene flow remains an intra-specific phenomenon in Europe because there is no wild relative (teosinte) existing. Volunteers have just a regional importance. In regions where winter temperatures do not usually fall to low enough levels to kill off volunteers (e.g., Mediterranean countries like Spain), they are common. The influence of the volunteers' presence on adventitious presence depends on the number of volunteers that reach the flowering stage and the flowering coincidence with the subsequent crops (Pla *et al.*, 2006).

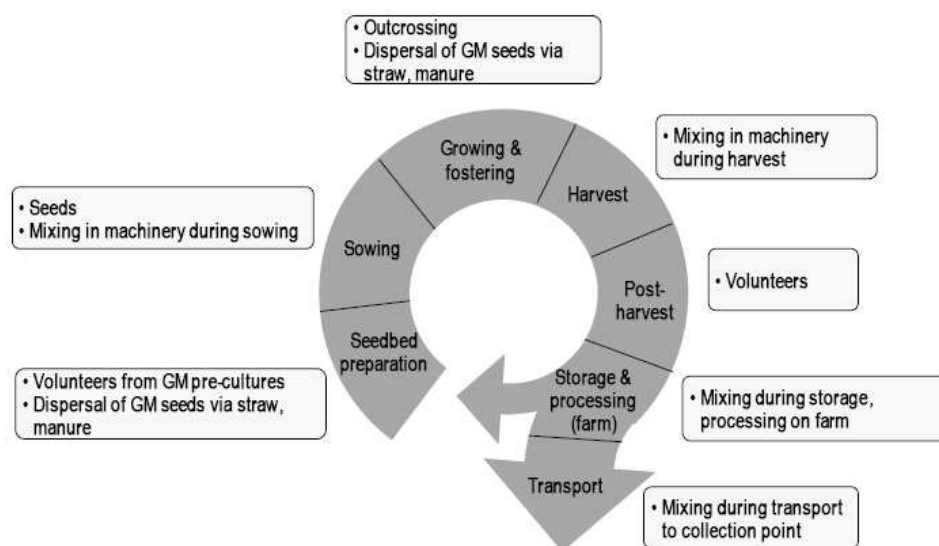


Figure 1.1: GM-dispersal routes in agricultural production systems.

1.6 Pollen mediated gene flow

Understanding pollen-mediated gene flow is crucial for the development of coexistence measures for farming systems with and without GMO. There are many factors involved in the process of pollen-mediated gene flow in maize: distance between donor and recipient fields,

wind direction, wind speed, flowering synchronization between donor and recipient plants, field topography, size and orientation of donor and the recipient fields, furthermore weather conditions like rain, temperature and air humidity (Devos *et al.*, 2005).

Because of pollen characteristics (e.g. weight and size), pollen cannot be transported to further distances (Raynor *et al.*, 1972). Di-Giovanni *et al.* (1995) reported that maize pollen settled nearly ten times faster than other pollen types. Raynor *et al.* (1972) showed that maize pollen is not transported by wind as far as smaller pollen grains, does not disperse as widely horizontally or vertically, and settles to earth more quickly, much of it around the source itself. Most maize pollen will fall within the first 5 m after the field edge (Pleasants *et al.* 1999; Sears and Stanley-Horn, 2000). At 60 m from the source, in downwind direction, maize pollen concentrations of less than 1% were measured. The majority of pollen could be found between the rows and directly near the maize field. The possibility to find a small amount of pollen at larger distances from the maize plot is given (Garcia *et al.*, 1998). Jarosz *et al.* (2005) measured pollen deposition rates from 0.001 to 0.0002 grains m⁻²s⁻¹ at distances of 800 m to 1000 m. One possibility of pollen to be transported over large distances is realised through convective air surface. Convective and turbulent conditions will improve the capacity of pollen to travel over large distances through the air (Aylor *et al.*, 2003). The distance travelled by pollen depends mainly on wind velocity.

One of the main factors affecting pollen-mediated gene flow in maize is the synchrony in flowering times. Angevin *et al.* (2001), Bock *et al.* (2002) and Westgate *et al.* (2003) stated that the closer the synchrony between anthesis of the pollen donor and silking of the recipient, the higher the probability for cross-pollination. One week difference in sowing between the donor and the recipient may reduce the level of cross-pollination in the first row of the recipient field by 50% (Devos *et al.*, 2005). By a sowing difference of on average three weeks, the cross-pollination would be reduced up to 75% (Brooks *et al.*, 2004, Ortega Molina, 2004).

Also wind direction is crucial for cross-pollination. As reported by Devos *et al.* (2005) and Henry *et al.* (2003) there is a high correlation between cross-pollination and wind direction. Della Porta *et al.* (2008) and Langhof *et al.* (2008) showed that the cross-pollination rate was higher downwind from the pollen source than upwind.

Shape and orientation of pollen source and recipient field are of great importance in the process of pollen mediated gene flow. According to Ingram (2000) and Meier-Bethke and Schiemann (2003), the amount of cross-pollination can easily double with elongated recipient fields compared to rectangular ones of the same surface.

The size and ratio of pollen source and recipient field influences also the level of cross-pollination. Messeguer *et al.* (2003) and Ortega Molina (2004) showed that with a pollen donor field of 0.25 hectare, the cross-pollination rate decreased from 1.77 % to 0.83% when the size of the recipient field was increased from 0.25 to 1 hectare. In other words: the deeper the recipient field, the less the cross-fertilisation level of the harvest product (Devos *et al.*, 2007).

Weather conditions influence the viability of pollen and silk. Pollen wash down with rain has been reported by Jones *et al.* (1950). Rain could be the reason for low levels of cross-pollination. Drought can alter the timing of silk emergence in relation to pollen shed resulting in poor kernel set (Bruce *et al.*, 2002) and thus giving the opportunity to foreign pollen to fertilize the ear (Aylor *et al.*, 2003). Because the tip of the ear will be fertilized at last, it is more exposed to foreign pollen.

1.7 Cross-pollination studies

Cross-pollination studies between adjacent maize fields have been conducted all over the world using either a colour marker system or Bt maize in combination with an isogenic line. The main focus of these studies was to gather information's about adequate separation distances to ensure coexistence and about the dependence of cross-pollination on the distance within the maize field (e.g. Bannert and Stamp, 2007; Ma *et al.* 2004; Pla *et al.* 2006; Della Porta *et al.* 2008; Langhof *et al.* 2008, Weber *et al.* 2007). Only a few studies deal with aspects like multiple GM pollen sources (Messeguer *et al.* 2006) under real coexistence conditions, the influence of the crop species grown between GM and non-GM maize (e.g. Eder, 2006; Langhof *et al.* 2008; Vogler *et al.*, 2009) and influence of all relevant climatic data as well as observations in flowering synchrony (Bock *et al.*, 2002; Vogler *et al.*, 2009; Westgate *et al.*, 2003).

For example, Bannert and Stamp (2007) investigated the effectiveness of distance in preventing out-crossing in maize. They could show that the cross-pollination rate was between 3% and 15% at 0.8 m from the donor. The level of cross-pollination decreased with increasing distance from the donor source and was below 0.9% at the distance of 15.2 m. Della Porta *et al.* (2008) found cross-pollination rates of 5.72% between 0 and 10 m, 0.35% between 10 and 25 m, 0.23% between 25 and 50 m and 0.19% above 50 m. A study conducted in the United Kingdom with 55 locations over three seasons showed average cross-pollination rates below 0.9% at a distance of 25 m. It was concluded that 80 m and 258 m were sufficient to reach levels of 0.3% and 0.1% (Henry *et al.*, 2003). The results of the "Erprobungsanbau" (Weber *et al.*, 2007) conducted at 18 sites in Germany gave similar results with cross-pollination rates of 1.15% at distance between 0 and 10 m, 0.24% at distance between 20 and 30 m and 0.15% between 50 and 60 m. Bénétrix (2004) obtained 1 - 2% at distances lower than 10 m. Behind a distance of 185 m, Gracia *et al.* (1998) found no cross-pollination. Eder (2006) showed that cross-pollination rate was below the 0.9% threshold at a distance of 75 m.

Jones and Brooks (1952) demonstrated that a barrier of trees beside the field was effective in reducing the amount of cross-pollination by 50 % directly behind the barrier when compared to the cross-pollination obtained at the same distance without a barrier. But this tree barrier was less effective at greater distances. Pla *et al.* (2006) concluded that GM adventitious presence of GM-maize was significantly lower when non-GM maize of the same variety was planted adjacent to the donor-field than in the absence of any plant barrier. Della Porta *et al.* (2008) came to same results. They stated that buffer maize plants that shed non source pollen were the most efficient barrier against cross-pollination. Langhof *et al.* (2008) conducted field

experiments to compare a tall sunflower crop vs. a short clover–grass crop with regard to their ability to reduce cross-pollination when grown as crop species between pollen donor and recipient maize plots. They found no significant difference between the cross-pollination rates when comparing these crop species as gap crops. Vogler *et al.* (2008) assumed that pollen flow over long distances could depend on convective air movement. They found no differences in the amount of pollen deposition above different thermal surfaces.

Hoyle and Cresswell (2007), Della Porta *et al.* (2008), Langhof *et al.* (2008), Ma *et al.* (2004) and Vogler (2008) stated that the cross-pollination rate was significantly higher downwind than upwind from the pollen source. The results of Melé (2004) showed a cross-pollination rate < 0.9% at 10 m downwind and < 0.9% at 2 m upwind. Della Porta *et al.* (2008) observed that cross-pollination depends on flowering synchronization. Little or no reduction of pollen flow was observed when there were only up to 3 days difference in flowering time between pollen source and recipient. By 4 to 5 days difference a 25% reduction of pollen flow was recorded. A difference of 6 days reduced the pollen flow to approx. 50% and a difference of 7 days caused no pollen flow. These results were also supported by Bannert and Stamp (2007) and Ma *et al.* (2004).

The main result from the various studies is that the bulk of cross-pollination occurs within very short distances, less than 25 m from the source. On average, almost all maize pollen travels not further than 100 m and nearly all potential cross-pollination between fields on non-GM maize occurs within 25 m of the source. Pollen flow at larger distances (several hundred meters) and over fragmented landscapes is very low but could occur due to special wind conditions, field heterogeneity or genetic impurity (Devos *et al.*, 2007). The distance between the fields, flowering coincidence and orientation to prevailing horizontal wind speed have been defined as the major factors affecting cross-pollination (Devos *et al.*, 2005; Hüsken *et al.*, 2007).

1.8 Detection methods

Several methods are available to detect or quantify the GM content in the harvest product. GMOs can be quantified by detecting either the inserted genetic material at DNA level, the mRNA transcribed from the newly introduced gene, the resulting protein, metabolite or phenotype and on a seed to seed basis (visual detection) using e.g. different inherited kernel colours. Within the EU, the analytical tests on raw materials are generally carried out with the polymerase chain reaction (PCR method) detecting the inserted DNA. So far, only real-time PCR has found broad application in GMO detection as a generally accepted method for regulatory purposes in food and feed material. On the contrary, the GM quantification by seed is largely used for the control of seed production (Remund *et al.*, 2001). It is generally assumed that GM contents in seed lots should be expressed on a seed to seed basis (percent GM seed in non-GM seed lot).

Real-time PCR is a system based on the continuous monitoring of PCR products. This is done via the fluorometric measurement of an internal probe during the reaction. For relative quantification an endogenous reference gene is quantified in parallel to serve as internal control for the quantity of the extracted DNA, and as estimation of the total amount of plant

specific DNA present in the sample (Pla *et al.*, 2006). Each series of analysis includes the analysis of a full set of standards, giving rise to a standard curve. The standard curve is plotted with the Ct values of the standards against the log of the amount of known copy number or concentration. The Ct values of the sample are compared to the standard curve to determine the amount of target and endogenous sequence present. The percentage target sequence is calculated as percentage of the amount of endogenous sequence present. The results are expressed as: “the percentage of GM-DNA copy number in relation to target taxon specific DNA copy number, calculated in terms of haploid genomes” (Regulation (EC) No 2004/787). Cross-fertilization was usually detected in former times qualitatively based on xenia or on the presence of phenotypic off-types in the progeny. Seed to seed characterisation allows an easy visual detection of the presence or absence of a specific trait. The endosperm of maize kernels can be yellow or white. These colours are easily observable and can be used as markers in genetic studies or tools for evaluating cross-fertilization. In the past several studies were realised (Bannert and Stamp, 2007; Langhof *et al.*, 2008; Ma *et al.*, 2004; Vogler, 2008) simulating the GM content in the harvest product. The yellow-kernel colour is dominant over white-kernel colour. Number of yellow kernels developing on white kernel maize ears were counted, and the percentage of yellow kernels in relation to the mean total kernel number of a white-kernel maize was calculated (% seeds). The yellow colour is a homozygous trait; 100% of the pollen carries the yellow-colour genes. Therefore the percentage of yellow kernels is often divided by two, taking into account that commercial transgenic hybrids are usually hemizygous for the transgene.

So far, the results of real-time PCR quantification are not smoothly convertible to results obtained by phenotypic markers (Devos *et al.*, 2007). This is due to different measurement units (real-time PCR based vs. seed based), due to the ploidy level of each kernel tissue and due to the extractable amount of DNA in each type of tissue (endosperm, embryo and plant material) and their ratio in various products.

1.9 Aims of the study

Since maize is a wind pollinated crop species, the main source of GM impurities in maize seed lots and harvest products is pollen-mediated gene flow. In addition, GM admixtures may be also caused by mixing in seeding and harvest machines as well as during transport, shipping and storage. Pollen-mediated gene flow processes have been investigated all over the world using kernel colour and real-time PCR methods to quantify the level of cross-pollination. These studies have identified the distance to pollen source, the flowering synchronization of pollen donor and receptor, and the prevailing wind direction as the most modifying factors for the rate of cross-pollination; but nevertheless, reliable studies taking into account the consequences of GM seed admixture on the level of cross-pollination in the harvest product, the influence of crop species grown in the field between GM-maize and conventional maize and whether the climatic conditions and start of female flowering are surrounding circumstances or causal factors are still lacking. These sources of variation were the focus of the studies conducted here.

The goal of the first study was to quantify the consequences of GM seed admixture on the harvest product using varieties belonging to different maturity classes. In addition, two different measurement units (visual GM- and real-time PCR-detection) were applied. The consequences of seed impurities in the supply chain were often considered as a key factor to cause a considerable effect on the level of adventitious presence in the harvest product, but experiments which validate these effects are still missing. Furthermore, to maintain comparable results throughout the food chain, there is a need to translate the seed-to-seed quantification (% seeds) in haploid genomes percentages (% DNA).

In a second study the effects caused by different crop species between GM and non-GM maize fields on the level of cross-pollination were analysed. Field experiments with different thermal surfaces (barley stubble and clover-grass) were conducted to identify appropriate separation distances and to generate information about the potential of gap crops (wet vs. dry microclimatic conditions) in increasing or reducing the cross-pollination rate in the recipient field. So far, literature data focus mainly on the implications on pollen deposition, data investigating the effect on the level of cross-pollination are still lacking.

In a third study, the impacts of all relevant climatic data like wind speed, wind direction, humidity, and precipitation as well as observations in start of female flowering on the level of cross-pollination were investigated. Although the impact of climatic factors on cross-pollination was often mentioned, detailed flowering observations as well as precise weather recording are quite rare in many studies so far.

2. The impact of GM seed admixture on the non-GM harvest product in maize (*Zea mays* L.)

2.1 Abstract

Recently, the introduction of GM maize in agricultural production in the EU and elsewhere has raised the issue of adventitious presence of GM seeds in conventional seed lots. Adventitious presence may occur in all arable farming, and at any step in the production of seeds or grain, or in processing of harvested product in the food/feed chain. As of today, there are no official thresholds governing the adventitious presence of GM seeds in conventional seed lots in Europe. But nevertheless, it is assumed that GM admixture in seed lots could have a considerable influence on the level of adventitious presence in the non-GM harvested product. The experiments highlighted in this chapter aim to the consequences of adventitious presence of GM maize seeds in conventional seed lots. It is shown for varieties belonging to the same maturity group that the final GM rate (% seeds) in the harvest product is similar to the seed admixture (% seeds). The variation depends principally on the flowering coincidence, the site and climatic conditions. Furthermore, a comparison between the visual GM seed detection and real-time PCR detection was done. It is evident that the result of the real-time PCR detection method has a more variable uncertainty associated with its results than the visual seed testing method. The accuracy of prediction from % GM seed to % GM DNA depends on the reference material used for calibration curves.

2.2 Introduction

Varietal purity in maize (*Zea mays* L.) seed production is necessary for agronomic uniformity and to enable potential market segregation. International standards for seed lot purity, as established in the “OECD Schemes for the varietal certification of seed moving in international trade” do not address genetically modified (GM) seed admixture so far and their general purity standards vary at levels between 0.1 and 2 percent. Adventitious presence of GM seeds in non-GM seed lots can arise through various pathways. These include cross-pollination between adjacent fields, seed dispersal from volunteers and mechanical transfer at sowing, harvesting, transport and storage operations (Brookes *et al.*, 2004). Seed lots are starting points in an ever increasing supply food chain. The EU commission has proposed that directives on seed material should contain limits on GM seeds in non-GM seed lots assuming that the admixture in seed lots could have a considerable influence on the level of adventitious presence in the non-GM harvested product. Estimated average potential rates of adventitious presence within the 0.9% threshold set by the EU labelling legislation (Brookes *et al.*, 2004) are 0.3% for seed admixture for cross-pollinating species like maize, 0.5% for crop to crop cross-pollination and 0.1% for all other sources like volunteers, machinery etc. (Scientific Committee on Plants, 2001).

The current EU maize seed production does not satisfy the EU's maize seed demand. On average, around 30% of the maize seeds used in the EU originate from imports (European Seed Association, 2006). These seed imports largely come from the USA, Argentina and Chile. Most of these countries have extensive GM maize seed and crop production shares. For example, in 2000 some maize seed lots imported into France from South America were found to have low levels (< 0.2%) of adventitious GM presence (European Seed Association, 2006). In addition, GM varieties cultivated in South America often belong to later maturity groups compared to the European varieties (FAO 170 till FAO 350). This could have a considerable

influence on the level of GM cross-pollination in the non-GM harvest product, because one of the most important factors affecting the level of cross-pollination rate in the harvest product is the flowering coincidence between different maize varieties (Hüsken *et al.*, 2007). Growing seed lots containing two or more varieties with equal maturity groups will result in a close flowering synchrony and successful cross-pollination. On the other hand, growing seed lots containing two or more varieties with different maturity groups will result in differences between the start and duration of the flowering period. As a consequence, asynchronous flowering made recipient plants more susceptible to their own pollen and led them to be non-receptive to foreign pollen (Aylor *et al.*, 2003; Devos *et al.*, 2005).

Another important factor that should be considered in the context of GM seed admixture is that the EU regulation frame (EC recommendation 2004/787) recommends that the quantitative results of GM material detection in food and feed should be expressed as "the percentage of GM DNA copy number in relation to target taxon specific DNA copy number, calculated in terms of haploid genomes". Despite the fact that still no specific legislative framework has been established for the labelling and analysis of GM seeds, it is generally assumed that GM contents in seed lots should be expressed on a seed-to-seed basis. In order to maintain comparable results throughout the food chain, there is a need to translate the seed to seed quantification (% seeds) in haploid genomes percentages (% DNA). For GM varieties which are homozygous this is not a problem, because seeds with 100% GM DNA (both haploid genomes GM) will also be 100% GM seeds. However, a large proportion of maize in the world is produced by using hybrid seeds. For hemizygous GM seeds with complex genomes like maize results will be inconsistent using different units. For example, the quantitative analysis of a seed sample based on haploid genomes percentages will differ depending on the origin of the transgene (paternal, maternal) and on the relative representation of the three seed tissue types in the sample (Trifa and Zhang, 2004; Zhang *et al.*, 2008). This does not necessarily mean that the adventitious presence found with the PCR DNA quantification method will reflect the real GM content (% of GM seeds in total number of seeds).

The objectives of the present study are to verify the consequences of GM seed admixture on the non-GM harvest product using different maize maturity groups. Furthermore a comparison between the visual GM seed detection and real-time PCR detection is done to produce new evidence.

2.3 Materials and Methods

2.3.1 Location description

Field trials were conducted in three locations in 2007 and two locations in 2008: one in France, in Pau (2007, 2008; 48° 18'N, 0° 22'W, altitude 199 m), two in Germany, in Sickte (2007, 2008; 52°13'N, 10°39'E, altitude 94 m) and Brunswick (2007; 52°17'N, 10°26'E, altitude 76 m). The locations of Brunswick, Sickte and Pau belong to those with temperate climatic conditions. The soil type in Sickte and Brunswick is pseudogley and loam-muddy in Pau. The average precipitation per year was 812 mm (2007), 618.4 mm (2008) in Sickte, 752.3 mm (2007) in Brunswick and 938 mm (2008) in Pau. The average temperature was

10.6°C (2007), 10.3°C (2008) in Sickte, 10.2°C in Brunswick (2007) and 13.2°C in Pau (2007/08).

2.3.2 Seed varieties

GM cross-pollination due to seed admixture was detected by using selected maize varieties with different inherited kernel colour. In Germany, the varieties PR39V17 (seed admixture (SA) variety) and DSP 17007 (seed lot (SL) variety) were used in both years. PR39V17 is a MON810 variety, homozygous for the kernel colour yellow and hemizygous for the transgenic event *Cry1Ab*. DSP 17007 is a white kernel variety. Both varieties belong to the same maturity group. Cross-pollination of SL plants by pollen from SA maize resulted in yellow kernels on otherwise white ears.

In France, GM seed admixture was simulated by using a conventional blue grain maize variety; cross-pollination is clearly visible on yellow grain receptor maize. Two varieties (heterozygous blue, made with a blue late inbred crossed by either a yellow late dent inbred or a flint early yellow inbred) were used as SA components and DKC5783 (homozygous yellow variety, late maturity group) and A258 (homozygous yellow variety, early maturity group) were used as SL components in the year 2007. In 2008, the homozygous blue variety Adonis (SA) and the isogenic yellow variety (SL) were used. Cross-pollination of SL plants by pollen from SA maize resulted in blue kernels on otherwise yellow ears.

2.3.3 Field trial design

Maize was grown in Germany at seed densities of 10/m² and with a row spacing of 0.75 m. Seed material was protected against soil borne pathogens by standard seed treatment fungicides (TMTD 98% Satec active ingredient (a.i.) thiram, Satec Elmshorn, Germany; Maxim XL, a.i. fludioxinil and metalaxyl, Syngenta, Maintal, Germany) and by bird repellent (Mesurool, a.i. Bayer, Monheim, Germany) Soil preparation, plant protection measures and fertilizer applications were performed according to the recommendation of the region to reach a yield of 80dt/ha. In France the seed density was 8.5/m² with a row spacing of 0.80 m. Seed material was protected against soil borne pathogens by Influx XL (a.i. mefenoxy and fludioxinil, Syngenta, France). Soil preparation, plant protection measures and fertilizer applications were performed according to the recommendation of the region to reach a yield of 100dt/ha.

The field design to assess the consequences of GM seed admixture is shown in Figure 2.1. The size of the field was 12 m x 14 m. Three replications were carried out at each location and in each year. In 2008 in Sickte, the field was surrounded by 12 white maize rows to avoid cross-pollination from another field situated about 60 m further.

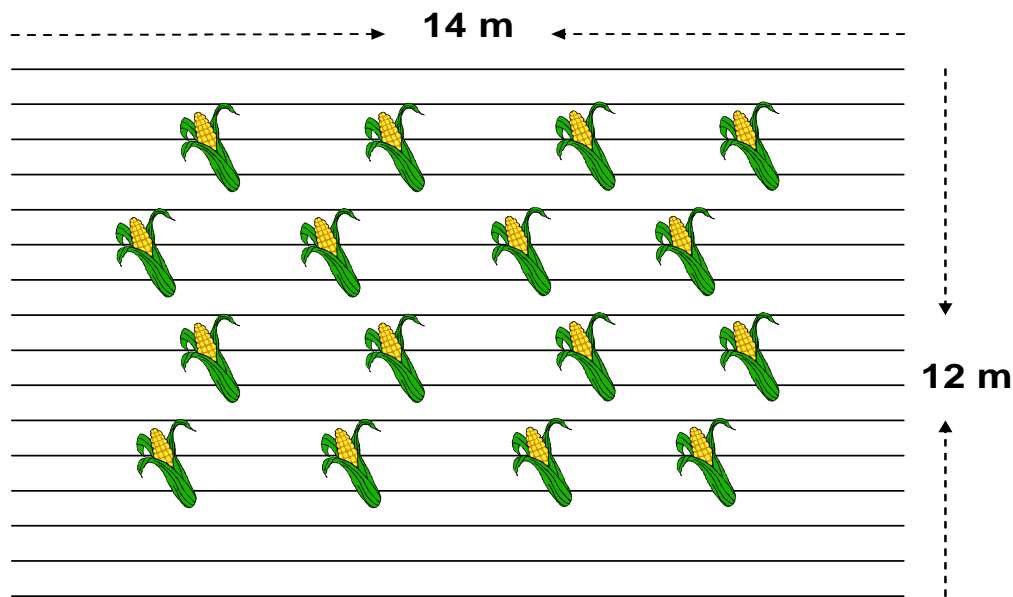


Figure 2.1: Field trial design. GM seed admixture is represented by yellow ears and the black lines are representing the sown seed lot.

After sowing the seed lot, white (yellow (France)) maize kernels were replaced by yellow (blue (France)) kernels in order to arrange an intended seed admixture of 1%. SA seeds were replaced with a minimum distance of 2 m between each SA plant and between SA plants and borders. The SA plants were labelled in order to determine the phenology of the plants during the growing season.

2.3.4 Phenology monitoring

During the flowering period, the full flowering of the SA plants and the recipient plants were monitored as the day at which each SA male flowers were at the stage of full flowering (50%) and the day at which 50% of the SL plants were at the full anthesis and silking stage.

The external anthesis silking interval of SA male flowers and SL female flowers (ASI) were calculated as day difference at which 50% of the SA male and SL female were at the stage start of flowering.

2.3.5 Sampling strategy

To estimate the GM cross-pollination rate in the non-GM harvest product, each ear per plant were harvested at full maturity stage (BBCH 89). Ears were harvested separately and labelled depending on the row and the position in the row to determine the cross-pollination at ear level over the field. The donor plants were harvested separately in both years. Visual analysis of cross-pollination rates per ear was done on the basis of undried ears directly after harvest. Afterwards, ears per replication were dried down to approximately 10% moisture content in a seed dryer and shelled to a pooled kernel sample. Samples were stored in a room at 20°C and about 30% relative humidity. From this pool samples (3x 3000 kernels) were taken from each replication for visual and qPCR (only done in Germany) detection.

2.3.6 Detection methods

Two methods were used to quantify the GM seed content in the harvest product: the visual detection method and the qPCR analysis. The visual detection was based on the assumption that the colour yellow (blue (France)) is dominant over the colour white (yellow (France)). After successful cross-pollination, kernels will become yellow (blue (France)).

For visual detection, 3 x 3000 kernels per replication were taken and the percentage of yellow (blue (France)) kernels determined. Since the Bt-maize (Germany) is homozygous for the character colour and hemizygous for the *cry1Ab* all cross-pollinated kernel would be yellow, but only 50% of the yellow kernel would harbour the transgene. Therefore, the number of yellow kernels was divided by two to determine the GM cross-pollination rate. In France two varieties (heterozygous blue (2007), homozygous blue (2008)) were used for simulating GM seed admixture. For the homozygous blue variety the results were divided by two, for the heterozygous variety the results were not divided by two because only 50% of the pollen will harbour the colour trait blue.

The real-time PCR analysis was based on the detection and amplification of the DNA target sequence. 3 x 3000 kernel samples per replication were ground using the ZM 200 mill (Retsch, Germany) and homogenized for 2 hours using the Turbula Typ T2 F (Willy Bachofen AG, Maschinenfabrik Germany). DNA extraction and qPCR analysis were carried out according to a previously published validated real-time PCR method (Zagon *et al.* 2006).

2.3.7 Data analysis

For each location and year, the average seed admixture rate, external anthesis silking interval, average cross-pollination rate in the harvest product and corresponding standard deviations were calculated.

Cross-pollination data per ear and plot were gridded and contoured with the program Originpro8 (OriginLab Corporation, USA). This technique employs a weighted moving average interpolation (extrapolation) method that minimizes the estimated variance of a predicted point (grid nodes) from the weighted average of its neighbours. The weighted value is determined by the spatial correlation structure of the original data.

A regression analysis was performed to compare the visual detection with the real-time PCR analysis.

Data were analysed using the statistics software Statgraphics version XV.I (StatPoint Technologies, INC. USA).

2.4 Results

2.4.1 Distribution of the cross-pollination level over the field

In general the number of cross-pollination kernel was highest on ears located next to the donor plant and decreased with increasing distance from the donor plant (Fig. 2.2a-b). The highest cross-pollination rate detected on ears of proximal plants next to the donor plant was

14.44% (Sickte, 2008). The mean percentage of cross-pollination on ears harvested in a 1.5 m circle around the SA plants was across all replications 0.63% in the year 2008 (data not shown). Irrespective of the year and site, as distance increases further away from the pollen source, a low level of background cross-pollination exists and remains constant at less than 0.1% out to the edge of the field.

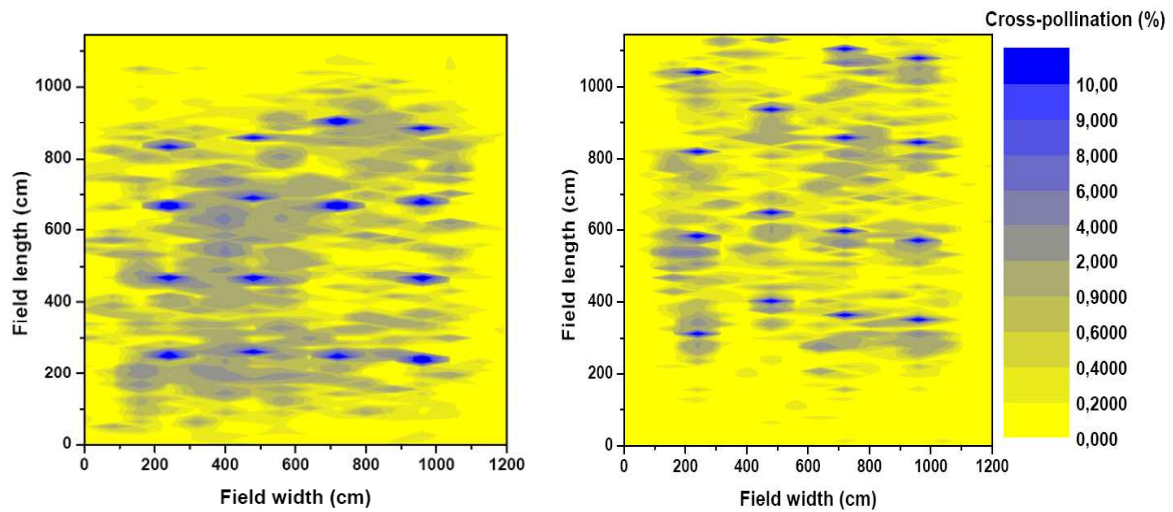


Figure 2.2a: Distribution of the level of cross-pollination over the field in Pau in the year 2007 (Left: Early blue x early yellow variety; Right: Late blue x late yellow variety).

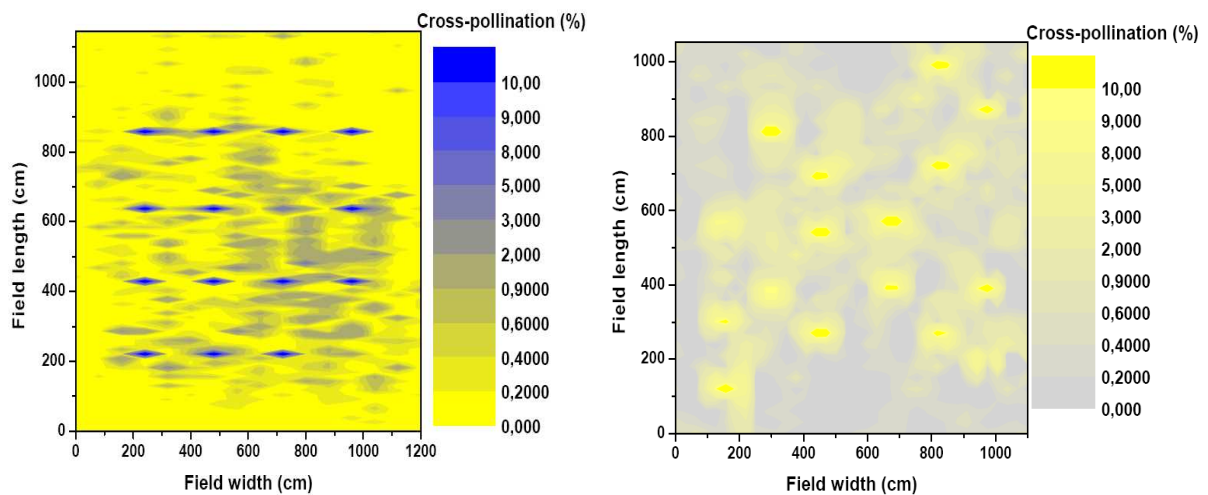


Figure 2.2b: Distribution of the level of cross-pollination within the stand in the year 2008. Left: Early blue x early yellow variety (Pau); Right: Mid early white x mid early yellow variety (Sickte).

2.4.2 Cross-pollination rate in the harvest product (visual detection)

The cross-pollination rate using varieties belonging to the same maturity group was similar to the seed admixture rate in the seed lots at all locations and in both years (Table 2.1). Donor flowering across each replication was relatively uniform, with low deviation from the field average (data not shown) and donor pollen was present during the main silking period of the recipient.

Table 2.1: Average seed admixture rate, ASI and average cross-pollination rate in the harvest product using varieties with the same maturity group

Year	Location	n	SAR (%) \pm SD	ASI (days)	\emptyset CPR (%) \pm SD
2007	Brunswick	3	1.15 \pm 0.10	2.33 \pm 0.58	1.13 \pm 0.03
	Sickte		1.27 \pm 0.07	1.67 \pm 1.15	1.31 \pm 0.13
	Pau		1.08 \pm 0.05	1.33 \pm 0.58	0.93 \pm 0.05
2008	Sickte	3	1.17 \pm 0.01	5.33 \pm 0.58	1.19 \pm 0.25
	Pau		1.15 \pm 0.0	3.33 \pm 0.58	0.90 \pm 0.04

SAR = seed admixture rate; ASI = external anthesis-silking interval; CPR = cross-pollination rate

To determine the effect of the maturity group on the cross-pollination rate, we compared the level of cross-pollination using early SA x early SL (ExE), early SA x late SL (ExL) and late SA x late SL (LxL) varieties in France (2007). The results obtained are shown in Table 2.2. Varying hybrid maturity times (ExL) resulted in asynchronous flowering and the chance for cross pollination was reduced. The cross-pollination rate in the harvest was significantly lower (0.59%) than the seed admixture rate (1%) in the seed lots.

Table 2.2: Effect of maturity group on the cross-pollination rate (Average seed admixture rate, ASI and average cross-pollination rate in Pau)

Year	Maturity group	n	SAR (%) \pm SD	ASI (days)	\emptyset CPR (%) \pm SD
2007	ExE	3	1.08 \pm 0.05	1.33 \pm 0.58	0.93 \pm 0.05
	ExL		1.06 \pm 0.09	- 1.50 \pm 0.87	0.59 \pm 0.06
	LxL		1.08 \pm 0.03	- 0.77 \pm 0.06	0.99 \pm 0.07

E = Early variety; L = Late variety; SAR = seed admixture rate; ASI = external anthesis-silking interval; CPR = cross-pollination rate

2.4.3 Comparison between the visual GM seed detection method and real-time PCR

To compare the visual detection method with the qPCR analysis, we performed a regression analysis. We consider the number of seeds as independent variable. We obtained a strong relationship between both detection methods ($R^2 = 0.89$) and a slope of 0.4034 at a 95%

confidence interval (Figure 2.3). But nevertheless, the qPCR results were consistently lower than the results obtained by visual detection

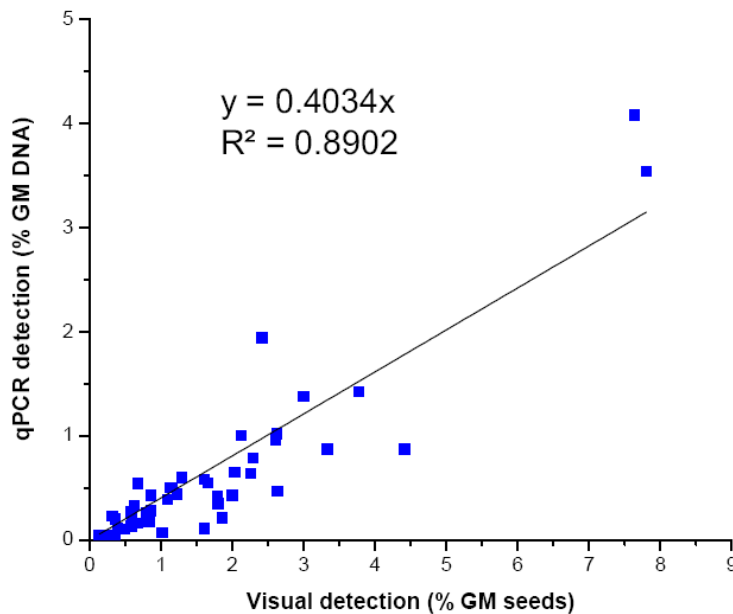


Figure 2.3: Correlation between visual and qPCR detection (n=44).

2.5 Discussion

2.5.1 Observed levels of cross-pollination within stands

One of the most important factors affecting the level of cross-pollination in maize is the flowering synchronization between the donor and the recipient plants. It determines the pollen competition within stands and therefore the degree of cross-pollination for single plants (Aylor *et al.*, 2003; Devos *et al.*, 2005). Optimal cross-pollination is obtained by an ASI of 3 to 4 days (Bassetti and Westgate, 1994; Bock *et al.*, 2002). By simultaneously planting donor and recipient maize in the current study, synchronization of anthesis in the pollen donor and silking in the recipient was possible, and thus, the prerequisite for cross-pollination was given at each site. On average, using varieties with the same maturity group, the full flowering stages of male donor and female recipient flowers overlapped for 3 d. The results show that the greatest amount of cross-pollination occurred in proximal plants and decreased with increasing distance. This would be expected as the dispersion of maize pollen is influenced by its large size and rapid settling rate and filtering within the stand. In addition, the effective pollen dispersal was not uniform in all directions. Variations among the experiments depend on the flowering coincidence between donor and recipient plants, the site, the year and climatic conditions (e.g. wind direction). Although several studies on pollen mediated gene flow in maize exist, studies taking into account the cross-pollination potential of single plants within a stand are rare. Paterniani and Stort (1974) conducted trials using one central plant with dominant yellow endosperm colour in each of 4 plots of white endosperm maize ranging

in size from 15 x 20 m to 40 x 40 m. They could show that in the smaller fields much more effective pollination from the central plant occurred than in the larger ones. This is an indication that the pollen from many plants is well mixed in the air, and that the pollen of the central plant meets with greater competition as the number of plants of the field increases. Furthermore, pollen from the central plant could travel in small plots to the edges of the plot despite the filtering and impact effects of flowering phenology and climatic conditions. This is in agreement with our results. Another interesting result was that the distance measured in number of plants is more important in assessing the efficiency of cross-pollination than the distance in meters.

2.5.2 Cross-pollination rate in the non-GM harvest product

For varieties belonging to the same maturity group, it is shown that the final GM seed rate (% seeds) in the harvest product is similar to the seed admixture rate (% seeds). This corresponds to Hardy-Weinberg expectations. The variation (\pm SD) depends principally on the flowering coincidence between donor and recipient, the site and climatic conditions. When hybrids with varying maturity times are used, the level of cross-pollination in the harvest product is reduced. Varying hybrid maturity times reduces the chance for cross-pollination from the GM plant to the adjacent earlier-maturity non-GM hybrid within the stand. In addition, flowering delay interacted with distance to the pollen donor source and further reduced GM pollen flow in all cases. According to Della Porta *et al.* (2008), a 25% reduction of cross-pollination was recorded at a 4-5 days difference between donor pollen shed and recipient silk appearance. When the time difference was 6 days, they observed a 50% reduction, and at a time difference over 7 days, the cross-pollination was about 0%. In our study, differences in maturity were not large enough to ensure that the flowering periods of hybrids did not coincide, therefore the cross-pollination rate in the harvest product was reduced but not zero. Larger differences in maturity between GM and non-GM hybrids may not be great enough to ensure that the flowering periods of the hybrids will not overlap, especially when climatic conditions accelerate or delay flowering.

2.5.3 Comparison between the visual detection and the real-time PCR

The results from real-time PCR (% GM DNA) correlated closely with phenotypic estimation of the number of seeds (% GM seeds). The calculated slope was 0.4034, the expected slope of such regression is 0.5 (hemizygous GM seeds). Thus, real-time PCR results were consistently lower than expected. Possible explanations for the discrepancy include that the used IRMM (Institute for Reference Materials and Measurements) reference material for the real time PCR calibration carries the transgene by the female parent, whereas the transgene in our samples is paternally inherited. Due to maize kernel constitution, the consequences of not taking into account the effect of biological factors would lead to incorrect GM haploid genome copy numbers during the calibration curve. This results in a consistent underestimation of the GM content in all samples (Zhang *et al.*, 2008). Furthermore the IRMM reference material has been prepared by mixing flours of GM seeds in the flour of non-GM seeds (% GM weight) which contains always an unknown level of bias due to the associated biological uncertainty and may not accurately reflect the genome percentage in each sample. This illustrates the complexity of GM content determination and confirms that the use of homozygous GM

material or plasmids as certified reference material is more reliable. A first certified reference material for MON810 on the basis of plasmids is available since June 2008 (JRC, 2008).

2.5.4 Conclusion

If GM maize is grown within a stand of conventional maize, the maximum contribution that GM plants could make to the pollen flow from the mixed stand would be in the ratio of the plants grown (Hardy-Weinberg-principle). This could be considerably less depending on the maturity group but would not decrease to zero even if there were only a few GM plants with varying maturity times and these were in the centre of the plot. In order to maintain comparable results throughout the food chain, there is a need to translate the seed-to-seed quantification (% seeds) in haploid genomes percentages (% DNA). The accuracy of prediction from % GM seed to % GM DNA depends on the reference material used for calibration curves. There is no generic conversion factor between % seeds and % DNA copies. However in certain cases with appropriate knowledge specific conversion between the different units of expression is possible.

3. Cross-pollination from genetically modified maize (*Zea mays* L.): Impact of isolation distance and different gap crops on the cross-pollination rate

3.1 Abstract

The commercial cultivation of genetically modified (GM) crops in the EU is restricted to Bt maize resistant to the European corn borer. In the EU, a labelling threshold of 0.9% is regulated by the EC 1830/2003. So far, separation distances are mainly used for ensuring coexistence between GM and non-GM maize production fields additionally fixed in good farming practice of Bt maize. In the framework of “The German Federal Research Programme on Coexistence”, the effectiveness of different separation distances, the edge effect and the impact of the crop type planted between two adjacent maize fields on cross-pollination rates has been investigated over three years at one location in a worst case scenario: the field alignment was based on prevailing wind blowing from pollen donor to pollen recipient.

The extent of pollen-mediated gene flow in maize is strongly dependent on distance, climatic conditions as well as maize pollen characteristics. Though the cross-pollination rates varied between the years, it is shown that the cross-pollination rate is highest at border rows facing the donor (edge effect) and decreases within the recipient field rapidly with increasing distance from the donor field. The rate of cross-pollination to distance could not be described by a general function, it changed substantially from year to year and between the field experiments. Therefore, it is assumed that the vertical pollen transport over distance was more dependent on strong and predominant wind conditions than on different thermal conditions induced by different gap crops. It is evident that an appropriate separation distance alone cannot account for keeping crops completely separated, due to the high interception of foreign pollen of the field edge. Thus, separate edge harvest may be an interesting additional measure. The removal of the first few rows of a field facing a GM maize field prior to harvest might be worthwhile to reduce the total level of cross-fertilisation in the recipient field.

3.2 Introduction

Maize is one of the world’s most important crops based on production volume and area cultivated (FAO, 2006). The introduction of genetically modified (GM) traits through maize crops into commodity markets has been increasing constantly during the last 12 years (James, 2008). In spite of the increasing area grown with GM maize worldwide, the growing area in the European Union (EU) is still limited. According to the EU guidelines (EC, 2003), farmers should be free in choosing GM, conventional or organic crops for cultivation. Co-existence depends on the development of recommendations to meet adventitious presence below the EU labelling threshold of 0.9% (Devos *et al.*, 2007) in the harvest product. Since maize is a wind pollinated and mainly cross-pollination species (only ~ 5% selfing, Messeguer *et al.*, 2006), distances required to separate non-GM from GM production have to be considered. Cross-pollination in maize occurs only within members of the genus *Zea* (Estham and Sweet, 2002). Maize pollen grains are relatively large (average diameter of 90 µm) and heavy (0.25 µg) (Di-Giovanni *et al.*, 1995; Aylor *et al.*, 2003) and have been reported to be dispersed over considerable distances (1000 m) downwind from maize crops (Jarosz *et al.*, 2005). Di-Giovanni *et al.* (1995) and Aylor (2002) found that maize pollen usually has a high settling speed and a quick deposition. At distances further than 30-50 m, the levels of pollen

dispersion are very low. There is no clear cut-off distance beyond that a zero level is reached (Devos *et al.*, 2005). During the last years several studies have been conducted to understand the dynamics of pollen flow, cross-pollination and distance (Byrne and Fromherz, 2003; Henry *et al.*, 2003; Hoyle *et al.*, 2006; Kuparinen *et al.*, 2007; Langhof *et al.*, 2008; Ma *et al.*, 2004; Messeguer *et al.*, 2006; Pla *et al.*, 2006). Data from field experiments in France have shown a decrease in cross-pollination in the whole non-GM field below the EU labelling threshold of 0.9% at distances greater than 25 m from the donor field (Fabié, 2004). Common to all studies is that many factors influence the cross-pollination rate between maize production fields such as distance between donor and recipient fields, field size, orientation of the fields, flowering synchrony between donor and recipient plants and climatic conditions (Devos *et al.*, 2005). Though the cross-pollination rates varied between different experiments and years, it is obvious that the cross-pollination rate is highest at border rows facing the donor (edge effect) and decreases rapidly with increasing distance within the recipient field from the donor field.

In addition to distance and edge effect, the effect of different gap crops between donor and recipient fields on the cross-pollination rate has been investigated. Langhof *et al.* (2008) conducted field experiments to compare a tall sunflower crop vs. a short clover-grass crop with regard to their ability to reduce cross-pollination when grown as buffer between pollen donor and recipient maize plots. They found no significant difference between the cross-pollination rates when comparing these crop species as gap crops. Ganz *et al.* (2007) concluded that maize, in contrast to potatoes, grass and summer barley, was the only gap crop that could reduce significantly the cross-pollination rate between a GM and a non-GM maize field. Maize produces the same pollen and this would therefore lead to the dilution of pollen coming from the donor field. The hypothesis that gap crops may influence the microclimatic conditions between maize production fields has never been proven. Previous studies have shown that the air over a harvested wheat field warms up more than the air over a green meadow (Noilhan and Planton, 1989). The rising warm air carries some of the pollen to higher layers of the atmosphere (Vonwahl *et al.*, 1991). The average wind speed increases as altitude increases, carrying the pollen over greater distances (Irwin and Thresh, 1988). Depending on the gap crop, this should result in conserving or reducing the pollen viability or in transport of pollen over distance in the air (Devos *et al.*, 2005).

The objective of the present study was to verify appropriate separation distances for ensuring coexistence between GM and non-GM maize production fields and to quantify the extent of cross-pollination into donor-facing field edges of maize recipient plots. Moreover, the effect of different gap crops (wet vs. dry microclimatic conditions) on the cross-pollination rate between two adjacent maize fields has been investigated. Field experiments were conducted within the framework of the “German Federal Research Programme on Coexistence”.

3.3 Materials and Methods

3.3.1 Location

Field experiments were performed in the years 2005, 2006 and 2007 at the site Wendhausen in Germany (52°17' N, 10°26' E; elevation: 76 m). The soil type is pseudogley with 45% clay, 25% loam and 30% sand. The location is characterised by temperate climatic conditions. The annual precipitation was variable depending on the year with 464.3 mm (with a maximum of 90.7 mm in July) in 2005, 392.6 mm (with a maximum of 62.8 mm in August) in 2006 and 752.3 mm (with a maximum of 112.2 mm in September) in 2007. The annual temperature in average was 9.82°C in 2005, 2006 and 10.29°C in 2007 with the maximal value of 18.57°C, 22.73°C and 18.28°C, in July.

3.3.2 Plant material

In 2005 and 2006, cross-pollination between maize varieties was determined using the Bt-maize variety PR39V17 (Pioneer, Europe) as donor and the near isogenic variety Sandrina (Pioneer, Europe) as recipient; In 2007, the Bt-maize variety PR39F56 was used as donor and the near isogenic variety PR39F58 as recipient. The Bt-maize varieties PR39V17 and PR39F56 contain the transgenic MON810 event, which expresses a bacterial *Bacillus thuringiensis* toxin (Bt toxin) gene, poisonous to the insect pest European corn borer (*Ostrinia nubilalis*).

3.3.3 Field trial

Maize was grown at seed density of 100.000/ha and with a row spacing of 0.75 m. Seed material was protected against soil-borne pathogens by standard seed treatment fungicides (TMTD 98% Satec a.i. thiram, Satec Elmshorn, Germany; Maxim XL, a.i. fludioxinil and metalaxyl, Syngenta, Maintal, Germany) and by bird repellent (Mesurol, Bayer, Monheim, Germany) Soil preparation, plant protection measures and fertilizer applications were performed according to the recommendation of the region to reach a yield of 80 dt/ha.

The field design to measure cross-pollination between the donor and the recipient field is shown in Figure 3.1. The size of each donor field was 102 m x 230 m in the years 2005, 2006 and 2007. The recipient field was made up of 5 compact partial plots situated at different separation distances from the donor. The sizes of the recipient fields were: 38 m x 237 m (partial plots 1 and 5, separation distance 24 m), 38 m x 210 m (partial plots 2 and 4, separation distance 51 m) and 78 m x 183 m (partial plot 3, separation distance 78 m) in 2005 and 2006. In 2007 field sizes were 38 m x 210 m (partial plots 1 and 5, separation distance 51 m), 38 m x 183 m (partial plots 2 and 4, separation distance 78 m) und 78 m x 156 m (partial plot 3, separation distance 102 m). The space between the donor and the recipient was either planted with clover-grass (2005, 2006, 2007; field design 1) or with spring barley (2006, 2007; field design 2) In order to obtain barley stubbles (dry microclimatic conditions) as a gap crop, the barley field was harvested before beginning of the anthesis-silking of donor and recipient maize. 50 m wide hemp (*Cannabis sativa* L.) was sown between the two field experiments in the year 2006 and 2007 to avoid cross-pollination from one field experiment to the other. At the beginning of the flowering period Hemp was taller than maize and was established to act as pollen barrier between the two field experiments. In general, all field

experiments were designed in a worst case scenario: the Bt maize field was established downwind of the maize recipient in respect to the prevailing wind direction.

3.3.4 Weather recording

During the flowering period, meteorological data were recorded by an on-site weather station. Temperature and relative humidity were recorded hourly using a thermo-hygrograph. Precipitation was measured with a Hellmann-type rain gauge. A Woelfl e-type wind recorder detected wind speed and direction at a height of 2 m (all meteorological instruments, Lambrecht, Göttingen, Germany). Meteorological data recording was done by the German National Meteorological Service.

3.3.5 Flowering data

In order to assess coincidence of pollen shedding of the donor plants and silking of the recipient plants as well as coincidence of anthesis and silking in the recipient, 86 record points in the recipient and 20, (2005), 60 (2006, 2007) in the donor field were selected (Fig. 3.1). At each record point 20 maize plants were labelled. Start of flowering, full flowering and end of flowering were monitored every day for the male and female flowers as recommended by Westgate *et al.* (2003). In 2005, only the full flowering of recipient female flowers was monitored every 2 days.

3.3.6 Sampling strategy

To estimate the cross-pollination rate in the recipient field, 20 ears per sample point were harvested at full maturity stage (BBCH 89). Altogether 20 ears each from 222 (2005, clover-grass), 222 (2006, clover-grass), 294 (2006, barley stubbles), 294 (2007, clover-grass), and 312 (2007, barley stubble) sample points were harvested. Ears were collected directly into labelled bags to avoid mixing. After being dried down to approximately 10% moisture content in a seed dryer, each sample was shelled to a pooled kernel sample of approximately 6000 to 8000 seeds. One-half of each pooled sample was used for real-time PCR analysis; the remainder acted as retain sample. Samples were stored in a seed storage room (18°C, 30% relative humidity) before real-time PCR analysis.

3.3.7 Sample analysis

The percentage of MON810-specific DNA relative to an endogenous maize gene in each sample was quantified by real-time PCR. Real-time PCR was done by a DIN EN ISO/IEC 17025 accredited laboratory (JenaGen GmbH, Jena, Germany (2005) and Planton GmbH, Kiel, Germany (2006, 2007)) according to a validated MON810 event-specific protocol (Kuribara *et al.*, 2002; Shindo *et al.*, 2002).

3.3.8 Data analysis

For the analysis of weather data collected during the period of flowering, the time between 8 am and 6 pm was chosen as recommended by Jarosz *et al.* (2005).

To create the wind rose diagram, average wind speed was taken for each wind direction. Rayleigh's test was performed to determine whether there was a predominant wind direction during the flowering period of each year or if wind direction was random (Batschelet, 1981).

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Cross-pollination data were gridded and contoured with the program Originpro8 (OriginLab Corporation, USA). This technique employs a weighted moving average interpolation (extrapolation) method that minimizes the estimated variance of a predicted point (grid nodes) from the weighted average of its neighbours. The weighted value is determined by the spatial correlation structure of the original data. The statistical analysis was done using the Statistic Software OriginPro8 (OriginLab Corporation, USA).

For each distance and partial plot, the minimum, maximum, as well as average cross-pollination rate, standard deviation and variance was calculated. To check for any probable significant differences between the partial plots 1 and 5 (distance 24 m) as well as partial plots 2 and 4 (distance 51 m), cross-pollination data per row at equal distance to the recipients' field border were compared separately using a t-test. In the case of non-significant differences the partial plots 1 and 5 as well as partial plots 2 and 4 were treated as one field, resulting in three (partial plots 1+5, partial plots 2+4, partial plot 3) instead of five part fields per year and gap crop.

Regression analysis was performed using linear and non-linear functions for the partial plot 1+5, 2+4 and 3.

To compare the effects of clover-grass and barley stubble as gap crops on the amount of cross-pollination, the average cross-pollination rate of the partial plots 1 + 5, 2 + 4, 3, first and last 4 sample rows were chosen and compared. The data were analysed using a nonparametric Wilcoxon test to determine whether clover-grass or barley stubbles caused differences in cross-pollination.

To determine the edge effect, average cross-pollination data in the first row of the recipient partial plot as well as within the field at sample points with the same distance from the field edge were compared. For the comparison, a nonparametric Wilcoxon test was performed to see if there is any significant difference between the average value of the data at the edge and the data at equal distances to the recipients' field border.

The statistical analysis was done using the statistic software Statgraphics version XV.I (StatPoint Technologies, INC. USA).

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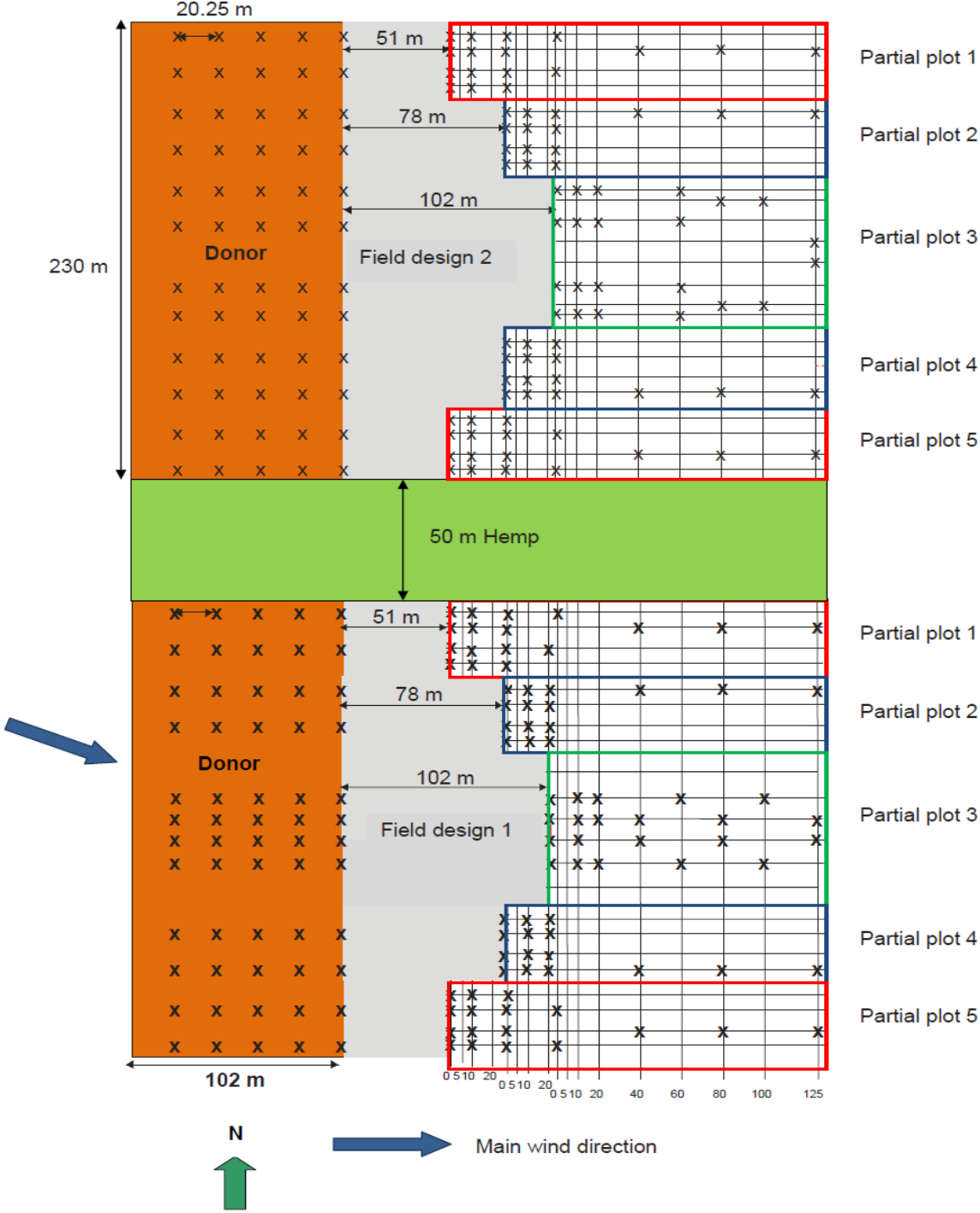


Figure 3.1: Field design for determining the impact of distance, edge effect and gap crops on the cross-pollination rate in the recipient fields' total harvest (gap distances 24, 51, 78 m (2005, 2006) and 51, 78, 102 m (2007)). The gap between the donor and the recipient was sown either with barley stubble (field design 2) or with clover-grass (field design 1). A 50 m wide hemp strip separates both field experiments; horizontal lines in the recipient field indicate sampling rows, joint points of vertical and horizontal lines represent sampling points and crosses represent points where flowering was monitored (in 2006 and 2007).

3.4 Results

3.4.1 Weather conditions

The weather conditions during the flowering period varied considerably depending on the year (Table 3.1). The year 2006 was generally more droughty than the years 2005 and 2007, with soil showing remarkable drought indications. The precipitation during the anthesis-silking period was more evenly distributed in the years 2005 and 2007. In the year 2006, the precipitation was highest at the end of the flowering period.

Table 3.1: Climatic conditions during the flowering period in the years 2005, 2006 and 2007

	temperature (°C)		rel. humidity (%)		precipitation (mm)	average wind direction (°)	average wind speed (m/s)
	min.	max	min.	max.			
2005	12.22	22.32	66.91	93.16	0.55	248.5	1.67
2006	13.32	26.75	51.04	92.75	0.35	227	1.84
2007	12.98	25.59	56.54	97.91	0.68	240.5	2.17

3.4.2 Field trial phenology

Comparison of flowering information at the observed individual sampling points showed a relative good synchrony with low deviations from the field average (data not shown). In the recipient field, the anthesis-silking coincidence lasted on average 4.12 days (± 2.9 SD, clover-grass 2005), 1.87 days (± 1.64 , clover-grass 2006), 2.61 days (± 2.00 , barley stubble 2006), 2.20 days (± 1.17 , clover-grass 2007) and 4.02 days (± 1.87 , barley stubble 2007). Overlap of anthesis in the donor field with silking in the recipient field was on average 1.83 (± 1.64 SD, clover-grass 2005) days and 3.78 (± 2.11 SD, clover-grass 2006) days, 3.76 (± 2.11 SD, barley stubble, 2006) days, 4.94 (± 2.49 SD, clover-grass 2007) days and 5.60 (± 2.73 SD, barley 2007) days.

3.4.3 Distribution of cross-pollination within the recipient field

In general, cross-pollination rates were higher at the border facing the donor field than within the field and decreased with increasing distance from the donor field (Fig.3.2a-b). The effect of the distance on the level of cross-pollination was found to be highly significant ($p < 0.0001$) by correlation analysis (data not shown). Irrespective of year and gap crop the first row of the recipient partial plots with the lowest distance to the pollen source had the highest cross-pollination rates. The observed maximum cross-pollination value was 11.63% (2005 at 24 m, partial plot 1), 4.50% (2006 at 24 m, partial plot 5) for field design 1 and 3.60% (24 m, partial plot 3) for field design 2 (2006). In 2007, the highest values were 22.26% (51 m, partial plot 1) for field design 1 and 14.57% (51 m, partial plot 5) for field design 2 (data not shown). The average level of cross-pollination in the first recipient row of each partial plot and for both field designs in 2007 was greater than the average cross-pollination rate in the

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first recipient row in 2005 and in 2006. Comparing the cross-pollination over the whole field there was a significant difference at the level $p \leq 0.01$ between the cross-pollination rate in the year 2005 and the cross-pollination rates in the years 2006 and 2007 for field design 1. The comparison of the whole fields' cross-pollination in 2006 and 2007 showed also a significant difference at the level $\alpha \leq 0.01$ for field design 2 (data not shown).

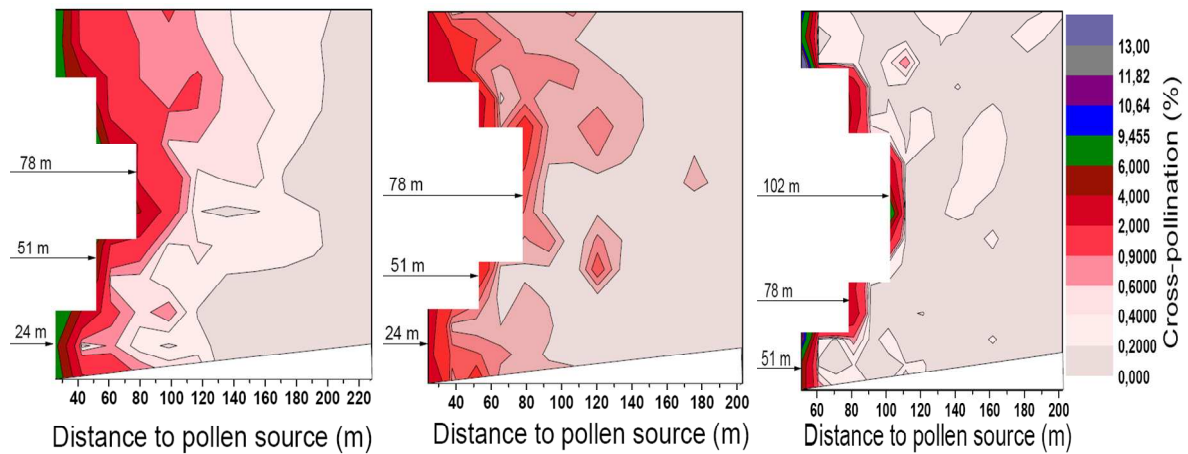


Figure 3.2a: Cross-pollination results of the year 2005 (left), 2006 (middle) and 2007 (right) for the field design 1. The arrows indicate the gap distances to the pollen source.

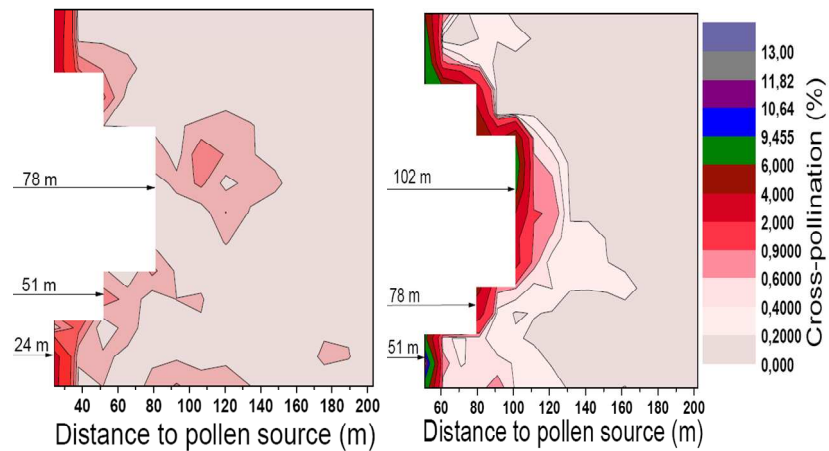


Figure 3.2b: Cross-pollination results of the year 2006 (left) and 2007 (right) for the field design 2. The arrows indicate the gap distances to the pollen source.

When comparing the average values of cross-pollination per sampling row for partial plots 1 and 5 (gap distance: 24 m (2005/06), 51 m (2007)) and partial plots 2 and 4 (gap distance: 51 m (2005/06), 78 m (2007)) there was no significant difference, irrespective of year and gap crop. For this reason, the results of the partial plot 1 and 5 (partial plot 2 and 4), were treated in the following as one field (partial plot 1+5; partial plot 2+4).

The distances within the partial plot at that the average cross-pollination rate per row dropped first time below the EU labelling threshold of 0.9% (Figure 3.3a-c) in 2005 was 78 m for the

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partial plot 1 + 5; 71 m for the partial plot 2 + 4, and 118 m for the part field 3. In 2006 the distances were 44 m, 29 m for the partial plot 1 + 5; 61 m, 51 m for the part field 2 + 4, and 78 m, 83 m for the partial plot 3 (field design 1, field design 2, respectively). In 2007, the distances within the partial plot at that the average cross-pollination rate per row dropped first time below the EU labelling threshold of 0.9% (Figure 3.3a-c) were 61 m, 61 m for the partial plot 1 + 5, 88 m, 88 m for the partial plot 2 + 4, and 107 m, 112 m for the partial plot 3 (field design 1, field design 2, respectively).

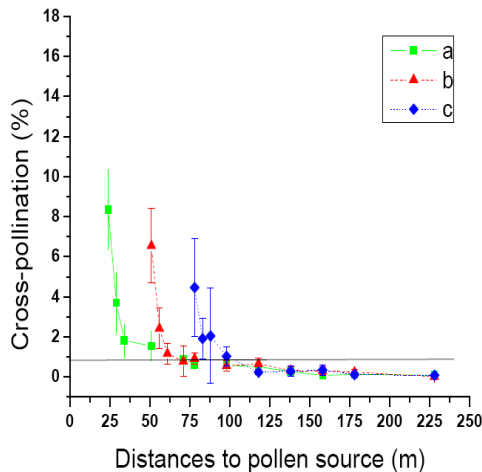


Figure 3.3a: Cross-pollination rate (% , \pm SD) within the recipient field using the field design 1 (2005): a = partial plot 1 + 5, b = partial plot 2 + 4, c = partial plot 3, vertical bars indicate standard deviation; the horizontal line indicates the 0.9% EU labelling threshold.

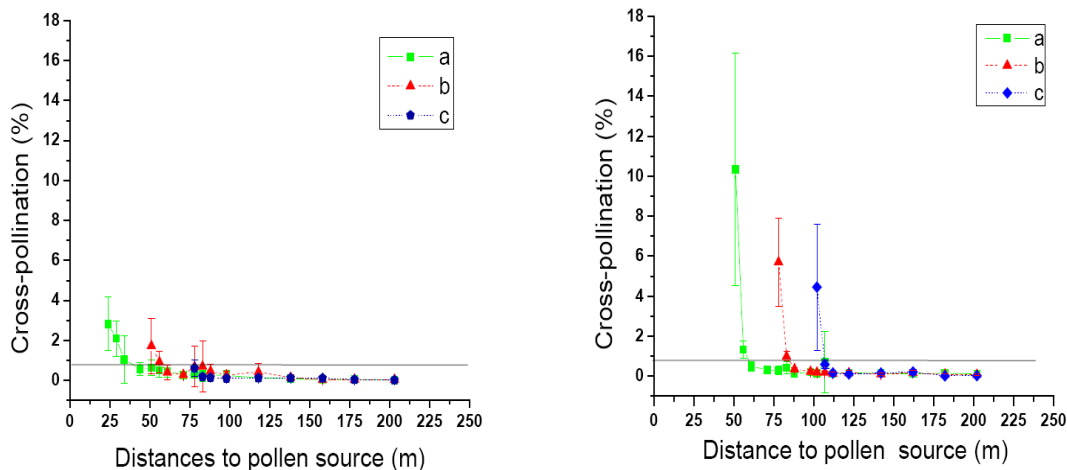


Figure 3.3b: Cross-pollination rate (% , \pm SD) within the recipient field using the field design 1 (2006 and 2007): a = partial plot 1 + 5, b = partial plot 2 + 4, c = partial plot 3, vertical bars indicate standard deviation; the horizontal line indicates the 0.9% EU labelling threshold.

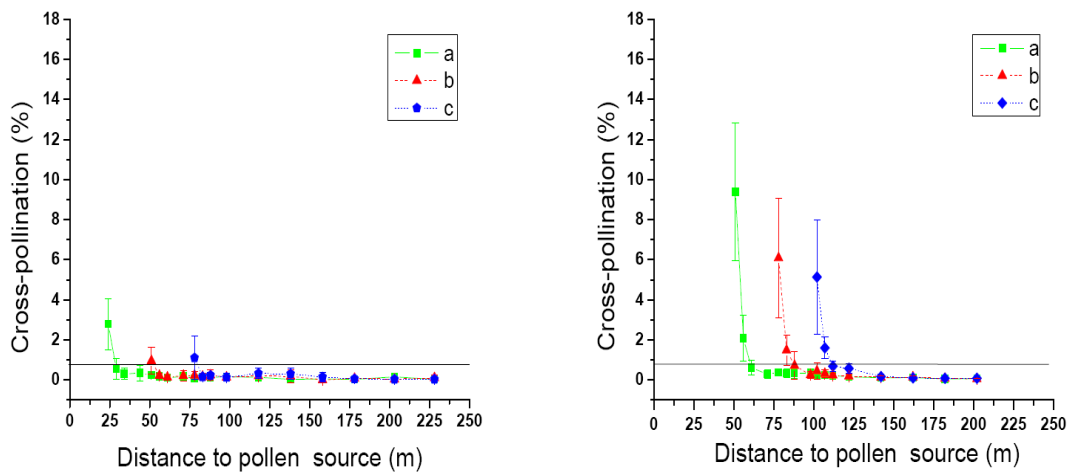


Figure 3.3c: Cross-pollination rate (% , \pm SD) within the recipient field using the field design 2 (2006 and 2007): a = partial plot 1 + 5, b = partial plot 2 + 4, c = partial plot 3, vertical bars indicate standard deviation; the horizontal line indicates the 0.9% EU labelling threshold.

Assuming an exponential decrease of cross-pollination with increasing recipients' field depth, non-linear least-squares regression was done. The ratio of cross-pollination to distance could not be described by a general function, it changed substantially from year to year and between the gap crops and part fields (data not shown).

3.4.4 Edge effect

Generally, the average cross-pollination rate of the sampling points facing the donor field at edge was significantly higher than the average cross-pollination rate of the sampling points within the field at the same distance from the donor (Table 3.2a-b).

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Table 3.2a: Comparison of the cross-pollination rate at recipient edge (first row) with the cross-pollination rate within the recipient field at different equal distances from the donor field (field design 1)

distance (m)	2005		2006		2007	
	edge	within the field	edge	within the field	edge	within the field
51	6.55 ± 1.86**	1.55 ± 0.73	1.73 ± 1.38 ⁺	0.68 ± 0.38		
78 ^a	4.47 ± 2.42**	0.91 ± 0.27	0.61 ± 0.39*	0.27 ± 0.13		
78 ^b	4.47 ± 2.42*	0.61 ± 0.24	0.61 ± 0.39 ⁺	0.68 ± 1.02	5.71 ± 2.20**	0.29 ± 0.20
102 ^a					4.46 ± 3.15**	0.14 ± 0.08
102 ^b					4.46 ± 3.15**	0.19 ± 0.10

^{a, b} denotes the average cross-pollination rate for the partial plot 1 + 5, 2 + 4; ⁺ = $p \leq 0.1$; * = $p \leq 0.05$; ** = $p \leq 0.01$

Table 3.2b: Comparison of the cross-pollination rate at recipient edge (first row) with the cross-pollination rate within the recipient field at different equal distances from the donor field (field design 2)

distance (m)	2006		2007	
	edge	within the field	edge	within the field
51	0.66 ± 0.38 ⁺	0.20 ± 0.06		
78 ^a	1.30 ± 1.20 ⁺	0.05 ± 0.04		
78 ^b	1.30 ± 1.20 ⁺	0.23 ± 0.23	6.09 ± 2.99**	0.38 ± 0.11
102 ^a			5.13 ± 2.85**	0.25 ± 0.16
102 ^b			5.13 ± 2.85**	0.45 ± 0.42

^{a, b} denotes the average cross-pollination rate for the partial plot 1 + 5, 2 + 4; ⁺ = $p \leq 0.1$; ** = $p \leq 0.01$

In 2007, the average cross-pollination rate at edge differed highly significant from the average cross-pollination within the field at different equal distances for each gap distances 51 m, 78 m. This was not always the case in 2005 and 2006.

3.4.5 Comparison between clover-grass and barley stubbles as gap crops

The comparison of the effect of clover-grass and barley stubble as gap crops on the average cross-pollination rate for the whole partial plots 1 + 5, 2 + 4 and 3 in the years 2006 and 2007 is shown in Figure 3.4.

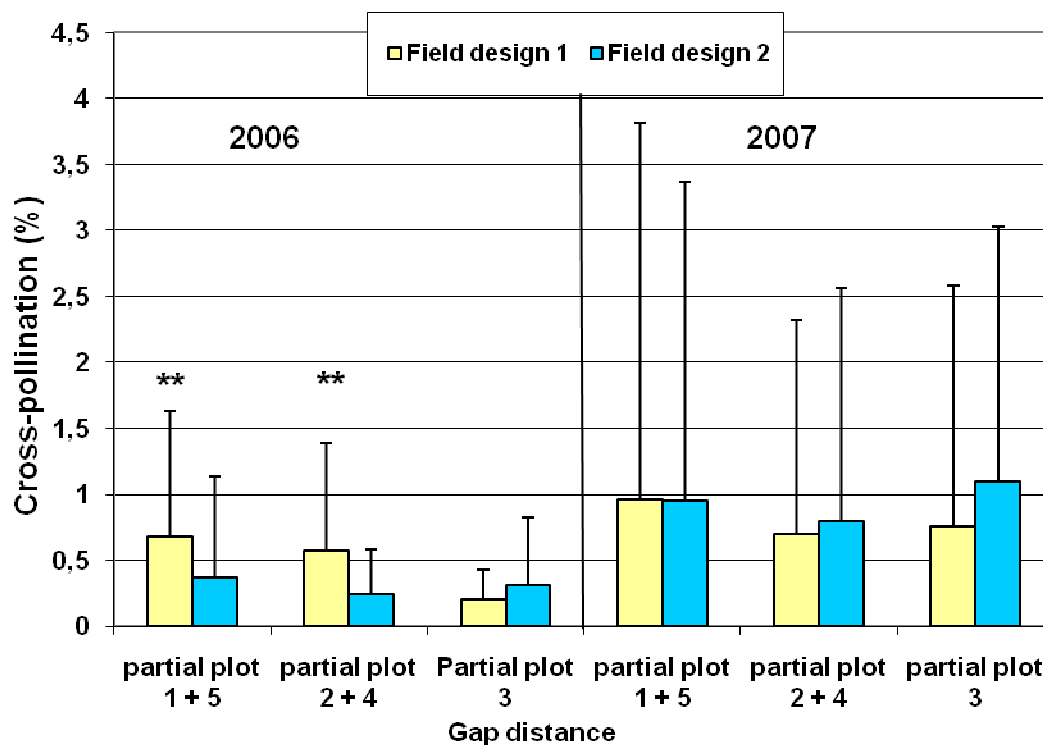


Figure 3.4: Comparison of the average cross-pollination rates (% , \pm SD) of field design 1 (yellow) and field design 2 (blue) for the partial plots 1 + 5, 2 + 4 and 3 (2006, 2007). Bars indicate the standard deviation, ** denoted a significant difference at the level $p \leq 0.01$.

The average cross-pollination rate of the partial plots 1 + 5 and 2 + 4 for the field with the field design 1 and 2 were statistically significant different in the year 2006. The comparison of the partial plots 3 (2006), partial plots 1 + 5 and partial plots 2 + 4 in 2007 showed no significant difference.

The comparison of the cross-pollination rate in the first and last 4 sampling rows (Table 3.3) showed a significant difference between the gap crops in 2006 for the partial plot 2 + 4 for the first 4 rows, in 2007 for the partial plot 1 + 5 for the last 4 rows. The comparison of the first and last 4 rows showed no significant difference in the average cross-pollination rate in 2006 and 2007 for the partial plot 3.

Table 3.3: Average cross-pollination rate of the first and last 4 rows in comparison for field design 1 and 2 in 2006 and 2007

	year	first 4 rows		last 4 rows	
		field design 1	field design 2	field design 1	field design 2
partial plot1 + 5	2006	1.63 ± 1.31	0.99 ± 1.25	0.03 ± 0.02	0.06 ± 0.10
	2007	3.12 ± 5.07	3.09 ± 4.13	0.13 ± 0.08*	0.06 ± 0.04
partial plot 2 + 4	2006	0.83 ± 0.93*	0.38 ± 0.48	0.07 ± 0.09	0.05 ± 0.07
	2007	1.82 ± 2.69	1.82 ± 2.53	0.11 ± 0.08	0.10 ± 0.07
partial plot 3	2006	0.26 ± 0.28	0.45 ± 0.74	0.14 ± 0.20	0.09 ± 0.18
	2007	1.73 ± 2.62	2.64 ± 2.58	0.11 ± 0.12	0.11 ± 0.07

* denotes a significant difference at $\alpha \leq 0.05$.

3.5 Discussion

3.5.1 Distribution of cross-pollination within the recipient field

Previous cross-pollination studies (reviewed in Devos *et al.*, 2005) often chose experimental conditions not representing practical agricultural conditions (e.g. small recipient fields). Only Messeguer *et al.* (2006), Weber *et al.* (2006), and Della Porta *et al.* (2008) carried out field experiments under real agricultural conditions. In this respect, our field experiment was designed to simulate conditions near agricultural practice using large donor and recipient fields separated by different distances and gap crops. Generally, our results showed cross-pollination rates decreasing with increasing distance from the donor field. In comparison to other studies on pollen dispersal and cross-pollination, the results presented here are similar to those obtained by Bannert (2006); Eder (2006); Ganz *et al.* (2007); Henry *et al.* (2003); Langhof *et al.* (2008); Ma *et al.* (2004); Messeguer *et al.* (2006); Pla *et al.* (2006) and Vogler (2008).

The decrease of cross-pollination with distance can be attributed to many factors such as pollen characteristics, pollen competition and environmental variation. Maize pollen grains are relatively large and heavy (Di-Giovanni *et al.*, 1995; Aylor *et al.*, 2003), therefore maize pollen has a high settling speed and rapid deposition. Consequently, the concentration of GM pollen will decrease with increasing distance from the pollen source. Jarosz *et al.* (2005) observed pollen deposition rates ranging from 10 to 100 grains/m² at 10 m downwind from the pollen source. Ca. 95-99% of the released pollen will settle down within a distance of 30-50 m. At downwind distances of 800 and 1000 m, the pollen deposition rates decreased to 0.001-0.0002 grains/m². Some pollen grains might reach greater distances due to special wind conditions, however this pollen will be confronted to strong pollen competition from pollen shed in the recipient field, thus the chance of successful cross-pollination is low.

Irrespective of the two compared gap crops, the cross-pollination rate over the field was randomly distributed and varied substantially between years. This suggests that variations in climatic conditions can result in a major shift in cross-pollination. The results obtained in the year 2006 showed the lowest cross-pollination rate for both gap crop field experiments. The weather conditions during the anthesis-silking period had a great influence on the effective cross-pollination rate and distribution within the recipient field. The year 2006 was characterized by the lowest precipitation and the highest temperature. The unusual drought in 2006 resulted in unsynchronized pollen shed and silking, thus reducing the opportunity for cross-pollination. Furthermore, if the air is too dry, pollen viability will be quickly lost and maturation of silking is also affected (Ma *et al.*, 2004). Aylor *et al.* (2003) and Jarosz *et al.* (2003) confirmed in their studies the effect of the weather conditions on the pollen viability and cross-pollination. Moreover, wind from non-prevailing directions as evidenced in all years, mostly pronounced in 2006, may have had an additional impact on the level of cross-pollination. Ma *et al.* (2004) stated that instantaneously changing wind direction at the time of silking of the recipient affected cross-pollination more than the overall prevailing wind direction. Cresswell *et al.* (2004) figured out that crops with short full flowering periods like maize are likely to be most influenced in cross-pollination rates by changing wind conditions.

Hot spots of cross-pollination were recorded in the year 2006 at unexpected distances from the pollen source. Convective airflow resulting from the warmed up air above a crop may explain the aerial concentrations of pollen above maize fields and the occurrence of cross-fertilisation hot spots over longer distances than expected (Raynor *et al.*, 1972). Another explanation for the cross-pollination hot spots may be the occurrence of late developing plants (e.g. due to genetic impurity or to field heterogeneity) provided that there is a good synchrony between recipient silking and late donor anthesis. Due to the smaller pollen clouds above the recipient field, late developing plants are more prone to cross-fertilisation (Della Porta *et al.*, 2008; Ma *et al.*, 2004).

Comparing the cross-pollination rate at multiple sample points within the recipient field, higher values were detected in 2005 than in 2006 and 2007. In fact, this can be explained by the combination of factors like the gap distance between the donor and the recipient field which in 2005 and 2006 was lower than in 2007, the wind direction, the temperature and the relative air humidity which were favourable for pollen production and viability in 2005 and 2007. We suppose that the cross-pollination rate within the field in the year 2007 would have been approximately the same as the cross-pollination rate in 2005, if in 2007 the gap distances were equal to those in the years before.

The highest single sample points' cross-pollination rates were observed in the year 2007, in spite of greater gap distance. The average wind speed during the flowering period was considerably higher in 2007 compared to 2005 and 2006. Moreover, a predominant wind direction was observed, thus the chance of successful cross-pollination was increased. The possible sensitivity to wind conditions means that occasionally large amounts of pollen could be dispersed over greater distance. Furthermore, it is assumed that the changed field size ratio might have additionally increased cross-pollination. Due to the longer gap distances in 2007 the recipient field was smaller in the year 2007 than the one in 2005 and 2006. This led to a reduction of the non-GM pollen mass above the recipient field resulting in higher pollen

competition and therefore higher cross-pollination was probable. These findings have also been reported by several authors: For a pollen donor field of a given size, the levels of cross-pollination decrease as the size of the recipient field increases (dilution effect) (Méle, 2004; Messeguer *et al.*, 2003; Ortega Molina, 2004).

Furthermore, the common assumption for wind-pollinated plants that gene flow is leptokurtic (Levin and Kerster, 1974; Moyes and Dale, 1999; Vekemans and Hardy, 2004) was not supported by our results. All non-linear equations tested did not model well the decrease of cross-pollination with increasing recipients' field depth. Curve performance differed significantly between the years.

Estimated distances to reach the 0.9% labelling threshold was in a 29 m to 118 m range. In the presence of large variations in the level of cross-pollination and occasional extreme climatic conditions it occurs that 0.9% labelling threshold distances cannot be reliably established within 100 m from the pollen source. The key point to note is that the present study operates with a 'worst case' scenario and that the estimated distances to reach the 0.9% labelling threshold were based on average values obtained from particular distances within the recipient field including the applied gap distances. Data from other studies have shown that the cross-pollination rate beyond the initial 30 m of the recipient field was in most cases below 0.9% (Devos *et al.*, 2005). But nevertheless, the level of cross-pollination in total fields harvest is most relevant for co-existence. Some studies (Benetrix, 2004; Fabié, 2004; Foueillassar and Fabié, 2003; Melé, 2004) observed an overall level of cross-fertilisation between 0.2 and 0.83 % respectively for the recipient fields planted adjacently to or surrounding the pollen source. In a recipient sampling area of 5 and 23.3 ha adjacent to a source of 23.3 ha, Ortega Molina (2004) reported a GM content of 0.54 and 0.26 %, respectively.

3.5.2 Edge effect

Cross-pollination rates for field design 1 and 2 were higher at the recipient field border facing the donor field than within the field at equal distances from field edge. Most of the pollen coming from the donor field was intercepted by the first few maize rows. At the field edge the recipient pollen concentration may be lower than the donor pollen concentration due to the chosen field orientation in respect to direction of the prevailing wind; therefore, higher cross-pollination occurs. The first few border rows act as a barrier for pollen coming from the donor field (Devos *et al.*, 2005). Paterniani *et al.* (1974) found that the plants near the source tend to receive a greater quantity of pollen than those in a greater distance. The border rows may also act as windbreaker permitting pollen coming from the donor field to settle down and so increase the chance for cross-pollination (Du *et al.*, 2001). A range of recent studies confirmed the finding of our work (Henry *et al.*, 2003; Ganz *et al.*, 2007; Langhof *et al.*, 2008; Ma *et al.*, 2004; Bannert and Stamp, 2005; Méle *et al.*, 2005; Weber *et al.*, 2005). We denoted in our study that the level of significance comparing the cross-pollination rate at edge with the cross-pollination rate at specific sample points within the field at equal distances varied over the years. This may be explained by the fact that the flowering coincidence determines the level of cross-pollination (Devos *et al.*, 2008). The year with the lowest flowering coincidence (2006) showed the lowest significance level for the edge effect. In general, due to the high interception of foreign pollen at the field margin and the decrease of cross-pollination with increasing field depth, the total field harvests' GM content is mainly

determined by cross-pollination beyond a certain distance in the recipient field. Finally, the finding of this study may be helpful for recommending measures to reduce cross-pollination rates between fields. For example border rows with the highest cross-pollination rate could be harvested separately.

3.5.3 Effect of the gap crop between the donor and the recipient field

In general, the used gap crops permitted unhindered pollen transport. The nature and characteristics of different surfaces alter the air temperature above the ground. In a humid environment (e.g. above clover-grass fields), the availability of water for evaporation from vegetation is given. The possibility of evaporation is one of the main factors involved in air temperature variations. In dry environments (e.g. barley stubbles) evaporation is reduced by the lack of available water, as a result the air over the ground will heat up during the day and thermal up-winds are initiated (Noilhan and Planton, 1989), mainly during noonday. During these meteorological conditions, convection cells may develop and pollen can be transported to greater distances than usual. To date little is known about the behaviour of pollen above different thermal surfaces that can influence pollen transport outside maize fields (Loos et al., 2003). But nevertheless, the hypothesis that gap crops influence the microclimatic conditions between maize fields and consequently the cross-pollination rate was not supported by our results. One possible explanation may be that the peak pollen shed usually occurs in mid-morning, when thermal up-winds cannot develop due to the relative high humidity at this time. Moreover, maize pollen may be too heavy to be lifted up by thermal up-winds and transported further than a few metres away from the pollen source. Vogler *et al.* (2009) suggested that high temperatures and low wind speeds may favour the generation of thermals above hot surfaces, capable of lifting up maize pollen grains. High wind speeds could reduce or prevent the generation of thermal convective cells. The results from other studies (Eder, 2006; Ganz *et al.*, 2007) showed that gap crops like potatoes influence the cross-pollination level due to thermal conditions between donor and recipient. But nevertheless, they suggested that maize is the best culture reducing considerably the cross-pollination rate between a donor and a recipient field.

3.5.4 Conclusion

In general, the cross-pollination rate decreased with increasing distance from the donor source. The vertical pollen transport over distance was more dependent on strong and predominant wind conditions than on different thermal conditions induced by different gap crops. Considering the general distribution of cross-pollination rates within the field, it could be concluded that the implementation of appropriate separation distances (100 m) is recommended for ensuring coexistence between non-GM and GM maize production fields. Moreover, it is evident that an appropriate separation distance alone cannot account for keeping crops completely separated due to the high interception of foreign pollen at the field edge. The removal of the first few rows of a field facing a GM crop prior to harvest might be worthwhile to reduce the total level of cross-pollination in the recipient field.

4. Cross-pollination from genetically modified maize (*Zea mays* L.): Effect of flowering time and climatic conditions

4.1 Abstract

Cross-pollination is a complex process depending on factors like flowering synchronization between pollen source and pollen recipient and climatic conditions. With the introduction of genetically modified (GM) crops, there is a major concern related to the possibility of unwanted transgenic inputs into non-GM crop production systems. In the framework of the “German Federal Research Programme on Coexistence”, the effect of flowering synchronization and weather conditions on the level of cross-pollination has been investigated for two years at one location in a worst case scenario: the field alignment was based on prevailing wind pattern.

The results of the study showed no clear correlation of the flowering time and the climatic conditions with the cross-pollination rate. There is evidence that cross-pollination per ear is not simply a function of start of female flowering. Environmental factors are known to alter the floral development in time, but nevertheless a certain independence of cross-pollination with respect to meteorological optima exists.

4.2 Introduction

Cross-pollination in maize is a complex process depending on pollen release, silk receptiveness, flowering coincidence and climatic conditions (Aylor *et al.*, 2003; Devos *et al.*, 2008; Lizaso *et al.*, 2003; Westgate *et al.*, 2003). Several field studies have examined the dynamics of maize flowering (Banner and Stamp, 2007; Pagano *et al.*, 2007; Vogler, 2008). It was shown that the greatest volume of pollen per tassel is typically shed during the second and third day after emergence of anthers. Peak pollen shed usually occurs in mid-morning. A second peak of pollen shed occurs in late afternoon or evening (Jarosz *et al.*, 2005). Pollen shed is not a continuous process; it stops when the tassel is too wet or too dry, and begins again when temperature and moisture conditions are favourable. Therefore, pollen release depends directly on short term meteorological incidents. Moreover, the optimal temperature or relative humidity needed for the release of pollen deviates from the climatic optimum needed for the ripening of pollen in the anthers (De Vries, 1971). The climatic conditions during the morning hours are most responsible for the extent of pollen release. During the day, the ripening of pollen in the anthers depends on weather conditions favourable for the anthers. High temperature from tassel initiation to cross-pollination shortens the duration of pollen shed (Struik *et al.*, 1986). Pollen shed throughout the whole day occurs only under relatively cool and cloudy conditions (Nielsen, 2007). Furthermore, the viability of pollen depends on temperature and relative humidity (Aylor *et al.*, 2003). Purseglove (1972) stated that pollen is viable for about 24 hours, but loses its viability more rapidly (< one hour) during very hot and dry weather conditions (Fonseca and Westgate, 2005). At anthesis, the pollen is dehydrated and continues to dehydrate as it moves through the atmosphere until it intercepts a silk (Hoekstra, 1986; Kerhoas *et al.*, 1987). Cool temperature and high relative humidity appear to be important factors in extending the time of pollen viability (Aylor, 2003).

For successful cross-pollination, silks must be receptive during the time that pollen is shed and viable. Silks are most receptive one day after exertion (Aylor *et al.*, 2003; Westgate *et al.*, 2003) and remain receptive, however, with decreasing receptiveness, up to 10 days after silk

emergence. Thus silks are more affected by long term climatic incident (Westgate *et al.*, 2003), drought and low relative humidity can make them to be non-receptive to pollen germination.

In the context of coexistence between GM and non-GM maize, attention has turned in recent years towards a more detailed knowledge of possible relationships between cross-pollination rate, start of female flowering and meteorological optima (Vogler, 2008). Therefore, this study was undertaken to investigate whether the level of cross-pollination is correlated with the flowering time of the recipient silking on one hand and with hourly recorded weather conditions during maize flowering conditions on the other hand using data from two cross-pollination field experiments over two years. Field experiments were conducted within the framework of the “German Federal Research Program on Coexistence”.

4.3 Materials and Methods

4.3.1 Location

Field experiments were performed in the years 2006 and 2007 at the site Wendhausen in Germany (52°17' N, 10°26' E; elevation: 76 m). The soil type is pseudogley with 45% clay, 25% loam and 30% sand. The location is characterized by temperate climatic conditions. The annual precipitation was variable depending on the year with 392.6 mm (with a maximum of 62.8 mm in August) in 2006 and 752.3 mm (with a maximum of 112.2 mm in September) in 2007. The annual temperature on average was 9.82°C in 2006 and 10.29°C in 2007 with the maximal monthly average of 22.73°C and 18.28°C, in July.

4.3.2 Plant material

In 2006, cross-pollination between maize varieties was determined using the Bt-maize variety PR39V17 as donor and the near isogenic variety Sandrina (both Pioneer, Europe) as recipient. In 2007, the Bt-maize variety PR39F56 was used as donor and the near isogenic variety PR39F58 (both Pioneer, Europe) as recipient. The Bt-maize varieties PR39V17 and PR39F56 contain the transgenic MON810 event, which expresses a bacterial *Bacillus thuringiensis* toxin (Bt toxin) gene, poisonous to the insect pest European corn borer (*Ostrinia nubilalis*).

4.3.3 Field trials

Maize was grown at a seed density of 100.000 seeds/ha and with a row spacing of 0.75 m. Seed material was protected against soil borne pathogens by standard seed treatment fungicides (TMTD 98% Satec a.i. thiram, Satec Elmshorn, Germany; Maxim XL, a.i. fludioxinil and metalaxyl, Syngenta, Maintal, Germany) and by bird repellent (Mesurol, Bayer, Monheim, Germany) Soil preparation, plant protection measures and fertilizer applications were performed according to the recommendation of the region.

The field design to measure cross-pollination between the donor and the recipient field is shown in Figure 3.1. The size of each donor field was 102 m x 230 m in the years 2006 and 2007. The recipient field was made up of 5 compact partial plots situated at different separation distances from the donor. The sizes of the recipient fields were: 38 m x 237 m (partial plots 1 and 5, separation distance 24 m), 38 m x 210 m (partial plots 2 and 4,

separation distance 51 m) and 78 m x 183 m (partial plot 3, separation distance 78 m) in 2006. In 2007 field sizes were 38 m x 210 m (partial plots 1 and 5, separation distance 51 m), 38 m x 183 m (partial plots 2 and 4, separation distance 78 m) und 78 m x 156 m (partial plot 3, separation distance 102 m). The space between the donor and the recipient was either planted with clover-grass (field design 1) or with spring barley (field design 2) in both years. In order to obtain barley stubbles (dry microclimatic conditions) as a gap crop, the barley field was harvested before beginning of the anthesis-silking of donor and recipient maize. 50 m wide hemp (*Cannabis sativa* L.) was sown between the two field layouts in both years. At the beginning of the maize flowering period hemp was taller than maize and was established to act as pollen barrier between the two field experiments. In general, all field experiments were designed in a worst case scenario: the Bt maize field was established upwind of the recipient maize in respect to the prevailing wind direction.

4.3.4 Weather recording

During the flowering period, meteorological data were recorded by an on-site weather station. Temperature and relative humidity were recorded hourly using a thermo-hygrograph. Precipitation was measured with a Hellmann-type rain gauge. A Woelfl e-type wind recorder detected wind speed and direction at a height of 2 m (all meteorological instruments, Lambrecht, Göttingen, Germany). Meteorological data recording was done by the German National Meteorological Service.

4.3.5 Flowering data

In order to assess coincidence of pollen shedding of the donor plants and silking of the recipient plants as well as coincidence of anthesis and silking in the recipient, 86 record points in the recipient and 60 in the donor field were selected (Fig. 3.1). At each record point 20 maize plants were labelled. Start of flowering, full flowering and end of flowering were monitored every day for the male and female flowers as recommended by Westgate *et al.* (2003).

4.3.6 Sampling strategy

To estimate the cross-pollination rate in the recipient field, 20 ears per sample point were harvested at full maturity stage (BBCH 89). In total ears from 222 (2006, field design 1), 294 (2006, field design 2), 294 (2007, field design 1), and 312 (2007, field design 2) sampling points were harvested. Ears were collected directly into labelled bags and after being dried down to approximately 10% moisture content in a seed dryer, each sample was shelled to a pooled kernel sample of approximately 6000 to 8000 seeds. One-half of each pooled sample was used for real-time PCR analysis for MON810 event; the remainder acted as retain sample. Samples were stored in a seed storage room (18°C, 30% relative humidity) before real-time PCR analysis.

4.3.7 Sample analysis

The percentage of MON810-specific DNA relative to an endogenous maize gene in each sample was quantified by real-time PCR. Real-time PCR was done by a DIN EN ISO/IEC 17025 accredited laboratory (Planton GmbH, Kiel, Germany) according to a validated MON810 event-specific protocol (Kuribara *et al.*, 2002; Shindo *et al.*, 2002).

4.3.8 Data analysis

For the analysis of the weather data collected during the flowering period, the time between 6 am and 6 pm was chosen as recommended by Jarosz *et al.* (2005).

Wind direction and wind speed were analysed using the Originpro8 software (OriginLab Corporation, USA). With this program, the dominant wind pattern for a particular area can be determined. Rayleigh's test was performed to determine whether there was a predominant wind direction during the flowering period of each year or if wind direction was random (Batschelet, 1981).

The Spearman rang correlation for cross-pollination with the start of female flowering (50% of plants at the stage "start of flowering") as days after planting was calculated for each sample point. The Spearman rang correlation for cross-pollination with weather parameters was calculated with climatic variables following the day at which 50% of the plants were at the stage "start of flowering" for each sampling point: maximal and minimal relative humidity, maximal and minimal temperature, maximal precipitation; as well as minimal and maximal wind speed, average relative humidity and average wind direction.

The Spearman rang correlations were calculated separately for each partial plot and distance from the donor field. At least 4 sampling points per distance were necessary to perform the calculation.

We defined the days of anthesis silking synchrony within the recipient maize variety Sandrina as ASI1 and the days of anthesis silking synchrony within the recipient female and the donor male flowers as ASI2. ASI1 and ASI2 were calculated as day difference at which 50% of recipient male and female at each sample point reached the stage "start of flowering" (ASI1) and the day difference at which 50% of the donor male and recipient female reached the stage "start of flowering" (ASI2).

Statistic analysis was carried out using the statistic software Statgraphics XV.I (StatPoint Technologies INC. USA).

4.4 Results

4.4.1 Precipitation, relative humidity, temperature

Precipitation, relative humidity and temperature data recorded during the period of maize flowering are shown in Figure 4.1a-b. In 2006, the weather was dry and sunny. The daily average temperature was high ($> 20^{\circ}\text{C}$) with no precipitation at the beginning of the flowering period. The relative humidity was low (around 60%) and increased only at the end of flowering with a mean of 74.3%. In 2007, the daily mean temperature was around 20°C at the beginning of the flowering period and decreased until the end of the flowering period. The precipitation was well distributed over the period of flowering. The relative humidity ranged between 56% and 97% with a mean of 77.4%.

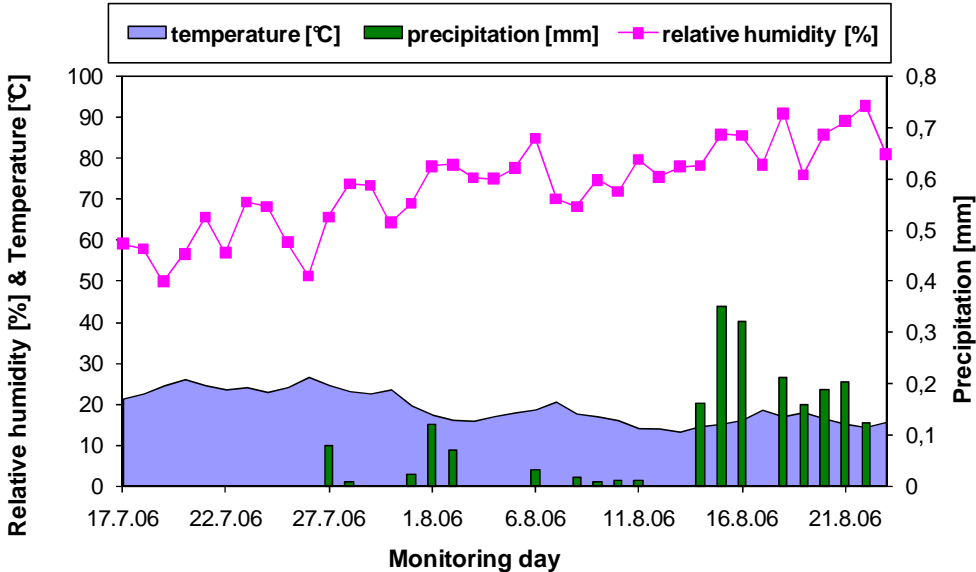


Figure 4.1a: Average daily temperature, relative humidity-, and precipitation during the maize flowering period in 2006.

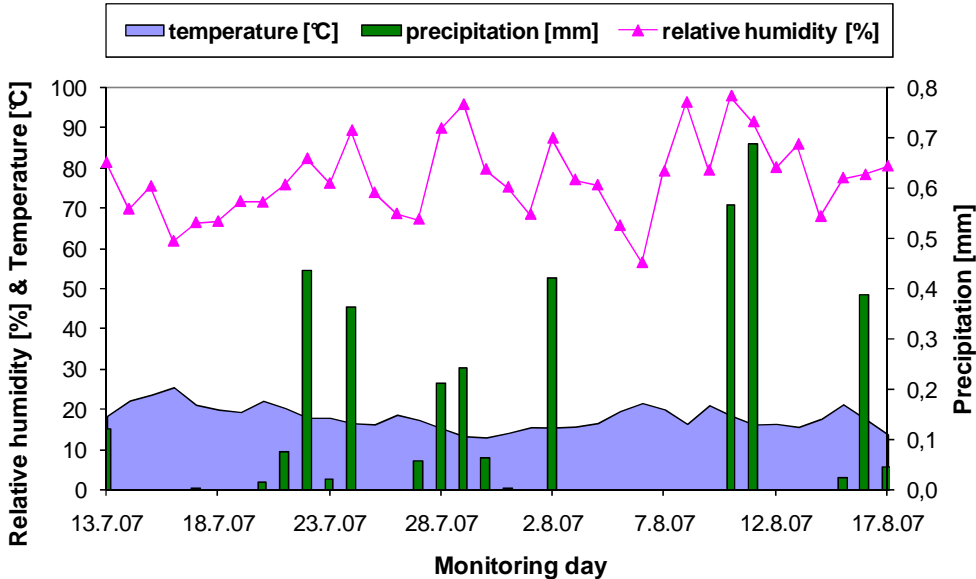


Figure 4.1b: Average daily temperature, relative humidity-, and precipitation during the maize flowering period in 2007.

4.4.2 Wind pattern during the flowering period

Wind direction and wind speed recorded during the assumed hours of pollen shed (6:00 am to 6:00 pm) are shown in Figure 4.2. Predominantly, wind came from westerly directions. The average wind direction was 227° (SD. 86.6°) in 2006 and 240.5° (SD. 91°) in 2007. Wind speed was variable with 5.3 m/s as highest value recorded in 2007. The average wind speed

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was 1.8 m/s (max. 4.4 m/s, min. 0.2 m/s) in 2006 and 2.2 m/s (max. 5.3 m/s, min. 0.1 m/s) in 2007.

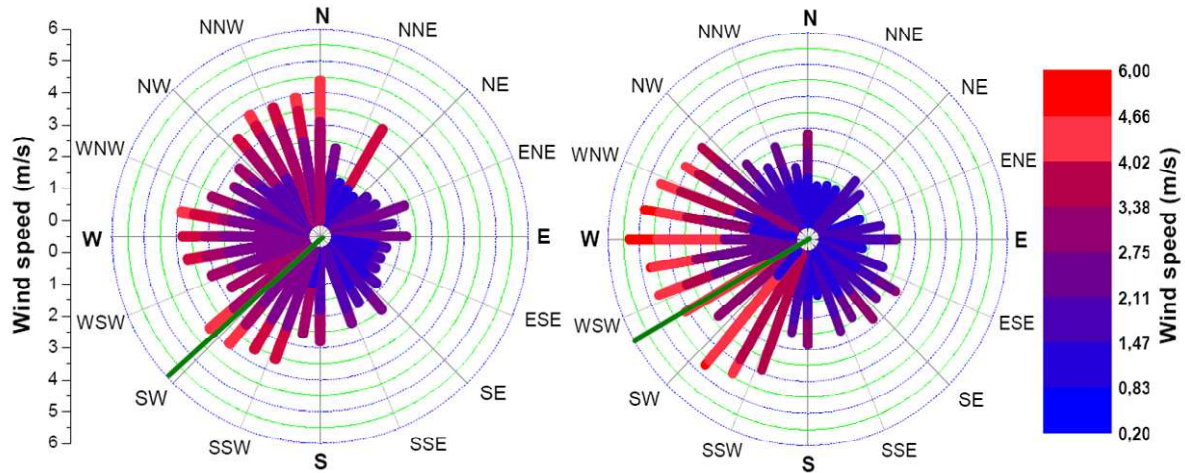


Figure 4.2: Wind rose diagram showing frequency of wind direction and wind speed during the flowering period in 2006 (left) and 2007 (right); the green line indicates the calculated main wind direction.

4.4.3 Flowering dynamics

Maize flowering dynamics differed between the field experiments and years. The Figures 4.3a-b show the flowering dynamic of the donor male and the recipient male and female flowering in 2006 and 2007. Flowering at each sample point was relatively uniform and did not show a great deviation from the field average.

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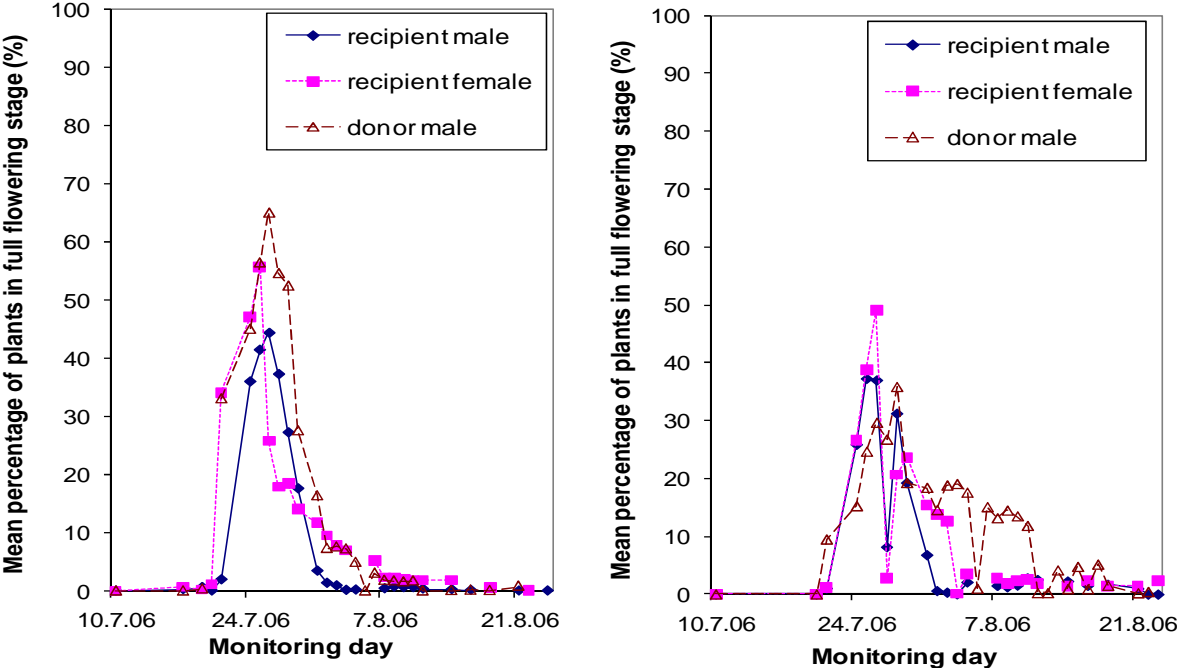


Figure 4.3a: Flowering dynamics of the male and female recipient flowers and male donor flowers for the field design 1 (left) and field design 2 (right) in 2006.

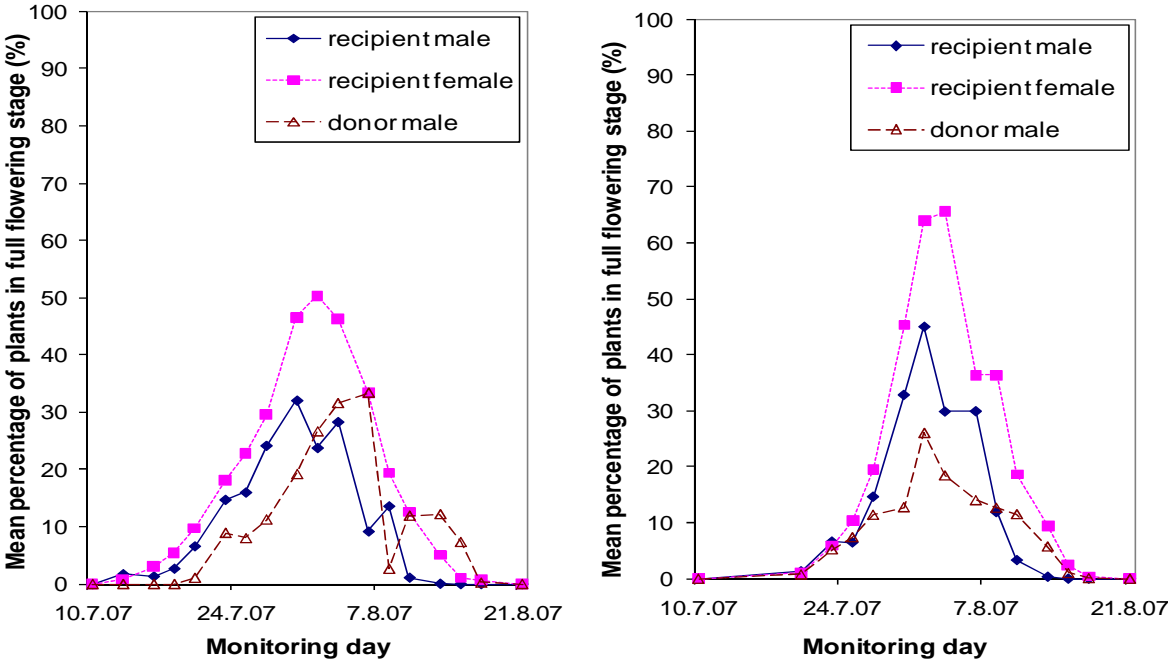


Figure 4.3b: Flowering dynamics of the male and female recipient flowers and male donor flowers for the field design 1 (left) and field design 2 (right) in 2007.

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The ASI2 (Table 4.1) was significantly larger than the ASI1 in both years and for both field experiments. Donor pollen was present during the main silking period of the recipient.

Table 4.1: Internal and external anthesis silking interval for the field design 1 and 2 in 2006 and 2007 (mean \pm SD)

	2006		2007	
	field design 1	field design 2	field design 1	field design 2
ASI1* (days)	1.98 \pm 1.65	2.61 \pm 2.02	2.20 \pm 1.18	4.02 \pm 1.87
ASI2* (days)	3.78 \pm 2.11	3.76 \pm 2.11	4.94 \pm 2.49	5.60 \pm 2.73

*: Internal (ASI1) and external (ASI2) anthesis silking interval

The analysis of all measured flowering data showed high correlation between the recipient female full flowering and the recipient male full flowering, the recipient female full flowering and the ASI1 and also a high correlation of the recipient female full flowering with the ASI2 (data not shown). Only the recipient stage “begin of female flowering” was used in the next step to assess the correlation between the level of cross-pollination and start of female flowering.

4.4.4 Correlation of cross-pollination with flowering time

The Spearman rang correlation for the level of cross-pollination with flowering time, using the cross-pollination rate of each sample point and the day at which at each sample point 50% of the silk were at the stage start of flowering, is shown in Table 4.2. There was no trend detectable.

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Table 4.2: Spearman correlation coefficients (rs) between the start of female recipient flowering and cross-pollination at different distances from the pollen donor for field design 1 and 2 in 2006 and 2007

partial plot	distance (m)	2006		2007	
		field design 1	field design 2	field design 1	field design 2
1+5	24	0.42	0.89*		
	34	-0.32	0.62		
	51	-0.81*	0.29	0.16	-0.07
	61	n.d.	n.d.	-0.009	-0.26
	78	0.56	-0.68	0.64	0.18
	102	n.d.	n.d.	0.55	0.12
	2+4	51	0.49	0.29	
61		0.74*	-0.31		
78		0.60	-0.68	0.44	-0.16
88		n.d.	n.d.	-0.07	0.28
102		n.d.	n.d.	0.66	-0.33
3	78		0.63		
	88		-0.21		
	98		0.31		
	102		n.d.	-0.71	0.88
	112		n.d.	-0.73	-0.36
	122		n.d.	0.65	-0.51
	138		0.6		

* = $p \leq 0.05$; n.d. = not determined ($n < 4$)

4.4.5 Correlation of cross-pollination with weather pattern

A casual relationship between climatic conditions and the level of cross-pollination could not be proven on the basis of hourly recorded weather data. As an example, the Spearman rang correlation associating each sample row with its corresponding weather data one day after silk appearance (DAP + 1) is shown in Table 4.3-4 for the average daily mean relative humidity and the minimal/maximal temperature.

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Table 4.3: Spearman correlation coefficients (rs) between the average daily mean relative humidity and cross-pollination at different distances from the pollen donor for field design 1 and 2 in 2006 and 2007

partial plot	distance (m)	2006		2007	
		field design 1	field design 2	field design 1	field design 2
1+5	24	0.30	0.74		
	34	-0.16	0.34		
	51	0.002	-0.69	-0.06	-0.18
	61	n.d.	n.d.	0.57	0.27
	78	0.56	-0.66	0.15	-0.27
	102	n.d.	n.d.	0.55	-0.48
	2+4	51	-0.58	-0.69	
61		0.52	-0.25		
78		0.64	-0.66	0.06	0.60
88		n.d.	n.d.	-0.19	0.43
102		n.d.	n.d.	0.83**	0.25
3	78	n.d.	n.d.		
	88	n.d.	n.d.		
	98	n.d.	n.d.		
	102		n.d.	0.27	-0.33
	112		n.d.	-0.73	0.53
	122		n.d.	0.30	-0.18
	138		0.58		

** = $p \leq 0.01$; n.d. = not determined ($n < 4$)

Cross-pollination from genetically modified maize (*Zea mays* L.): Effect of flowering time and climatic conditions

Table 4.4: Spearman correlation coefficients (rs) of the daily minimal und maximal temperature and cross-pollination at different distances from the pollen donor for field design 1 and 2 in 2006 and 2007

partial plot	distance (m)	2006				2007			
		field design 1		field design 2		field design 1		field design 2	
		°C min.	°C max.	°C min.	°C max.	°C min.	°C max.	°C min.	°C max.
1+5	24	-0.28	-0.31	-0.93+	-0.79				
	34	0.20	0.39	-0.91	-0.29				
	51	0.14	-0.02	-0.63	-0.07	-0.13	-0.28	0.03	0.03
	61	n.d	n.d	n.d	n.d	0.58	-0.28	0.39	-0.23
	78	-0.56	-0.56	-0.42	0.27	0.23	-0.08	-0.11	0.04
	102	n.d	n.d	n.d	n.d	0.55	-0.55	-0.21	-0.36
	2+4	51	0.60	0.41	-0.63	-0.07	-0.15	-0.05	0.59
61		0.31	-0.58	0.28	0.29	0.05	-0.17	0.17	-0.40
78		-0.10	0.31	-0.42	0.27	0.86**	-0.79*	0.49	-0.12
88						n.d	n.d	n.d	n.d
102						n.d	n.d	n.d	n.d
3	78			0.21	-0.63				
	88			0.21	-0.63				
	98			0.31	0.31				
	102			n.d.	n.d	0.26	0.80	-0.25	-0.22
	112			n.d.	n.d.	-0.73	0.73	0.17	0.49
	122			n.d.	n.d.	0.26	-0.87	-0.15	0.12
	138			-0.4	-0.4				

+ = $p \leq 0.1$; * = $p \leq 0.05$; ** = $p \leq 0.01$; n.d. = not determined ($n < 4$)

4.5 Discussion

In spite of a relatively synchronous flowering observed in 2006 and 2007 a clearly defined trend between the level of cross-pollination and start of female flowering could not be found. Results indicate that most of the silks exerted very early were only pollinated eventually. Furthermore, despite synchrony between pollen shed of donor and silk emergence, not all exposed silks were fertilized when the pollen shed period was short. This made recipient plants more susceptible to their own pollen and made them non-receptive to foreign pollen. Vogler (2008) found a positive correlation between the level of single plant cross-pollination and start of female recipient flowering. He assumed that late flowering plants are more prone to cross-pollination than early or average flowering plants. This is not supported by our study. Bassetti and Westgate (1994) showed that the level of cross-pollination decreased with decreasing uniformity of silking for the population.

In addition, no consistent correlation between the pattern of climatic conditions and the extent of cross-pollination was found. Some causal relationship between cross-pollination rate and meteorological optima may exist. For example, Vogler (2008) found that there was a negative correlation between the cross-pollination and the daily minimum temperature, but the one is not conclusively defined by the other. Despite this fact, the data collected in this study allow a discussion about the relationship between such factors. It can be assumed that the increase of temperature during the morning hours causes an increase in pollen release, regardless whether it takes place eventually at 16 °C or 20 °C. This peak of pollen release could then coincide with silking. Pollen shed is not a continuous process; it stops when the tassel is too wet or too dry, and begins again when temperatures and moisture conditions are favourable. In our study the climatic conditions varied considerably during the period of flowering, thus improving or deteriorating the ability of pollen to cross-pollinate receptive silks. Moreover, most viable pollen is released at 70 - 75% relative humidity. As a result the quantity available at a further decrease or increase of relative humidity is much reduced (Aylor *et al.*, 2003). Thus the level of pollen viability could be less than required for optimum pollination of receptive silks.

A similar line of thought is possible for the factor silking. It is possible that after 50% silk appearance the weather becomes better or worse influencing positively or negatively the process of cross-pollination. For successful cross-pollination, silks must be receptive during the time that foreign pollen is shed and viable. Silks also dry rapidly under hot and dry conditions and may not have enough moisture to support pollen germination and subsequent fertilization.

According to literature, temperature and relative humidity seem to have a major effect on cross-pollination, while the role of other rarely investigated factors (precipitation) on the level of cross-pollination has sometimes been differently interpreted. Vogler (2008) came to a negative correlation between the level of cross-pollination and the minimum temperature whereas a possible role for relative humidity and minimum night temperature could not be established. Emezc (1962) found that pollen shed in several grasses was hindered during wet weather conditions and with strong winds. However, these two factors seldom appear without a decrease in temperature and light intensity, therefore, their importance on the level of cross-pollination is difficult to assess under natural conditions. It is hard to say whether the climatic conditions are surrounding circumstances or causal factors.

4.5.1 Conclusion

This study demonstrated that because cross-pollination is a complex process depending on many factors such as climatic conditions and flowering synchrony between the donor male and the recipient female, it is not easy to predict the effect of start of female flowering on the cross-pollination rate. Furthermore, there is evidence that cross-pollination per ear is not simply a function of climatic conditions. Environmental factors are known to alter the floral development, but a certain independence of cross-pollination with respect to meteorological optima exists. In reality it means that it is possible to point out certain circumstances and weather conditions under which cross-pollination is relatively likely to occur, but nevertheless the estimation as causal factors is difficult under natural conditions and seems to be a more simultaneous effect of different environmental conditions on silk and pollen survival.

5. General Conclusions

This study was undertaken to investigate several factors affecting cross pollination and coexistence in farming systems with and without the use of genetically modified (GM) maize. We investigated specific questions of which some have never been investigated to date e. g. the consequences of GM seed admixture on the non-GM harvest product, the comparison of two GM detection methods: visual (% seeds) and real-time PCR (% DNA), the effect of two buffer crops with different climatic surfaces on the level of cross-pollination and the effect of start of female recipient flowering and weather conditions on the cross-pollination rate. The main conclusions drawn from the various investigations are summarized below.

5.1 Consequences of GM seed admixture on the non-GM harvest product

To date no study was focused on the effect of GM seed admixture on the non-GM harvest product. The necessity of investigating GM seed admixture has been given with the introduction of GM crops into agriculture. GM seed admixture is one of the main routes how adventitious presence in the non-GM harvest can happen. In our study, the GM content in the non-GM harvest product was similar to the GM input in the non-GM seed lot when both varieties belonged to the same maturity group. Using varieties with the same maturity group overlapping of the full flowering stages of male donor and female recipient flowers is given. The results show that the greatest amount of cross-pollination occurred in proximal plants and decreased with increasing distance. Differences between the in- and output were detected when the donor and the recipient plants belonged to different maturity groups. In this case, flowering asynchrony between the donor and the recipient plants lead to a lower cross-pollination rate in the harvest product.

5.2 GMO detection methods

The visual detection method (% seeds) was already used in the 20th century by plant breeders to determine seed purity (Jones and Newell, 1948) and with the time the system was established to investigate the process of cross-pollination (e.g. Bannert and Stamp, 2007; Langhof *et al.*, 2008; Ma *et al.*, 2004). With the introduction of GM crops into agriculture, the EU regulation frame (EC recommendation 2004/787) recommends that the quantitative results of GM material in food and feed should be expressed as % DNA (real-time PCR). On the other side, it is generally assumed that GM contents in seed lots should be expressed as % seeds. But nevertheless, the visual detection method doesn't necessarily reflect the real-time PCR method. It was hypothesized that the comparison between the visual GM detection and real-time PCR detection may result in a factor of 0.5 (Devos *et al.*, 2005; Pla *et al.*, 2006). The comparison of the two detection methods in our study showed that results from real-time PCR (% GM DNA) correlated closely with phenotypic estimation of the number of seeds (% GM seeds). The calculated slope was 0.4034. Furthermore, the visual detection leads to more accurate results than the real-time PCR. Real-time PCR results were consistently lower than expected. The real-time PCR variability was caused by the used certified reference material from the Institute of Reference Material and Measurement (IRMM) for the calibration of the

real-time PCR run. Possible explanations for the discrepancy include that the used IRMM reference material for the real-time PCR calibration carries the transgene by the female parent, whereas the transgene in our samples is paternally inherited.

5.3 Impact of isolation distance and different gap crops on the cross-pollination rate

In general, the cross-fertilisation rate decreased with increasing distance from the donor source because of maize pollen characteristics and pollen competition over the recipient field. In comparison to other studies on pollen dispersal and cross-pollination, the results presented here are similar to those obtained by Bannert (2006); Eder (2006); Ganz *et al.* (2007); Henry *et al.* (2003); Langhof *et al.* (2008); Ma *et al.* (2004); Messeguer *et al.* (2006); Pla *et al.* (2006) and Vogler (2008). It could be concluded that the implementation of appropriate separation distances (100 m) is recommended for ensuring coexistence between non-GM and GM maize production fields. Moreover it is evident that an appropriate separation distance alone cannot account for keeping crops completely separated due to the high interception of foreign pollen at the field edge. The removal of the first few rows of a field facing a GM crop prior to harvest might be worthwhile to reduce the total level of cross-fertilisation in the recipient field. Furthermore, the common assumption for wind-pollinated plants that gene flow is leptokurtic (Levin and Kerster, 1974; Moyes and Dale, 1999; Vekemans and Hardy, 2004) was not supported by our results. Curve performance differed significantly between the years.

Besides isolation distances between the donor and the recipient field, specific surface characteristics between the donor and the recipient field was mentioned as a solution to reduce the cross-pollination rate (Vonwahl *et al.*, 1991). Little is known about the behaviour of pollen over different thermal surfaces that can influence pollen transport outside maize fields (Loos *et al.*, 2003). In the experiment presented in chapter 3, the effect of clover-grass and barley stubble on the cross-pollination is compared. In general, the used gap crops enabled free passage of pollen transport. Clover-grass is characterized as wet and barley stubble as hot surface in agricultural landscapes (Noihan and Planton, 1989). No difference in the behavior of cross-pollination was detected in the recipient field, probably because of maize pollen weight. Maize pollen is too heavy to be influenced by thermal up winds. This study has demonstrated that gap crops with different thermal surfaces between two adjacent maize fields may not influence the level of cross-pollination. Proposed is always isogenic maize grown adjacent to donor field to reduce the level of cross-pollination.

A common conclusion from many cross-pollination studies is that most of the pollen coming from the donor field may be retained at the border rows of the recipient field (Devos *et al.*, 2005). Recipient border rows act as a barrier for donor pollen, thus the proportion of donor pollen within the recipient field will decrease. The results presented in this study are similar; cross-pollination rates were higher at the recipient field border facing the donor field than within the recipient field at equal distance. Most of the pollen coming from the donor field was intercepted by the first few maize rows. Harvesting separately the border rows facing the

donor field may be a helpful management strategy to reduce the level of cross-pollination in the recipient field.

5.4 Effect of flowering time of single plant and weather condition on the cross-pollination

From other studies it is known that weather conditions have a great influence on pollen viability (Aylor *et al.*, 2003) and therefore cross-pollination. In addition, flowering synchrony determines the level of cross-pollination (Westgate *et al.*, 2003). The effect of start of female recipient flowering and weather conditions on the cross-pollination rate was the focus of chapter 4. We associated the time at which 50% of plants at each sample point in the recipient field reached the start of female flowering with the cross-pollination rate, and the cross-pollination rates with climatic values (temperature, humidity, wind speed, wind direction) one day after 50% of the plants at each sample point in the recipient field reached the start of female flowering. In spite of a relatively synchronous flowering observed in 2006 and 2007, a clearly defined trend between the level of cross-pollination and start of female flowering could not be found. Results indicate that most of the silks exerted very early in pollen shed were only pollinated eventually. Furthermore, despite close synchrony between pollen shed and silk emergence, not all exposed silks were fertilized during rapid pollen shed. Weather affects pollen viability and therefore cross-pollination. In the case of our study, there was no clear trend between the cross-pollination rate and weather conditions.

Finally, we can conclude that a coexistence of farming systems with and without GM maize is possible. Our investigations have been done in the worst case scenario and thus it was possible to define a separation distance (100 m) where the cross-pollinations rate is under the 0.9% labelling threshold. In addition, our results showed that with some management strategies (separate harvest of the first recipient rows) the level of cross-pollination in the harvest product would be significantly reduced, because the highest cross-pollination rates are found in the first maize rows facing the donor field.

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