

**Simulation of transgenic pollen dispersal  
by use of different grain colour maize**

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Alberto Soldati was born in 1945 as son of a farmer in Lugano - Tessin and found his way to the Federal Institute of Technology Zurich. There, he became the well-known representative of the ETH Field Research Station in Eschikon-Lindau, which he helped to plan from the modest beginnings and which was efficiently managing until the sudden end of his career. Due to his scientific expertise and thanks to his friendly personality he always succeeded in a smooth and efficient working environment. Alberto Soldati initiated the maize cross-pollination research of the ETH Agronomy and Plant Breeding group in the year 2002 that resulted in the present thesis.



Alberto Soldati was my supervisor and supported me in scientific and personal questions. In 17. March 2004 his sudden death forced me to finish our project without him. But his working part should be never forgotten like also his person.

## Summary

Cross-pollination in maize was a topic that has been researched in former years by plant breeders in order to guarantee seed purity. Nowadays, the cultivation of transgenic maize varieties has rapidly increased worldwide; therefore, cross-pollination research has experienced an equally rapid comeback, this time in the context of managing coexistence of conventional and transgenic maize. This differs somewhat to the present plant breeding situation, where female plants get detasseled and are highly receptive to both the pollen from the male parent but also to adventitious pollen from other neighbouring fields. Maize is a wind pollinated crop that produces high amounts of pollen and favours cross-pollination by protandry of flowers and has therefore biologically the potential to cross-pollinate by pollen dispersal into neighbouring fields.

Some studies about cross-pollination in maize have been published in recent years across the world; but so far no experiments have been carried out for the Swiss alpine conditions that are different in constellations of potentially important influence factors like weather conditions and topography. In the years 2003 and 2004 22 field experiments were conducted, mainly in two different regions that are typical for Switzerland: In an alpine region of Kanton Uri and in the Swiss Midlands of Kanton Zurich. Cross-pollination was not measured directly by use of transgenic maize varieties. Instead of this a seemingly simple approach was used by simulating transgenic cross-pollination by maize varieties of different grain colours. In this visual marker system yellow grain maize is assumed to be transgenic and its cross-pollination of a white grain receptor maize can be immediately detected by xenia effect, in this case the transfer of yellow grain colour shows upon the white grain plant for every successful fertilisation. An experimental white grain test hybrid, DSP17007, was identified as comparable to modern hybrids, which was proven in pilot experiments. The method of measuring cross-pollination by counting the number of yellow grains was time- and cost-effective and enabled high sample rates, which made it possible to investigate complex cross-pollination patterns in high detail in order to optimize the sampling procedures as well as in order to analyze cross pollination events even at very low levels of incidences.

Cross-pollination was investigated at long distances of 50 m up to 4500 m in the alpine region of Kanton Uri. Thirteen white grain fields were arranged at different wind orientations to the yellow grain pollen donor. Cross-pollination rate of total fields was always below 0.02 %. Cross-pollination pattern were in most cases like randomly dispersed, mainly single cross-pollination events, with an average cross-pollination frequency of 1.8% of the sampled ears. In four fields, located in distances of 50 m up to 370 m in the main wind direction, a low but

marked cross-pollination at the field border was found; this indicated pollen dispersal by horizontal winds. The potential pollen dispersal distance was calculated according to the measured wind conditions in relation to settling height and speed; hereby it could be confirmed that most shed pollen will not reach distances beyond 50 m. A pollen take-off experiment corroborated that only a very small portion of pollen will move vertical above the field, due to special events like thermals or gusts; these results help to explain the low rates of long distance pollen dispersal. Some few “hot-spot” areas with higher cross-pollination rates existed; but whenever the surrounding plants were checked in detail, pure yellow grain contamination plants were found. Therefore “hot-spots” may be more often an effect of seed contamination than of extreme or special atmospheric events. This should be carefully taken in account for any cross-pollination experiment.

Short distance cross-pollination experiments in the Swiss Midlands of the Kanton Zurich focused on the situation that maize field are adjacent to each other; such situations may arise when a farmer intends to produce different maize types on the same field or when two neighbouring farmers are planting their maize crops without another separation crop in between. Moreover, such a situation is of scientific interest as an extreme situation.

With different field designs the variability of the cross-pollination rate was checked by different constellation of influence factors on cross-pollination rate in order to define “worst-case” situations of maximum cross-pollination. In a few wind exposed locations a clear effect of the main wind direction was found, in most other cases the wind effect was probably minimized by the varied topography of the Swiss Midlands, characterized by hills, scattered woods and settlings. An important effect of the size ratios between pollen donor and pollen receptor fields was hypothesized. These size ratios varied from about 4:1 to 1:8, however there was no impact visible on the cross-pollination gradients in distance to the pollen donor; only a small portion of the pollen anyhow dispersed beyond distances of 10 m. A strong effect was exercised by flower asynchrony. When the pollen donor field sheds pollen five days later than the emergence of silks in the receptor field the cross-pollination rate was below 0.9% even in the neighbouring row at 1 m distance, which is in accordance of observed wilted (already fertilized) silks around five days after emergence. But it could be proven that flowering asynchrony can be strongly modified by the synchrony or homogeneity of flowering within a receptor field. One pollen donor field was seven days later in flowering than the mid silk emergence of receptor field, therefore little or no cross-pollination should have occurred. A surprisingly high cross-pollination rate was due to a high number of yellow grains on small weak ears that were late in flowering when probably the white grain pollen

availability had become already quite low in relation to the inflow from the yellow grain donor. Although variations of cross-pollination were high at close distance to the pollen donor the rates decreased rapidly with distance and were in all experiments beyond 15 m below 0.9 %, except for some rare spots with higher rates.

The results of this Swiss study supported and complemented results of international studies in a detailed and reliable manner; in general cross-pollination rates were lower which might be a feature of special Swiss conditions for climate, topography and landscape patterns or the special flower biology of the used maize varieties.

\* All cross-pollination values were calculated to the hemizygous case of transgene varieties at present: Cross-pollination data were halved as yellow varieties were homozygous to the transferred yellow grain colour marker.

## Zusammenfassung

Schon vor langer Zeit untersuchten Züchter die Fremdpollenbefruchtung von Mais um die Reinheit von Saatgut garantieren zu können. Heute, bei einem raschen weltweiten Anstieg der Kultivierung von genetisch modifizierten Maissorten, ist die Fremdpollenbefruchtung von Mais erneut aktuell, um die Koexistenz von gentechnisch veränderten und konventionellen Mais zu regeln. Die Koexistenz Thematik unterscheidet sich deutlich von der Saatgutproduktion, bei der von den Mutterpflanzen die männlichen Blütenstände entfernt werden und diese dann sowohl für den Pollen der Vaterpflanzen als auch für Pollen von ausserhalb der Anlage sehr empfänglich sind. Mais ist eine windbestäubte Kulturpflanze und einige biologische Merkmale der Blüte, wie die Produktion von hohen Pollenmengen und die Protandrie (Vormännlichkeit), begünstigen Fremdpollenbefruchtung.

In den letzten Jahren wurden weltweit einige Studien über die Fremdpollenbefruchtung von Mais veröffentlicht. Aber bis jetzt gab es noch keine Untersuchungen unter den alpinen Bedingungen der Schweiz, bei der wichtige mögliche Einflussfaktoren wie Wetterbedingungen und Topographie anders sind. In den Jahren 2003 und 2004 wurden hauptsächlich an zwei unterschiedlichen Standorten, die typisch für Schweizer Verhältnisse sind, 22 Feldversuche durchgeführt: In der alpinen Region des Kantons Uri und dem Schweizer Mittelland von Kanton Zürich. Die Fremdpollenbefruchtung wurde nicht direkt mit transgenen Maissorten untersucht sondern durch die Verwendung von Maissorten mit unterschiedlicher Kornfarbe simuliert. Bei diesem visuellen Marker System simuliert gelbkörniger Mais die transgene Sorte und weisskörniger Mais die konventionelle Sorte. Befruchtet Pollen von gelbkörnigen Sorten in weisskörnigen Mais entsteht dort aufgrund des Xenien Effekts mit jeder erfolgreichen Befruchtung ein gelbes Korn. Als weisskörnige Sorte wurde die Test-Hybride DSP17007, die sich in Pilotversuchen als geeignet und vergleichbar mit heutigen Maissorten gezeigt hatte, verwendet. Diese Methode, die Fremdpollenbefruchtung durch einfaches Zählen von gelben Körnern auf der weisskörnigen Sorte zu ermitteln, ist bezüglich Zeit- und Kostenaufwand effektiv und ermöglichte hohe Stichprobenzahlen, um detaillierte Verbreitungsmuster zu untersuchen und auch seltene Fremdpollenbefruchtungen noch erkennen zu können.

In den alpinen Regionen des Kantons Uri wurde die Fremdpollenbefruchtung über lange Strecken zwischen 50 m und 4500 m untersucht. In beiden Versuchsjahren wurden zusammen dreizehn weisskörnige Versuchsfelder in verschiedenen Abständen und Wind Orientierungen zu den gelbkörnigen Feldern angelegt. Die Fremdpollenbefruchtung des gesamten Feldes blieb immer unter 0.02%. Die Verbreitungsmuster der Einkreuzungen waren hauptsächlich

durch zufällige über das gesamte Feld verstreute Einkreuzungen, meist einzelne Einkreuzungen auf durchschnittlich 1.8% der untersuchten Kolben, charakterisiert. Bei vier Feldern, die in Abständen von 50 bis 370 m in Hauptwindrichtung eines gelbkörnigen Feldes orientiert waren, konnten an dem zugewandten Feldrand in der Randreihe etwas höhere Einkreuzraten gefunden werden, die vermutlich den Polleneintrag durch horizontale Windverfrachtung zeigen. Die durchschnittliche mögliche Pollenverfrachtungs Distanz wurde aufgrund der Windmessungen in Bezug zur Sinkgeschwindigkeit des Pollens errechnet. Damit konnte gezeigt werden, dass der meiste Pollen innerhalb von 50 m Reichweite zu Boden geht. Ein Versuch zur vertikalen Verfrachtung des Pollens zeigte dass nur eine sehr kleine Menge des ausgeschütteten Pollens durch Windböen oder thermische Aufwinde in 4 m Höhe über das Maisfeld gelangt. Diese Ergebnisse bestätigen die gefundenen niedrigen Auskreuzungsraten jenseits 50 m Distanz. Einige „hot-spots“, Bereiche mit abweichend hoher Einkreuzung, wurden gefunden. In den Fällen, in denen die nähere Umgebung dieser „hot-spots“ genauer untersucht wurde, konnten gelbkörnige Verunreinigungspflanzen als Ursache festgestellt werden. Die auch von anderen Experimentatoren beobachteten „hot-spots“ sind daher vermutlich eher auf Saatgutverunreinigungen als auf extreme Wetterereignisse zurückzuführen.

Schwerpunkt am Versuchsstandort im Schweizer Mittelland im Kanton Zürich waren Versuche zur Fremdpollenbefruchtung über kurze Distanz bis 50 m mit dem Fall eines Anbaus von gelbkörnigem Mais direkt neben weisskörnigem Mais. Solche Situationen treten auf wenn ein Landwirt verschiedene Maissorten kultiviert oder wenn zwei benachbarte Landwirte ihre Maisfelder ohne einer anderen Feldfrucht dazwischen anlegen. Besonders aber ist diese Situation von wissenschaftlichem Interesse weil so die maximal mögliche Fremdpollenbefruchtung nachgewiesen werden kann.

Mit verschiedenen Feldversuchsanlagen wurde die Variabilität der Fremdpollenbefruchtung, verursacht durch die verschiedenen Konstellationen der Einflussfaktoren, untersucht, um „worst-case“ Situationen mit maximal hoher Fremdpollenbefruchtung zu ermitteln. Bei einem windexponierten Standort konnte ein klarer Effekt durch die Orientierung zur Hauptwindrichtung beobachtet werden. In den anderen Fällen war aber kein deutlicher Windrichtungseffekt zu erkennen, was auf die besondere Topographie des Schweizer Mittellandes mit Hügeln, verstreuten Waldstücken und Siedlungen zurückzuführen sein könnte. Ein wichtiger Einfluss des Feldflächen Verhältnisses von Pollendonator und Rezeptor wurde vermutet. In Versuchen wurden Verhältnisse von 4:1 bis 1:8 untersucht. Jedoch ergab sich dadurch kein erkennbarer Einfluss auf die Fremdpollenbefruchtung, was vermutlich

darauf zurückzuführen ist, dass nur sehr wenig Pollen über eine Distanz von 10 m hinaus verfrachtet wird. Einen starken Einfluss zeigte eine ungleiche Blütezeit. Wenn die Pollenausschüttung des Donor Feldes fünf Tage nach dem Erscheinen der Seiden im Rezeptorfeld mit der Pollenausschüttung begann war die Fremdpollenbefruchtung sogar in unmittelbarer Distanz von 1 m weit unter 0.9%. Dieser Befund stimmte auch mit der Beobachtung überein, dass die Seiden bereits fünf Tage nach ihrem Erscheinen verwelkt (bereits befruchtet) sind. Aber es wurde auch festgestellt, dass die Synchronie der Blütezeit von Feldern von der Gleichmässigkeit der Entwicklung der Pflanzen innerhalb eines Bestandes abhängt. Ein Pollendonor Feld begann sieben Tage nach dem Erscheinen der Seiden des Rezeptorfeldes mit der Pollenausschüttung und eine sehr geringe Fremdpollenbefruchtung wäre zu erwarten. Jedoch stieg die Fremdpollenbefruchtung wieder an aufgrund von einzelnen kleinen Maiskolben mit hoher Einkreuzung. Diese kleinen Kolben sind wahrscheinlich auf eine verzögerte Pflanzenentwicklung und Blüte zurückzuführen. Zu diesem Zeitpunkt der Blüte war vermutlich die Pollenausschüttung des eigenen Feldes im Verhältnis zur gerade beginnenden maximalen Pollenausschüttung des Pollendonor Feldes bereits reduziert. Obwohl es auf kurze Distanz, vor allem den ersten Metern, hohe Variabilität in der Fremdpollenbefruchtungsrates gab, nahm bei allen Feldversuchen die Rate mit der Distanz schnell ab und war ab 15 m bereits unter 0.9%. In seltenen Ausnahmefällen gab es auch jenseits der 15 m Distanz leicht erhöhte Werte („hot-spots“).

Die Ergebnisse der Studie in der Schweiz unterstützen und ergänzen bereits bestehende internationale Studien in detaillierter und verlässlicher Weise. Im Allgemeinen waren die Raten der Fremdpollenbefruchtung aber etwas geringer, was auf die besonderen Bedingungen in der Schweiz bezüglich Klima, Topographie und Landschaftsmuster sowie auf die besondere Blütenbiologie der verwendeten Maissorten zurückzuführen sein könnte.

\* Alle Fremdpollenbefruchtungswerte in dieser Studie wurden auf den heterozygoten Zustand derzeitiger transgener Sorten berechnet: Werte von Experimenten mit Kornfarben Marker, wie hier verwendet, wurden halbiert um den homozygoten Zustand der verwendeten gelbkörnigen Sorten zu berücksichtigen.

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## **CURRICULUM VITAE**

# 1 General Introduction

## 1.1 Development of plant breeding

Since agriculture was incepted more than 8000 years ago in different parts of the world, farmers choosed prospicious plant species and turned them into crops by adapting them slowly to their needs. Food supply often failed until 150 years ago in the developed world and up to present days in developing countries, causing severe hardships for affected people. Innovative progress in science and technology brought mankind in developed countries on the way to a society of wealth these days. One important step of it was the development of modern agriculture. As plants did not evolve to the needs of humans Plant Breeding based on Mendel's laws was a driving force for this development (*Odenbach, 1997*). Modern maize breeding started more than a hundred years ago in North America but the enormous yield boost in modern maize varieties was only achieved by the development of hybrid maize breeding 80 years ago. This was one driving factor to make maize one of the three most important crops worldwide. Genetic improvement led to increased productivity, earlier maturity, reduction of lodging by stronger root systems, shorter and stronger stalks and resistance to destructive disease pathogens and insect pests (*Poehlman and Sleper, 1995*). Thus yield could be increased 5-6 times by hybrid breeding (*Zscheischler et al., 1990*).

Breeding methods still have to be adapted these days according to new demands and goals for speciality crops in the developed world and for food security in developing countries. Many of these new demands and goals for agriculture at present and in future can not be achieved by classic breeding methods and depend on the introduction of sophisticated breeding technologies like plant biotechnology with a broad variety of different methods. Because of its effective large scale cultivation and its global economic importance maize became a main target for using plant biotechnology in breeding. Although in developed countries food production is high right now, farmers have to maintain or increase productivity and to cope with economic pressure. Also problems of large scale cultivation have to be solved in the most sustainable way. Important breeding targets are improvements of pest and herbicide resistance.

1996 the first transgenic plants were commercially sown in USA. Today, nine years later, transgenic plants grow on 81 million ha worldwide and are an important part of agricultural practice in some countries (*Transgen, 2005a*). GM maize is grown mostly in USA, Argentina,

Canada, Spain, South Africa, Uruguay and Philipines. For the year 2015 its cultivation is forecast for 30 countries and at a total level of 150 million ha (*Biosicherheit, 2005b*). Although there are a lot of different genetic modified cultivars in maize, at present there are only three different transgenic traits with global significance for agriculture: Herbicide resistance, BT-resistance to European corn borer (*Ostrinia nubilalis*) and BT-resistance to corn rootworm (*Diabrotica virgifera virgifera*).

Herbicide resistant maize produces through genetic modification an altered biosynthesis of an enzyme, thereby making it resistant to herbicides like “Roundup Ready” (glyphosate). These maize varieties have a great advantage to cope effectively with weeds in large scale cultivation. Beside economic benefits, by using these maize varieties new environment-friendly cultivation systems like conservation tillage systems and extended intercropping managment that improve soil fertility and reduce soil erosion can be implemented (*GKB, 2001*).

The BT-transgenic maize varieties are built up on an old concept of fighting pests. *Bacillus thuringiensis* (BT) species produce a group of proteins (BT-protein) that kill certain insect species. Since 1964 compounds of BT protein are used in organic farming. In transgenic BT-maize genes for the expression of BT proteins were transferred. Because of a high specificity, Bt-maize resistant against European cornborer and corn rootworm have different kinds of BT-protein (*Biosicherheit, 2005a*).

## **1.2 General acceptance, risk assessment and coexistence**

The current developments in agriculture using methods of biotechnology show some promising benefits on the one hand. But on the other hand there are a lot of public concerns about potential hazards, furthermore ethic reservations exist. There is a responsibility to use new technologies in such a way that hazards are avoided in a reasonable way. Besides concern on an impact of transgenes on environment (non target organism, horizontal gene transfer) and human health, there is a clear demand for a coexistence of conventional and GM-plants to guarantee free choice for farmers and consumers in Europe.

In many countries there are labelling requirements for products containing or derived from GMOs (genetic modified organism). In EU countries, the threshold for positive labelling of food and feed products containing or derived from GM crops is set at 0.9% at the moment (Regulation EC1829/2003 on GM food and feed; *EurLex, 2003*). In Switzerland labelling requirements are similar to the EU (*Transgen, 2005b*). In other countries labelling of

unintended GM content of plant products is quite different depending on the attitude to GM plants. For example in USA and Argentina GM labeling is voluntary but not compulsory, in Canada exist a voluntary threshold of 5%, a level that is mandatory in Japan (*Choices, 2003*). The countries that introduced mandatory-labeling legislation of GM foods have done so to give their consumers a choice in selecting the foods that they feel comfortable with (*Ahmed, 2002*).

Unwanted GM-contamination in conventional or organic production can result by events like seed lot contamination, volunteers, mixing at sowing, cross-pollination, harvest, and trade (*Sanvido et al., 2005*). Under Central European conditions contamination by volunteers in the field is unlikely, as maize kernels do not survive low winter temperatures north of the Alps; but it is possible in tropical regions, where seed companies usually continue breeding in winter time, e.g. Mexico, (*Hütter et al., 2000*). As maize is a wind pollinating crop, genetic material of GM-maize can be dispersed by pollen in adjacent conventional fields. The thesis will focus on this aspect of cross-pollination, as one crucial component of research for handling coexistence of conventional and genetic modified (GM) maize.

### **1.3 Flowering biology in maize**

Maize is a monoecious plant and develops unisexual male and female flowers in separated parts of the plant. The male florescence (tassel) arises from the shoot apical meristem, while the female inflorescences (ears) originate from the axillary bud apices. In every axillary bud of a leaf a female inflorescence is initiated, but only one, or in some cases two, will develop to a fertile ear (*Maiskomitee, 2003*). Abortion or growth cessation of a second ear usually occurs during or just after silking of the uppermost ear (*Tollenaar M., 1977; Harris et al., 1976*). Studies suggested that abortion of late-pollinated ovaries (e.g. from the tip of the apical ear or subapical ear) is triggered after the fertilization of the early pollinated ones, in what can be defined as a primigenic dominance (e.g. the dominance exerted by the organ formed first; *Bangerth, 1989*). Although many modern hybrids are prolific types, high yielding maize crops usually have only one ear and do not exhibit prolificacy and second ears at the common high plant density required for high yield (*Duvick, 1992*).

As the tassel is on the top of the plant and the silk at the middle part of the stem, the flowers are separated vertically by about 1-1.3 m on a plant that can become up to 3 m tall. This plant architecture favors outcrossing. There is usually protandry and the male inflorescence (tassel) appears around 2-4 days before silk emergence, favouring cross-pollination (*Maiskomitee, 2003*). Although modern breeding efforts have tended to reduce protandry (floral synchrony)

the tassel may begin to shed pollen before silks emerge. The degree of male and female floral synchrony is genotype specific and sensitive to plant population, soil fertility and environmental stress. Usually the tassels have emerged before pollen shed begins (*Burris, 2001*). Thus, maize is a naturally cross-pollinating crop; about 95% of the ovules on a shoot are usually cross-pollinated and 5% self-pollinated in a normal field stand (*Poehlman and Sleper, 1995*). Most of the pollen that pollinates an ear of maize comes from plants in the immediate vicinity, although the wind may carry pollen over large distances. In the fields there is often non-uniform plant growth (different plant size) and development (start of phenological stages of flowering) resulting in longer periods until flowering is completed, and a probably asynchronous pollination within the population (*Ma et al., 2004*).

Pollen is produced in the anthers of the tassel at the top of the plant. When mature, the anthers suspend on filaments, emerged from the tassel, and pollen is released from an opening at the tip of the anther. Even a slight breeze or vibration (e.g., as can be caused by gently tapping the stem) will release the pollen into the air when it is mature. The low wind speed threshold for maize pollen release has implications for its dispersal. Usually most pollen will be shed under low wind conditions with low potential traveling distances (*Aylor et al., 2003*).

The male inflorescence (tassel) can produce considerably more pollen grains than required for pollination of a single plant (*Schoper et al., 1987*). A single tassel from a normal plant may produce up to 25 million pollen grains or an average of 25 000 pollen grains for each female flower on an ear of 750 to 1000 kernels (*Poehlman and Sleper, 1995*).

*Jones and Newell (1948)* and *Raynor et al. (1972)* found production rates between 9 000 to 50 000 pollen grains per kernel set, depending on the genotype. This range is typical for wind-pollinated species (*Burris, 2001*). There is large variation in the estimates of pollen for tassels; *Burris (2001)* reports estimates ranging from 14 to 50 million in older varieties (*Miller, 1985*) to 2 to 5 million in modern hybrids. Because of selection for a reduced male dominance, the size of an average dent hybrid tassel has declined over the last three decades; this was due to a selection for closer spacing that demanded smaller tassel size and pollen production (*Duvick and Cassman, 1999*).

Changes in tassel size among maize hybrids have been observed since the 1930s as breeders focused on increased grain yield. Tassel dry weight has decreased by 36% from 1967 to 1991, improving the light interception into the canopy as well as reducing the demand on carbohydrate resources (*Duvick and Cassman, 1999*). This trend continues. *Mickelson et al. (2002)* described molecular markers for tassel traits. They suggest that the ideal maize genotype would have a smaller tassel at current plant densities, sufficient for reproduction and

seed set in a dense field stand. In conclusion, modern hybrids with reduced pollen shed contribute less to cross-pollination but their pollen competition to adventitious pollen is also lower.

The typical tassel may shed pollen for 2-14 days depending on genotypic and environmental factors with the major shed during a 5-8 day period beginning on approximately the third day after the tassel is expanded. During the shedding period the pollen is released for approximately four to five hours commencing approximately one hour after sun rise. The period may be delayed by one to two hours if the weather is cool and cloudy (*Burris, 2001*). Similarly, *Jarosz et al. (2003)* reported maize pollen shed for a period of 5-8 days, with the major portion of the daily release usually occurring during midmorning to midday. Also *Treu and Emberlin (2000)* and *Wolt et al. (2003)* reported that pollen anthesis continues for 5-8 days, but under favorable conditions the vast majority of pollen shedding may occur within a 2 day period. *Paterniani and Stort (1974)*, reported a flowering time of a tropical maize field of about 10 – 15 days.

Maize pollen is released mainly during dry and drying conditions. Additionally, maize pollen can be released during unsettled atmospheric conditions, preceding fronts or thunderstorms (*Aylor, 2003*); In experiments in France (*Jarosz et al., 2005*), pollen release started once the air vapor pressure deficit increased above 0.2 to 0.5 kPa. Dynamic of release was correlated with the dynamic of air vapor pressure deficit surrounding the tassels. The concentration at night did not fall to zero suggesting that pollen may have been resuspended (*Jarosz et al., 2005*).

To sum it up, there is quite some variation in pollen shedding, mostly depending on weather conditions and plant developmental stage.

#### **1.4 Pollination of maize**

Wind is the main agent in pollination of maize. Maize pollen grains are one of the heaviest and largest (about 90 – 100  $\mu\text{m}$  in diameter) among the wind-dispersed pollen grains, thus limiting the distance maize pollen can travel (*Raynor et al. 1972*). In a study of settling velocities of various pollen types *Di-Giovanni et al. (1995)* reported that maize settled nearly ten times faster than other pollen types. Under natural conditions, the majority of pollen grains from a plant normally fall on the soil surface within the row space; but it is assumed that a small amount of pollen can be transported over longer distances given favorable wind

speeds and appropriate humidity (*Kiesselbach, 1949; Garcia et al., 1998*). Washout by rain is disadvantageous for maize pollen dispersal. Fresh pollen grains have an internal osmotic potential of about  $-1.2$  Mpa (*Westgate and Boyer, 1986*). When placed in pure water, they take up water readily until they burst open, whence they are incapable of germination. It seems likely that washout by rain can severely limit maize pollen dispersal both by rapidly removing airborne pollen from the air and by preventing germination and fertilization (*Aylor et al., 2003*). Frequent rainy weather during flowering time can result in badly fertilized ears (*Maiskomitee, 2003*).

Honeybees collect pollen for feeding and breeding their larvae. They collect them around a distance of 2 km of their beehive. The female flowers of maize are not attractive to honeybees because they produce no nectar. Male and female flowers are spatially separated. During maize pollen collection at the top of the plants the bees usually do not come in contact with the female silks at the mid of the stalks. (*Transgen, 2005c*). *Kohl (1993)* reported that honeybees collected pollen of maize fields even when there were enough possibilities to collect pollen from other plants. During late maize flowering in mid July to end of July half of the collected pollen originated from *Zea mays* and *Helianthus annuus*. In Germany (*Transgen, 2005d*) field experiments were conducted on pollen collection by honeybees. When the bee populations were established before the onset of maize flowering, bees did not collect pollen of maize despite direct proximity they had already looked for more attractive flowers. In conclusion, honeybees usually do not contribute to fertilization and cross-pollination in maize (*Transgen, 2005c*).

### **1.5 Pollen viability**

In in-vitro tests it was found that UV-B treatment interferes with flowering (delay), pollen ultrastructure and anther maturation but there was no effect on pollen germination, regardless of treatment during plant development and/or pollen germination (*Santos et al., 1998*). During the ontogenic development of reproductive organs, pollen grains are enclosed in anthers and it has been demonstrated that anther walls attenuate UV-B radiation by at least 98% (*Flint and Caldwell, 1983*). Maize pollen was found to be much more resistant to UV-C than the relatively resistant conidia of *Venturia inaequalis* and the urediniospores of *Uromyces appendiculatis* (*Aylor, 2004*). Maize plants specifically, were classified as tolerant with respect to this stress condition (*Krupa and Kickert, 1993*).

Because pollen germination appears to be relatively insensitive to solar radiation *Aylor (2004)* concluded that dehydration is the main factor in maize pollen mortality. Several other studies have shown the sensitivity of pollen to dehydration (*Barnabas, 1984; Buitink et al., 1996; Luna et al., 2001*). Water is lost from a pollen grain at a rate that depends mainly on the conductivity of the pollen wall and to the drying power of the atmosphere, which depends on the energy input from the sun and the vapor pressure deficit of the ambient air (*Aylor, 2004*). There is a limit of 80% to the amount of water loss which maize pollen can sustain (*Barnabas, 1984*). According to *Kerhoas et al. (1987)* pollen is shed in partly dehydrated state at anthesis; at anthesis, the water content of maize pollen is 57-58%. Maize pollen can withstand the drying process until a water content of 28% is reached, at which point 60-80% of the individuals show a negative reaction in a fluorochromatic viability test. At this water content, there is no more crystallizable water and thus metabolism decreases, leading to oxidative damage. It is interesting to note that each grain in the pollen population develops independently according to its tolerance to drying.

The water content of maize pollen directly affects its dispersal in the atmosphere through its effect on settling speed and viability. Depending mainly on the vapour pressure deficit of the air, the water status of maize pollen can change from being fully hydrated to being nearly dehydrated within 1 – 4 h (*Aylor, 2003*). Several physical changes take place during drying: for example, the shape of maize pollen changes from a prolate spheroid to a crinkled, prismatic solid, its specific gravity increases by about 16%, and its settling speed decreases by about 34% (*Aylor, 2002*).

*Aylor (2004)* determined the survival of maize pollen for various lengths of exposure to atmospheric conditions outdoors on different days by in vitro germination tests. Initial germination percentages were on average about 35 %. The germination percentage of freshly collected pollen exposed to direct sunlight outdoors tended to increase initially, remained relatively high for 60-120 min, and then decreased more or less rapidly over the next 60-120 min. *Luna et al. (2001)* measured the longevity of pollen exposed outdoors in Mexico in the wintertime using a complete fertilization criterion. They found a relative loss in pollen viability of 80% in 1 h and a 100% loss in viability in 2 h, and suggested that pollen death was due mainly to dehydration, controlled primarily by the vapor pressure deficit of the air. However depending on the environmental factors, mainly temperature (*Goss, 1968; Schoper et al., 1987; Jemison and Vayda, 2001*), humidity (*Goss, 1968; Barnabas, 1984; Garcia et al., 1998; Traore et al., 2000; Jemison et Vayda, 2001*) and atmospheric water potential

(Luna *et al.*, 2001) it can remain viable for up to 24 h after shedding. Cool temperatures and high humidity favor pollen longevity.

Barnabas (1984) found, that during the dehydration process adhesion, germination percentage and length of the pollen tube decrease substantially. Obviously, during severe dehydration the pollen fitness is reduced and there is not only quantitative but also qualitative pollen competition.

Pollen that has travelled over long distances will certainly be in disadvantage, compared to freshly shed pollen of a field. Water capacity of soil or additional watering could also vary the pollen vitality and competition. Pollen transported in the atmosphere will generally experience cooler air. Also there is higher humidity on higher air layers for rehydration. Clouds show 100% relative humidity. So if hydration is the only limiting factor on viability pollen could theoretically survive quite long, and viable transport over long distances could be possible.

### **1.6 Silk viability and time of fertilization**

The silks are the styles of the female part of the flower. One silk directs the germ tube of a germinating pollen grain to one ovule, which must be fertilized in order for a kernel to develop. Silks emerge from the top of the ear and continue to grow until fertilized, some reaching lengths up to 15 cm or more (Bassetti and Westgate, 1993a). The spikelets close to the base of the ear produce the longest silks which become the first to protrude from the husk. Silks originating from the base or the middle of the ear emerge next followed by apical silks. The number of female spikelets per ear varies by genotype and environment but rarely all the spikelets are fertilized and develop into seed. The silks are receptive at emergence and can remain receptive for more than 10 days. Once fertilized the silk stops elongation and desiccates rapidly. If not fertilized, the silk will continue to elongate until it is fertilized or cellular elongation is complete (Burris, 2001). Silks follow a well-defined sequence of emergence, elongation, and senescence (Bassetti and Westgate, 1993a), demarking a definite time window for fertilization. Consequently, the ability of pollen grains to fertilize is also depending on a receptive state of the female silks and eggs (Aylor *et al.*, 2003).

Senescence of silks started 7 to 8 d after emergence from the husk, as evidenced by the collapse of the basal region of the silk (Bassetti and Westgate, 1993b). Pollen tubes are unable to grow through this senescing area, thus preventing fertilization and contributing significantly to ear barrenness, especially in open pollinated varieties where pollen supply is less limiting.

Water deficits also inhibit the growth of silks, especially during the day (*Herrero and Johnson, 1981; Westgate and Boyer, 1986*). Water stress delays the senescence of recently exposed silks, but accelerates it in silks already exposed for several days to the atmosphere (*Bassetti and Westgate, 1993c*). Pollen survival alone is not sufficient for effective gene dispersal since the hydration of the silk and ovules can have a large impact on seed set (*Westgate and Boyer, 1986*). Water deficit has been implicated in damage to the embryo sac of the developing ovary when imposed at the megaspore mother cell development stage, and has resulted in an 80 to 90% reduction in grain weight per plant (*Moss and Downey, 1971*).

The silks themselves do not readily retain pollen, unless all are captured on the trichomes (hairs) on the silks. On a crop canopy basis, however, the silk area density is only about 0.001-0.02 m. Clearly, many of the airborne pollen grains are intercepted by other plant parts or the ground surface and do not hit their intended target (*Aylor et al., 2003*). As pollen grains drift through the maize canopy the vegetative parts thus can filter most of the pollen from the air, competing with the silks for available pollen. But fresh maize pollen has a tendency to roll on plant surfaces and it can be easily dislodged from a leaf flapping in a breeze. Thus, if pollen lies on surfaces sloping downward toward the leaf tip it can readily roll-off the leaf and get a second chance to reach silks. However, if it was deposited on surfaces sloping downward towards the stem, it might wind up being trapped in the leaf axle. When fresh pollen arrives, very little pollen sticks to the silks themselves, but the hairs on the silks are covered as they are sticky and retain deposited pollen against the force of an air jet having speeds of 15-30 m s<sup>-1</sup> (*Aylor et al., 2003*). Only small points of a silk are receptive and for this reason it needs a lot of pollen to hit the right parts. Many pollen grains can be deposited on a given silk, but only one will fertilize the ovule to which it is attached; each silk has multiple deposition sites but only one fertilization site (*Aylor et al., 2003*).

### **1.7 Anthesis silking interval**

When maize is stressed at flowering because of deficits of water, light, nutrients, and sometimes by long photoperiods and lack of thermal adaptation, ear growth slows in relation to tassel growth and the interval between pollen shedding and silk emergence, the anthesis-silking interval (ASI), increases. This appears to be a general response of the plant to a reduction in photosynthate formed during this growth stage (*Edmeades et al., 2000*). If maize cultivars are grown outside their area of thermal adaptation, for example during breeding phases in tropical countries at wintertime, ASI may increase, probably because of poor

adaptation. When cultivars adapted to the cool highlands of Mexico were grown in hot lowland tropical environments, ASI increased from 2 to 7 days; conversely, when a lowland tropical population was transferred to the highlands, its ASI increased from 0.3 to 5 days (*Lafitte et al., 1997*). Thus introgression of new, exotic germplasm could result in higher cross-pollination susceptibility. Nitrogen deficiency results in delayed flowering in maize (*Hanway, 1962*) and in slow silk emergence from distal spikelets (*Lemcoff and Loomis, 1994*). When drought occurs at flowering, silking is delayed though anthesis and anther dehiscence may be accelerated slightly by the higher air temperatures and lower relative humidity (*Srinivasan et al., 1997*). *Herrero and Johnson (1981)* observed that drought during silking had a greater effect on female than male floral development. Silk delay at high plant densities has been known since long (*Kohnke and Miles, 1951; Lang et al., 1956*).

Anthesis silking interval (ASI) can be observed on an individual plant basis, but at the plot level it is usually measured as the date when 50% of plants have visible silks minus the date when 50% of plants first extrude anthers. The ASI is a ubiquitous indicator of the level of stress under which the crop is growing at flowering, and a good predictor of grain yield and barrenness under stress (*Edmeades et al., 2000*).

The adverse effects of drought at flowering have been known for many years. *Lonnquist and Jugenheimer (1943)* observed that heat and drought on tassels and silks resulted in poorly pollinated ears, *Kiesselbach (1950)* reported silk delay in response to drought. *Sadras et al. (1985)* determined that the period during which silks are exposed to pollen may condition final kernel set. According to *Schussler and Westgate (1991)* kernel number decreased of up to 99% with severe water stress occurring during silking. This could be explained by the fact that in maize the number of kernels is mainly related to the current flux of assimilates around silking that is negatively influenced by stress (*Edmeades and Daynard, 1979*). Early attempts to quantify the relationship between ASI and grain yield were led by *DuPlessis and Dijkhuis (1967)*, who reported an 82% decline in grain yield as ASI increased from 0 to 28 d under drought stress (*DuPlessis and Dijkhuis, 1967*).

Detailed studies by Hall and co-workers in Argentina (*Hall et al., 1980, 1981, 1982*) indicated that lack of pollen for late-emerging silks can be a significant cause of barrenness.

For individual plants of different cultivars *Hall et al. (1982)* reported a pollen shed period that varied from 7 to 9 days. In a stand of a modern hybrid, pollen shedding has been shown to occur over a period of 16 days, with 90% occurring over a period of around 10 days when pollen shed density exceeds  $100 \text{ grains cm}^{-2} \text{ d}^{-1}$ , an important pollen density for successful fertilization. At the peak of pollen production, more than  $500 \text{ pollen grains cm}^{-2} \text{ d}^{-1}$  are shed

(*Bassetti and Westgate, 1994*); grain set declines when pollen shed density falls below 100 grains  $\text{cm}^{-2} \text{d}^{-1}$ , in agreement with *Sadras et al. (1985)*.

*Bassetti and Westgate (1994)* noted that grain set in a maize crop declines because of pollen supply when ASI was  $< -5 \text{ d}$  or  $> 5 \text{ days}$ . Further losses could be expected with silks exposed for  $>6 \text{ days}$  because of silk senescence (*Bassetti and Westgate, 1993b*). When ASI exceeded 7 days in individual plants, grain set was limited by both pollen availability and silk exposure at the ear tipp, and the typical pattern emerged where grain set is restricted to the midbase of the ear. So under stress with increased ASI, there is a small period of possible cross-pollinations. Modern breeding efforts have tended to reduce protandry (floral asynchrony), reducing the tassel size concomitantly over the last three decades. To compensate this in production of pure hybrid seed increased border rows and increased male percentages are necessary (*Burris, 2001*). As mentioned above, a larger tassel size may be particularly important in stress environments where pollen shed is often reduced (*DuPlessis and Dijkhuis, 1967*). By selecting for tassel traits, breeders must balance the shading effect of the tassel with the need for adequate pollen, particularly in stress inducing environments where silking is delayed.

*Hall et al. (1982)* suggested the use of a mix of hybrids, including a late-pollinator, for reducing the negative effects of the lack of pollen. Although these studies imply lack of pollen is a primary cause of reduced kernel set under stress conditions, the addition of viable pollen to late-appearing silks does not guarantee an increase in final kernel number (*Otegui et al., 1995*). Pollinated ovaries of water-stressed plants are fertilized, but kernel abortion occurs after a few cellular divisions (*Westgate and Boyer, 1986*). *Bassetti and Westgate (1993a)* reported a decrease in kernel number caused by the loss of silk receptivity during water deficits, whereas *Otegui et al. (1995)* described this phenomenon to kernel abortion. *Westgate and Boyer (1986)*, addressing effects of water stress on the ASI-grain yield relationship, proposed that these causes could be grouped as a combination of several effects. Lack of pollen can be due to asynchrony, nonviability or because anthers do not exert. Damage to the embryo sac during megasporogenesis will normally prevent pollination, though silking may still occur (*Moss and Downey, 1971*). A slow rate of spikelet growth results in a large ASI, silk senescence, and abortion following pollination. *Westgate and Boyer (1986)* concluded that the reduction in grain number per plant under drought is almost always a combination of pollen supply and spikelet fitness.

Water deficit during silking reduces elongation (*Bassetti and Westgate, 1993d*) and promotes asynchrony between pollen shedding and silk appearance (*Hall et al., 1981, 1982*), which may

result in insufficient pollen availability for late-appearing silks from the tip of the ear. Thus, the tip of the ear is most susceptible to cross-pollination by foreign pollen.

These facts have consequences for cross-pollination. Whenever there is a perfect temporal alignment of pollen shed and silk emergence in a field pollen grains from local sources greatly outnumber (and out-compete) pollen from adventitious sources. On the other hand, if there is an imperfect temporal congruence of pollen release and silk receptivity there is an increased opportunity for pollen from outside sources to fertilize the ear. Adverse environmental conditions, such as drought, can alter the timing of silk emergence in relation to pollen shed, resulting in poor fertilization (*Hall et al., 1982; Bruce et al., 2002;*), allowing pollen from a distant field to have a greater competitive advantage than normally might be expected (*Aylor et al., 2003*). The importance of synchronous flowering in avoiding outcrossing in seed production is summed up in an old maize breeders adage: „If you have a perfect nick, then spatial isolation is easy, however, if you have an imperfect nick, then you can never have enough spatial isolation“ (*Aylor et al., 2003*).

## **1.8 Outline of the thesis**

Although there are different maize cultivation systems with different consequences for cross-pollination, the thesis will focus on the most common system of growing maize for silage or kernel harvest. Some results on cross-pollination are already available, but no study exists so far about cross-pollination under conditions of Alpine regions. It was presumed that the special climatic conditions, topography and landscape of Switzerland could result in different cross-pollination patterns.

In Switzerland, large scale field experiments with transgenic plants are not desirable today. Successful preliminary studies with different kernel colour markers, to simulate transgenic cross-pollination, were carried out. With a broad series of different field experiments at different locations the fluctuation margin of cross-pollination was to be examined and important factors of influence to be identified in order to develop data sets that allow for a more rational discussion of threshold levels.

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## 2 Measurement of pollen dispersal in maize by grain colour markers

### 2.1 Methods for measuring pollen dispersal

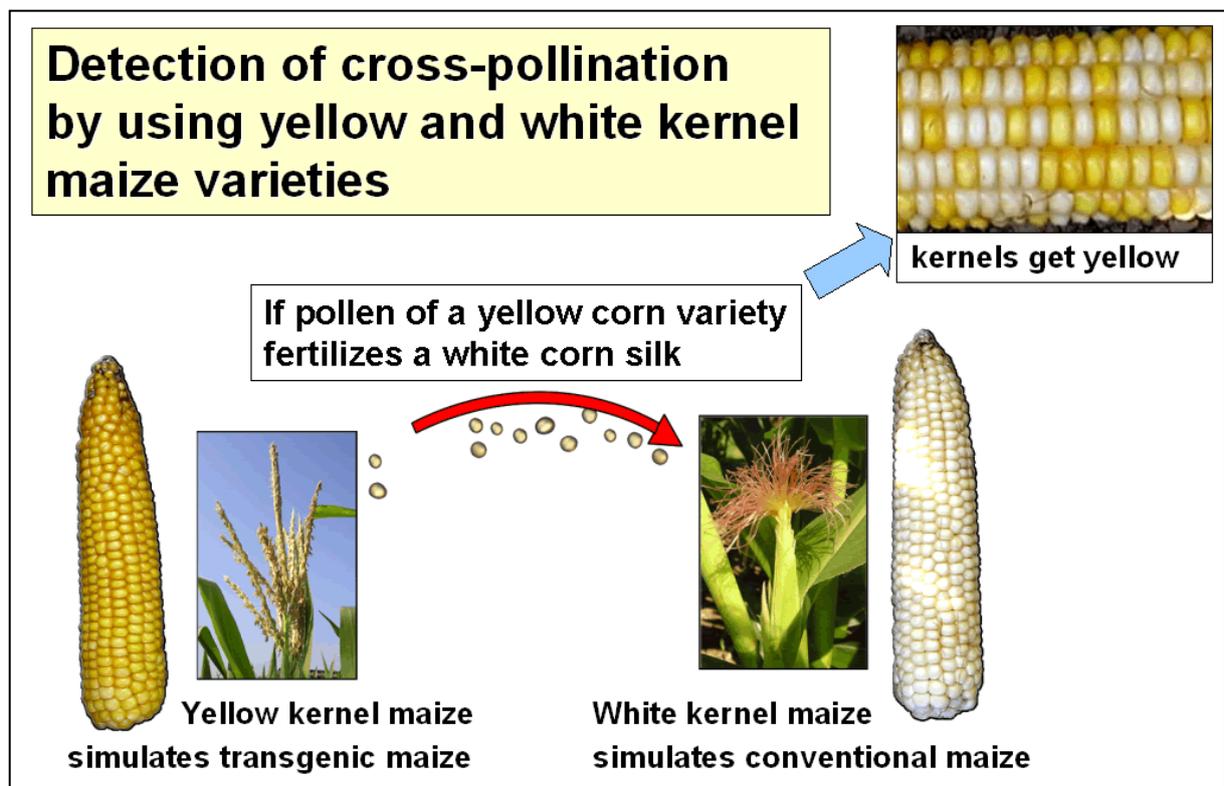
There are several methods available for measuring real or simulated transgenic cross-pollination in maize. Most common are PCR detection, germination assay of herbicide resistant maize, use of visual markers like waxy and sugary grain texture or grain colour markers.

PCR detection assays are based on the parallel amplification of transgene specific sequences and an endogenous reference gene, which serves as internal control for the quality of the extracted DNA. After amplification the total amount of target DNA present in the sample can be determined (*Hernandez et al., 2003*). The germination assay of herbicide resistance, that is sometimes used in combination with PCR detection, is based on transgenic herbicide resistant plants as pollen donors. If pollen of such field stands fertilizes plants in conventional field stands the grains will bear this transgenic information. When progenies from the latter will be sown, only seedlings with transgenic herbicide resistance will survive after herbicide treatment. Counting the rate of dead and surviving seedlings will show the cross pollination rate (*Biosicherheit, 2002*). Visual detection of cross-pollination on waxy maize is a possible tool, because conventional pollen cross-pollinating on waxy maize can be recognized by a simple colour test practised directly on the grains of the ear (*Fouellassar and Fabié, 2005*). A direct visual detection would be possible by using sweet corn maize as pollen receptor if varieties of suitable vigour are available.

Another approach is based, too, on a xenia phenomenon, i.e. grain colour transmission. In the case of dominance, the appearance of grain colour is characteristic of the pollen parent after fertilization. In maize, the seed coat is usually transparent and determined by the genotype of the mother plant, whereas pigmentation occurs in the aleurone tissue, and is thus determined by the genotype of both parents. In the case of dominance the male gene controls the colour over two copies from the female parent (*Coe, 2001*). These grain colours caused by pigmentation in the aleurone tissue of endosperm are easy to observe and can be used as markers in genetic studies or tools for evaluating cross-fertilization. The colours black, blue and red rely on anthocyan pigments, yellow is due to carotenoids, and white is the lack of any pigmentation. The system of dominant blue colour was used rarely (*Byrne and Fromherz 2003*), probably because of the complex genetics, instabilities of blue colour inheritance and lack of suitable hybrids for experiments in temperate conditions. But the system of dominant yellow pigmentation by pollen transfer on white grain maize is well known from a range of

experiments on cross-pollination so far (*Salamov, 1940; Jones and Newell, 1948; Jones and Brooks, 1950; Hutchcroft, 1958; Raynor et al., 1972; Paterniani and Stort, 1974; Garcia et al., 1998; Ma et al., 2004; Stevens et al., 2004*). In the earlier years this system was used to investigate cross-pollination in seed production to improve seed purity. Nowadays the system has been rediscovered in determination of cross-pollination of transgenic maize.

World production of white maize is estimated to amount to about 65 to 70 million tons, representing 12-13% of the annual world output of all maize. But in Switzerland commodity maize is uniformly of yellow grain colour. In this thesis the yellow grain hybrids act as dominant pollen donors to white grain hybrids which are serving as recessive receptors. The yellow grain hybrids simulate transgenic hybrids and the white grain hybrid the conventional one. If pollen of yellow grain hybrids fertilizes in a white grain hybrid the simulated transgenic contamination can be measured by simply counting the number of yellow grains on white grain ears. This is of great advantage comparing to the other methods, especially expensive PCR analysis, and enables high sample rates. Thus it is possible to gain knowledge of potential transgenic cross-pollination without using transgenic plants.



**Figure 2.1: System of cross-pollination detection by use of yellow and white grain maize**

## **2.2 Experimental set-ups with white grain maize**

A fitting white grain maize test hybrid, DSP17007, was bred by Delley Seeds and Plants (DSP) in Switzerland; it was first tested by *Weingartner et al. (2002)*. It is medium early for central European conditions and close the yield level of maize hybrids in this class. Given the fact that farmers are usually cultivating hybrids homozygous for yellow grain colour, pollen dispersal patterns can be studied in a network approach with farmers who agree to grow maize in desirable combinations with white maize test hybrids in selected field situations. Thus different geographical situations can be chosen and analyzed. Sowing in a region should be done preferably in collaboration with the local contractor in order to have a sufficient control on a complete cleaning of the machine before a new colour type is sown; this is one of the crucial error factors.

An important decision is how and how much to sample. Sample sizes must be big enough to detect contaminants at a predetermined expected threshold of cross-pollination. Ultimately, the optimum sampling strategy is a balance between sensitivity, cost and confidence (*Ahmed, 2002*). For pioneering scientific test evaluations one can go back to the technically highest possible sample number according to field size, available manpower and harvest time period. The number of samples can be limited by the short time for harvest in cooperations with farmers especially in the case of silage maize as the grain needs maturation until early dough stage before yellow grains can be distinguished from white grains.

To calculate the possible maximum of samples it has to be considered how many fields will be sampled, their total size, the quota of fodder maize with only a short period of time for harvest and the number of man hours available during this period. A technical solution to optimize the logistics can be to sample and store only ears with more than about ten yellow grains per ear, ears with less yellow ones can be preferably counted in the field. This enables to check up high numbers of ears per field in order to look on dispersal patterns if the question asks for a high spatial resolution.

## **2.3 Average grain number per ear and sample number**

The calculation of the cross-pollination rate is based on average grain number of the field. There are several factors of influence on final grain number of an ear that can lead to a considerable variability between ears of the same field. In maize, the number of potential grain ovules is established early in plant development and can be affected by carbon and nitrogen supply, while the number of these ovules that ultimately develop into mature grains is affected by the degree of pollination and extent of grain abortion (*Below et al., 2000*).

Under optimum conditions, grain sink capacity and number of grains is genetically determined (*Jones et al., 1996*). In general, small-grained hybrids (grain weight < 300 mg) have a larger grain number than do large-grained hybrids (*Otegui and Andrade, 2000*). Actual grain sink capacity under field conditions is clearly mediated by environmental perturbations such as high temperature and water deprivation (*Jones et al., 1996*). Presumably, starch content in maize ovaries supports reproductive growth and ovary starch levels are associated with final grain number (*Zinselmeier et al., 1985*). Also *Schussler and Westgate (1995)* reported that assimilate flux plays a key role during critical stage of reproductive development. Stress factors that limit assimilate flux will reduce final grain number. Water deficit around anthesis (*Westgate and Boyer, 1985; Schussler and Westgate, 1991; Ober et al., 1991; Quattar et al., 1987*), weed stress (*Tollenaar et al., 1997*) and N supply (*Mozafar, 1990; Uhart and Andrade, 1995*) will result in grain losses.

This response to stress is highly activated during a relatively narrow time-frame from early ear-shoot growth prior to pollination through the early period after pollination. By contrast, induction of storage product accumulation and later phases of grain fill are relatively insensitive to stress (*Ober et al., 1991; Quattar et al., 1987*). *Grant et al. (1989)* reported that the interval when grain number was sensitive to moisture stress began 2 to 7 days after silking and ended 16 to 22 d after silking and *Tollenaar (1977)* reported that grain numbers are determined during a period ranging from approximately 10 days before until 15 days after anthesis.

The main advantage of the use of the white grain colour marker is the fast assessment of yellow grains on an ear; their number will be mostly low when the experimental conditions are targeted to low contamination thresholds like 0.9%. The superiority of the system could not be maintained if the total grain number of an ear has to be counted in each case. Therefore a representative sampling pattern has to be devised first for the whole field or field parts in the case of large field to calculate an average grain number per ears; in relation to the homogeneity of a field stand the number of sampled ears per area unit will vary. On this average grain number per ear the percentage of the outcrossing can be based.

#### **2.4 Sample size and detection probability**

Sample sizes must be big enough to detect contaminants at the expected threshold. Too small sample sizes or improper sampling technique can result in “jackpot” distributions of positive and false negative results that will not represent the real situation (*Anklam et al, 2002*).

The USA Department of Agriculture provides a formula to determine sample size for single sample qualitative testing (USDA, 2000). Given the desired lot concentration and probability of detection, a sample size is computed with the following formula:

$$N = \log [1-(G/100)/\log(1-(P/100))]$$

N sample size (number of grains)  
 G probability in percent of rejecting a lot concentration  
 P Percent concentration in the lot

The relationship between the sample size and the probabilities of detection is exponential, so the sample size would have to be increased significantly to increase detection limit and probability. A table of the Ministry of Agriculture and Forestry (MAF) of New Zealand shows this relationship on an example of detection GM seeds (MAF, 2002).

Sample size Grain number (~g)	Chance of detecting 0.1% GM	Chance of detecting 0.05% GM	Chance of detecting 0.01% GM
1400 (~500)	75 %	50 %	13 %
3200 (~1000)	95 %	79 %	27 %
6400 (~2000)	99 %	95 %	47 %
10 000 (~3000)	99 %	98 %	63 %

**Table 2.1: Interplay of sample size, detection limit and detection probability**

To detect contamination of 0.1% GM a probability of 75 % can be achieved by 1400 grains, but for detection of 0.01 % checking 10 000 grains would achieve only 63 % probability (MAF, 2002). If the detection confidence is sought 95% at 0.1% contamination, then seed samples should be bigger than 4300 seeds and for 99% confidence bigger than 6600 (Heinemann et al., 2004). Very low concentrations of GM contaminations are always difficult to detect. For example to detect a 0.01% percent lot concentration with a 99% probability the required sample size is 46 050 grains. Increasing detection probability on low concentrations of GM contamination will increase sample size in a way it is not able to handle any more. So there has to be made a compromise between detection probability and sample size handling (USDA, 2000).

Variation found in field by checking the ears can decline during harvest, transport and storage due to different mixing processes. If in a PCR check of a maize harvest the raw materials are not systematically mixed during harvest and storage, the resulting strata can seriously invalidate assumptions associated with simple random sampling (Anklam et al., 2002). This

raises the additional issue of determining the number of samples (of the size recommended in the calculations described above) to quantify confidence limits associated with any heterogeneity because of incomplete mixing (*Heinemann et al., 2004*). But it can be expected, that heterogenous cross-pollinations in fields get more and more homogenous due to the different procedures starting from harvest to milling seed bulks for PCR analysis or product processing at the end, according to Boltzmanns physical law of entropy of all mixed matter.

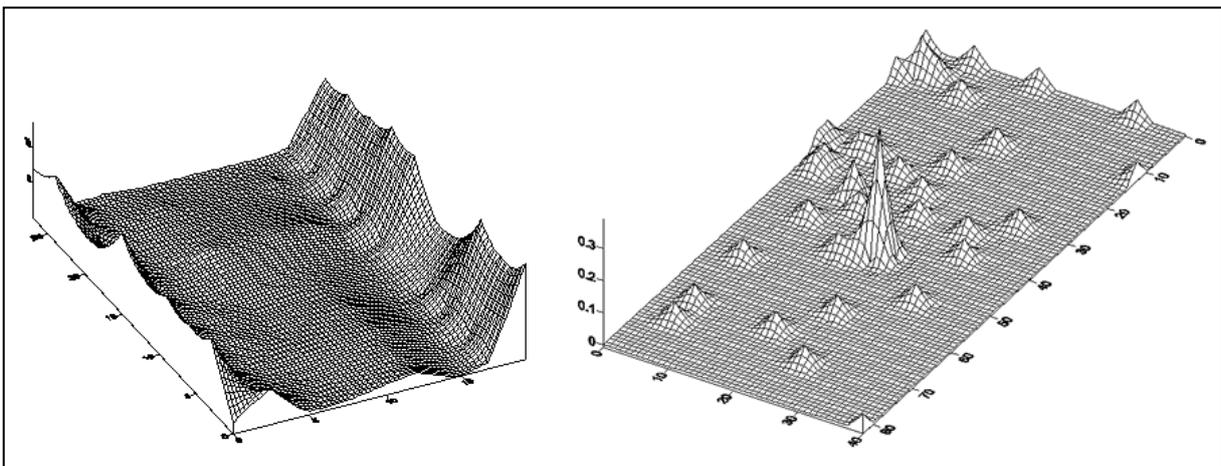
## 2.5 Evaluation of the colour marker system

In the present studies the formula (*USDA, 2002*) must be transformed to check which detection threshold is obtained on a probability of 95%, the standard discussed for seed control, according to recommendation of a tolerable 5% risk for consumers by the EU expert group of seed experts (*Kruse, 2004*).

$$P = - (10^{[\lg(1-(G/100))/N]} - 1) * 100$$

- P detectable cross-pollination concentration in lot
- N sample size (number of grains)
- G probability in percent of rejecting a lot concentration

Distribution of cross-pollination is different in short distance (distances up to 50 m) in comparison to long distance (distances greater than 50 m). On short distances there are strong gradients (Figure 2.2) of cross-pollination related to distance but on long distance cross-pollinations are more homogeneously distributed (Figure 2.3), probably as a result of a kind of fall-out of a “pollen-cloud” above the field.



**Fig.2.2 (left): Short distance cross-pollination: Strong gradients**

**Fig.2.3 (right): Long distance cross-pollination: Homogeneous distribution**

Such features have to be considered for calculation of detection thresholds in a different way for short distance and long distance. For long distances these calculations can relate to the whole field but for short distance the calculations can only relate to a certain distance within the gradient of similar cross-pollination levels.

As an example, detection threshold for a long-distance experiment ( $P_{LD}$ ) with distances above 50 m was calculated with the following assumptions: homogeneous distribution of cross-pollination (see figure 2.3) and sampled ears in a one ha field; 1000 ear samples per field with an average of 315 grains per ear resulting in 315 000 grain samples.

$$P_{LD} = - (10^{\lceil \log(1-(95/100))/315000 \rceil} - 1) * 100 = 0.00095$$

According to the formula above a homogeneous cross-pollination of about 0.001 % can be detected with a probability of 95%. This is a much lower detection threshold than the most sensitive PCR approaches reached so far (0.1 % technical detection limit).

Detection thresholds on short-distance experiments ( $P_{SD}$ ) would be principally the same as in long-distance experiments. But at short distance the distribution of cross-pollinations is more heterogeneous with strong gradients (see figure 2.2). For calculation of detection threshold only samples within similar distances to a foreign pollen source are representative for the cross-pollination in this area. Given these assumptions, a row with 100 sampled ears and an average ear grain number of 315 results in 31 500 investigated grains.

$$P_{LD} = - (10^{\lceil \log(1-(95/100))/31500 \rceil} - 1) * 100 = 0.0095$$

With the sample system there would be a detection threshold with probability of 95 % for whole field of 0.001 % cross-pollination and for a single row, with a certain distance, a detection threshold of about 0.01 %, which is quite sufficient for a scientific in depth analysis of cross-pollination rates at short distance. The same accuracy cannot be achieved at low cost by PCR analyses.

## 2.6 Comparison of colour marker results to PCR analysis results

For a comparison of cross-pollination values it has to be considered that PCR analysis methods and the GM background itself can be very heterogeneous. There are different copy

numbers of transgenes in different GM hybrids. For comparison often calculation factors are needed. Transgenic events are generated in maize lines selected for high transformation rates and other interesting traits for laboratory use and manipulation. Once a transformed plant is achieved, the transgenic locus is transferred through back-crosses to different existing lines. Thus, one transformation event can be introduced into many cultivars. In seed production the crossing of a homozygous transgenic parent with a non-transgenic one results in hemizygous hybrids for the gene of interest (*Trifa and Zhang, 2004; Vaughn et al., 2005*). For Bt11 and Mon810 hybrids, the two main types of BT maize at present, the cry1Ab gene for BT Toxin is expected in only half of the pollen and egg cells from hemizygous parents (*Chilcutt and Tabashnik, 2004*). As yellow hybrids are usually homozygous to yellow colour, 100% of the pollen will deliver yellow colour information on the white grain. For a comparison of transgenic outcrosses with the simulated ones by grain colour, the latter results have to be halved.

But maize hybrids with more than one transgene already exist, e.g. herbicide tolerance or different BT proteins affecting different pest (*Vaughn et al., 2005*). For these transgenic hybrids the probability of transgene occurrence in their pollen has to be calculated on an individual basis, according to the number of transgene copies in the parental lines.

## **2.7 Analytical error of PCR and grain colour marker assessments**

The analytical uncertainty of quantitative PCR methods is generally assumed to be around 25% for the best PCR methods available, it is difficult to estimate the uncertainty introduced by variation between different cultivars, harvests and grains (*Holst-Jensen et al., 2003*). During the German „Erprobungsanbau“ in 2004 a test comparison was done with four different certified laboratories; at low levels of contents results were similar but in the critical range around 0.9% there was a variation of sometimes more than factor two of the true value (*Weber et al., 2005*).

By using the grain colour marker system, contamination plants can have an impact but if the seed production has been done at sufficient care and the sowing procedures are sufficiently controlled the impact should be small; therefore this method is simple and robust, errors do not occur on a large scale. But in comparison with PCR analysed data the possible percentage deviation of the values and thus the range of values have to be kept in mind.

## 2.8 Tissue specific DNA ratio maternal / paternal

Whereas the grain colour marker just indicates the transgenic event in principle, the quantity of transgenes and the quantity of the transgenic product are subject to complex tissue dependent processes. Grains are composed mainly of three different kinds of tissues: embryo, endosperm and teguments. As the DNA origins are different for these tissues also the quantity of DNA and the number of copies of the genome vary. Whereas endosperms are triploid, resulting from the fusion of two maternal polar nuclei with one sperm nucleus, embryos are diploid, resulting from the fusion of one haploid maternal nucleus and one haploid male nucleus.

Teguments are diploid and of wholly maternal origin and contribute only negligible amounts of DNA to the grain (maximum 3.5% of total DNA). Microscopy studies have shown that embryos are made of small and dense cells, whereas most of the endosperm cells are larger and vacuolated (*Schel et al., 1984*). Results indicate that about half of the total DNA extracted from the maize grains originates from the endosperm tissue and the other half from the embryo tissue (*Trifa and Zhang, 2004*). Endosperm accounts for 80-90% of total grain weights (*Kowles and Phillips, 1985*) and the DNA amount contributed by the endosperm varies over a large range, from 36-60% (*Trifa and Zhang, 2004*). There are changes in DNA content of the developing endosperm during endoreduplication but not in the other tissues like the embryo. This results in changes of the DNA ratios of maternal / paternal origin could result in changes of PCR analytic depending on the developmental stage of the grain.

tissue	Transgene content	Relative DNA amount
teguments	0 %	max. 3.5 %
Embryo	50 %	95% (full maturity) – 5 % (endoreduplication)
Endosperm	33 %	1 % (full maturity) – 95% (endoreduplication)

**Table 2.2: Variation of transgene content and relative DNA amount by tissue**

On a grain in an earlier phase of development (endoreduplication) the endosperm will contribute considerably to the whole transgene DNA (33% transgene). But in fully mature grains with controlled cell-death (*Young et al., 2000*) there will be nearly no DNA in the endosperm, except the single layer aleurone tissue, and the total transgene DNA content will be determined mainly by the embryo (50% transgene). Thus in mixed samples the sum of all

grains and vegetative parts as defined by a certain developmental stage will finally determine the analytical transgene DNA-content.

In contrast, the transfer of yellow colour by pollen will always show a clear “yes” or “no” result. This has to be carefully respected when data of fodder maize and fully mature grain maize are compared. Furthermore, the grain colour is usually a homozygous trait, thus 100% of the pollen will contain the yellow colour but in most transgenic varieties at present only 50% of pollen contains the transgene. Thus the grain colour marker data indicate a maximum of transgenic outcrossing.

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### 3 Cross-pollination of maize in long distance

#### 3.1 ABSTRACT

Cross-pollination of maize was investigated in a 2 year study with 13 white grain test fields in Switzerland by using the occurrence of yellow grains as a marker of cross-pollination from neighboring fields. We investigated distances of 50 m up to 4500m between pollen donor and receptor fields. In no case the cross-pollination of the whole field was above 0.02%. Four fields, located in main wind direction, showed a low but marked cross-pollination at their borders in distances of 50 m to 371 m. In every field there were some low rate cross-pollinations dispersed around, mainly single cross-pollination events, on an average of 1,8 % of sampled ears. Horizontal wind speed measurements during flowering time in relation to settling speed of maize pollen showed a potential horizontal dispersal distance of up to 55 m. Observed cross-pollinations of longer distances could be due to gusts or by support of vertical wind movements (e.g. thermic or turbulence effects). In some fields spots of higher cross-pollinations were found that were in checked cases related due to a yellow grain seed contamination (0.004% of seedstock) of the white seeds.

#### 3.2 INTRODUCTION

Maize is a wind-pollinating crop and can cross-pollinate in neighboring fields. Research on maize pollen dispersal and cross-pollination was done since many years with the goal to improve seed purity (*Salamov A.B., 1940; Jones and Brooks, 1950; Burris, 2001*). Recently, with the introduction of genetic modified (GM) maize and labeling requirements in some countries there are requirements to establish defined thresholds of GM cross-pollinations to guarantee coexistence. The interest in long distance pollen transport was fueled by the discovery of transgenic sequences in maize landraces of Mexico, where cultivation of transgenic maize was forbidden to protect biodiversity (*Quist and Chapela, 2001*); but *Bellon und Berthaud (2004)* concluded that these transgenic sequences were due to the custom of small-scale Mexican farmers to incorporate improved commercial varieties into their open pollinated landraces.

Studies on maize cross-pollination generally show a rapid decrease on short distances up to 30 m, often accompanied with high variation, but a long tail with low cross-pollinations over several hundred meters (*Jones and Brooks, 1950; Raynor et al., 1972; Luna et al., 2001; Henry et al., 2003; Ma et al., 2004*), which is a general feature of aerobiological dispersal (*Westbrook and Isard, 1999*). Existing studies about long distance dispersal of maize are very

heterogeneous and difficult to compare, possibly due to the fact that the genetic material, the donor and receptor field sizes, the topography and the climatic situation are very diverse.

In older studies generally much higher cross-pollination rates are reported. Using different grain colours for detection, *Jones and Brooks (1950)* observed 5.8% in 75 m and 0.2% in 500 m in Oklahoma (USA) for two open-pollinated varieties; *Salamov A.B. (1940)* found up to 0.79% at distances beyond up to 800 m in North-Kaukasus (Russia). But he concluded in this much-cited study that the reason for this was a contamination of white grain with yellow grain maize leading to a variation independent of distance.

But cross-pollination rates considerably vary in recent studies as well. *Jemison and Vayda (2001)* used in USA a herbicide resistant maize field orientated upwind of predominant wind directions to the recipient field. In 1999 there was no cross-pollination at 350 m distance, but in 2000 cross-pollination of 0.7 – 1.4% took place at 100 m distance in a field with asynchronous pollen production. In a region characterized by low humidity and frequent high winds (Colorado, USA), using blue grain color and herbicide marker hybrids as pollen donor, cross-pollinations of 0.3% at 80 m and of 0.05% at 180 m were detected in organically cultivated maize (*Byrne and Fromherz, 2003*).

In Tsukuba and Tsumagoi (Japan) out-crossing rates of sweet corn varieties varied by years and locations between 0.1 to 1.2% at 50 m, and 0.04% at 400 m (*Matsuo et al., 2004*).

In recent multi- field trials, distributed over England, samples at 150 m into conventional maize stands showed evidence of cross-pollination in 19 out of 44 fields; of these, 12 had >0.1% GM DNA and 7 had >0.3% (*Henry et al. , 2003*). In several fields the levels of gene flow showed a marked increase at distances of 100 to 150 m from the GM source. This could have been due to the effects of the landscape and air movement over the crop. *Luna et al. (2001)*, too, found in Mexico more cross-pollinations at 200 m from the source than at the shortest distance of 100m.

The definition of long distance dispersal here was related to typical small scale farming patterns in alpine regions. Due to crop rotation requirements maize usually is not growing adjacent to it self but is separated by other crops with separation distances mostly greater than 50 m. According to an analysis of aerial photos of an eastern region in Switzerland the distance between maize fields varied between 75 and 149 m, with an average of 112 m (*Flury et al., 2004*). Therefore our pollen dispersal analysis was focused on distances between 50 m to 4500 m.

### 3.3 MATERIAL AND METHODS

#### 3.3.1 Site Descriptions

Field experiments were done in the years 2003 and 2004 in Urner Reusstal, Switzerland, Kanton URI (latitude  $8^{\circ}37'$  N, longitude  $46^{\circ}52'$  W, altitude = 449 m). The climate is temperate with an annual precipitation of about 1201 mm (with a maximum of 148 mm in July) and average temperature of  $8,6^{\circ}\text{C}$  with highest values in July ( $17,1^{\circ}\text{C}$ ).



**Fig.3.1: Experiment location Urner Reusstal, arrows show main wind direction**

The valley is characterized by grassland and forage maize cultivation with settlements between. It has a length of about 14 km, the width varies between 1.3 – 4 km. In this area there are around 20 maize fields with a size of 0,5 – 1,5 ha spread in distances of 50 m up to 4.5 km to the next field. High mountains around (up to 3000 m) act as barrier against pollen from outside sources, and a lake separates the next maize fields in northern direction by about 13 km. Thus long distance cross-pollinations should be due only from the field arrangements within the valley. As the topography forces wind in one main direction (from north-west to south-east) yellow and white grain fields could be arranged for different wind situations. The location is characterized by a mountain-valley and land-lake wind system which causes a change in wind direction from South-East over night within almost one hour between 6:00 and 9:00 am to North-West over day and again to South-East in the evening between 16:00

and 19:00 in most days. This wind systems and changes of wind direction were especially strong in the hot year 2003 which increased temperature differences due to different warming of different terrain with subsequent compensation by wind circulations. The soils in the valley are quite uniform; they are Eutric Cambisols with a sandy loam texture. Conditions for maize cultivation were slightly suboptimal for grain set in comparison to main arable land in the Swiss Midlands.

### **3.3.2 Maize varieties**

As low cross-pollination rates were expected, the visual yellow and white grain marker system was of advantage as it allows high sampling rates for the detection also of rare cross-pollination events. Each time pollen from yellow grain hybrids fertilizes a white grain ovary a yellow grain will develop indicating cross-pollination. For pollen receptor fields the white grain hybrid DSP 17007 (Delley Seeds and Plants company Switzerland) was used, which has comparable agronomic traits to the conventional hybrids cultivated at the test site in the two years: LG2185, LG2265, DK287 and Goldville. They are in early maturity group and similar in flowering time like DSP17007. The cross-pollination events could be assessed by simply counting the number of yellow grains on white ears.

### **3.3.3 Cultural Procedures and Crop Management**

Sowing was done by a special instructed local contractor to avoid seed contamination during sowing. The row distance was 80 cm and the final plant stands around 9 plants per m<sup>2</sup>. Sowing of all fields (yellow and white grain) was done in 2003 between 28.April to 9.May, a short timespan resulting in a good flower synchrony of the fields. In spring 2004 the weather was often cool and rainy and the sowing period was prolonged from 29.April to 28.May, resulting in some field situations with a flower asynchrony of up to seven days (see table 3.1). Agronomic practices were carried out according to the recommended levels of herbicide and fertilizer application, i.e. about 120kg N per ha.

### **3.3.4 Synchronization of flowering**

The flowering period was defined as the timespan between beginning of first pollen shedding and last wilted silks, these data were recorded for all white fields, whereas for yellow fields this was just done for the timespan of pollen shed. The main flower period of the white grain hybrid took about 10 days but due to variation of single plant development the complete flower period mostly encompassed 20 days on the field level. Difference in flower

synchronization was noted in number of days delay (+) or earliness (-) of the next pollen donor field (see table 3.1).

### 3.3.5 Meteorological Measurements

A meteorological station of MeteoSwiss at the experimental site delivered hourly measurements of wind direction, wind speed, air moisture, air temperature and global radiation. In both years a total pollination period was determined of around 22 days; for this period the hours with a vapor pressure deficit lower than 0.2 kPa were eliminated, as it has been observed that this value has to be passed for the start of pollen shedding (*Jarosz et al. 2005*). Furthermore, the evaluated hours were restricted to the time between 6:00 and 18:00, when nearly all pollen shedding occurs (*Jarosz et al. 2003*). This adjusted timespan was taken as the actual pollination period.

### 3.3.6 Sampling

The experiment fields were in most cases in the shape of a long rectangle with lengths around 200 m and widths around 40 m. The sampling points were distributed as follows: on the short side in every fourth row (every 2.5 m), on the long side at every 10 m. At every sample point 5 ears were taken and an average of yellow grains calculated. On a hectare basis this amounted to 1200 sample points and 6000 ear samples. The average ear grain number of a field was calculated on 30 ears that were randomly taken from a diagonal of the field. According to *Kruse (2004)* the detection limit can be calculated as ( $P_{LD}$ ):  $P_{LD} = - (10^{\log (1 - (95/100)^{315000}} - 1) * 100 = 0.00095$ . With this sampling system cross-pollinations down to 0.001% could be detected with a probability of 95%, which is in the range of the lowest level of cross-pollinations found here.

### 3.3.7 Data analysis

The cross-pollination rate of a sample point is the average of the yellow grains of the ears of the five adjacent plants to the sample point divided through the average number of the grains of ears of the field. It was important to take the average of 5 ears to compensate variations within a sample point.

$$CP_p\% = [ (SPE1+SPE2+SPE3+SPE4+SPE5) / (5 * AVK) ]$$

$CP_p\%$  Cross-pollination of a sample point

$SPE_x$  Ear number x at sample point (5 sample ears per sample point)

$AVK$  Average grain number of an ear of the field

The cross-pollination rate of the whole field is the ratio of the sum of all the yellow grains found on all samples divided through the total sum of grains of all ear samples (calculated on the average ear grain number of field).

$$CP_f\% = [ \text{sum (yellow grains of all samples)} / \text{sum (grains of all samples)} ] * 100$$

CP<sub>f</sub>% Cross-pollination rate of whole field

Sum of all grains of samples is calculated by the average ear grain number of the field multiplied by the number of sample ears. There was a yellow grain seed contamination of the white grain experimental seeds of about 0.004%, averaged over the two experimental years; the resulting few cross-pollination contaminations were not eliminated from the actual field to field cross-pollinations. Visualizations were done by geostatistic Software Surfer 8.0 ([www.goldensoftware.com](http://www.goldensoftware.com)) and gridding was done by the kriging method.

### **3.4 RESULTS**

#### **3.4.1 Rate and frequency of cross-pollinations**

The cross-pollination of a total single field was in no case higher than 0.02% (Table 1) with a minimum of 0.0002 % (one yellow grain in the whole field), although some single sample points had higher cross-pollination rates in other fields; but these cases were limited in number, for example, one yellow grain on average per ear would translate to 0.27% cross-pollination.

Although the years quite differed in meteorological conditions, an exceptional hot and dry summer in 2003 in contrast to moderately cool and wet conditions in 2004, there was no striking difference in cross-pollination between the years. Factors of influence (wind orientation and velocity, flower synchrony, surrounding landscape) varied of course as the fields could not be identically arranged in both years; thus only more obvious differences would have been recognizable at the very low cross pollination rates that characterized both years. These could be found only on 0.06 – 4.1% of sampled ears of a fields, with an average of 1.8% of samples in all fields. 89% of these cases were single-fertilization events, more than 5 cross-pollination per ear were found just on 4.9 % of the ears that showed cross-pollination.

**Table 3.1 : Cross pollination data for all fields in 2003 and 2004**

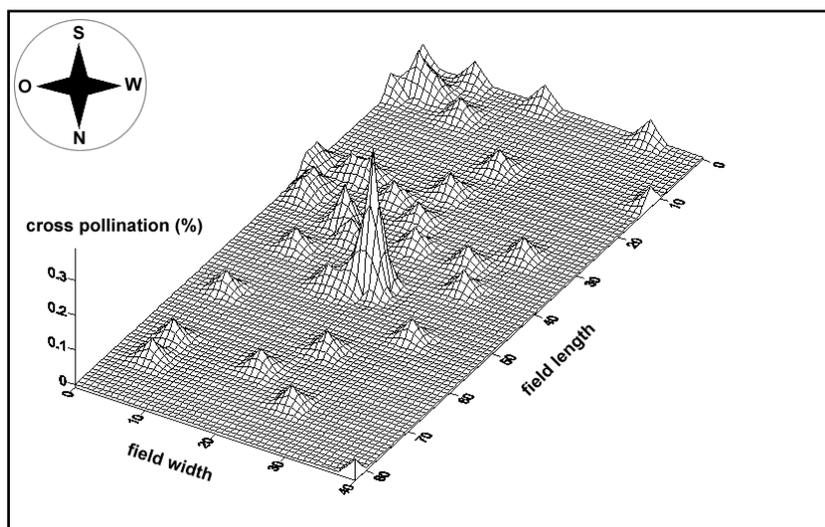
Arranged with respect to the distance from the closest yellow grain donor field.

field: field name; distance: to next pollen donor; wind: orientation to main wind directions (+ in direction, - parallel, - - against); flower: flower synchronization in days of mid pollen shedding of the donor to mid silking of receptor (e.g. -5 means 5 days earlier pollen donor; = means synchronous); %  $CP_f$  = total cross-pollination rate of whole field (calculated to hemizygous pollen traits).

Field	Year	Distance	Wind	Flower	$CP_f\%$
W7	2004	52 m	+	- 7	0,009
W12	2004	85 m	+	=	0,015
W2	2003	105 m	-	- 4	0,003
W3	2003	125 m	-	- 1	0,01
W10	2004	149 m	+	- 1	0,016
W5	2003	150 m	+	- 2	0,007
W1	2003	200 m	-	- 1	0,009
W9	2004	287 m	- -	- 4	0,005
W11	2004	371 m	+	- 5	0,008
W8	2004	402 m	-	- 1	0,005
W13	2004	458 m	+	- 7	0,0001
W6	2003	4125 m	+	- 1	0,006
W14	2004	4440 m	+	- 2	0,0005

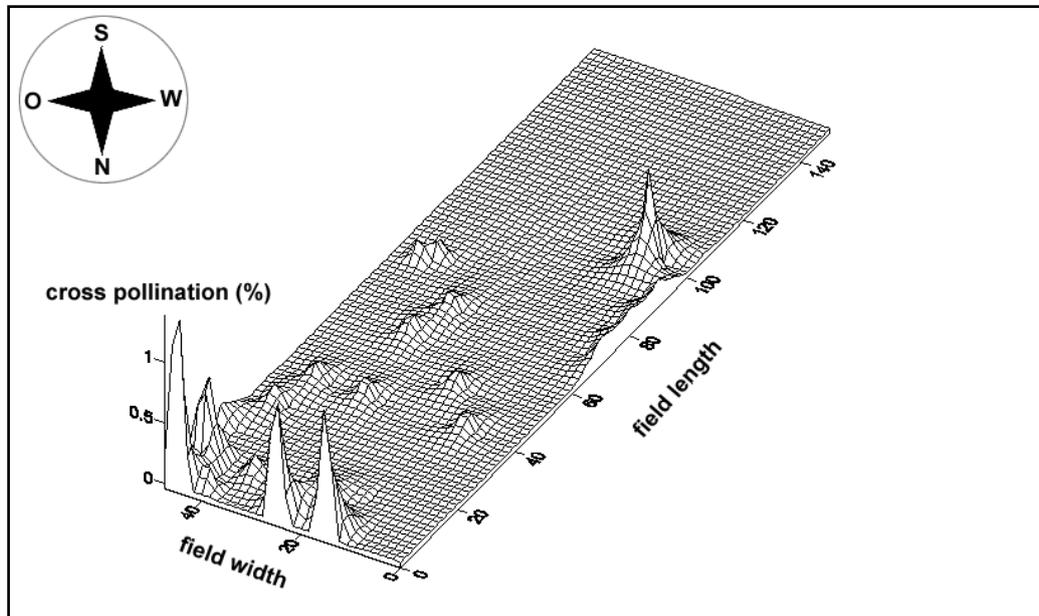
### 3.4.2 Distribution of cross-pollinations

Cross-pollinations were in most fields randomly distributed over the whole field (Example in Figure 3.2).



**Figure 3.2: Example of cross-pollinations randomly distributed (field W8); maximum value of 0.3%.**

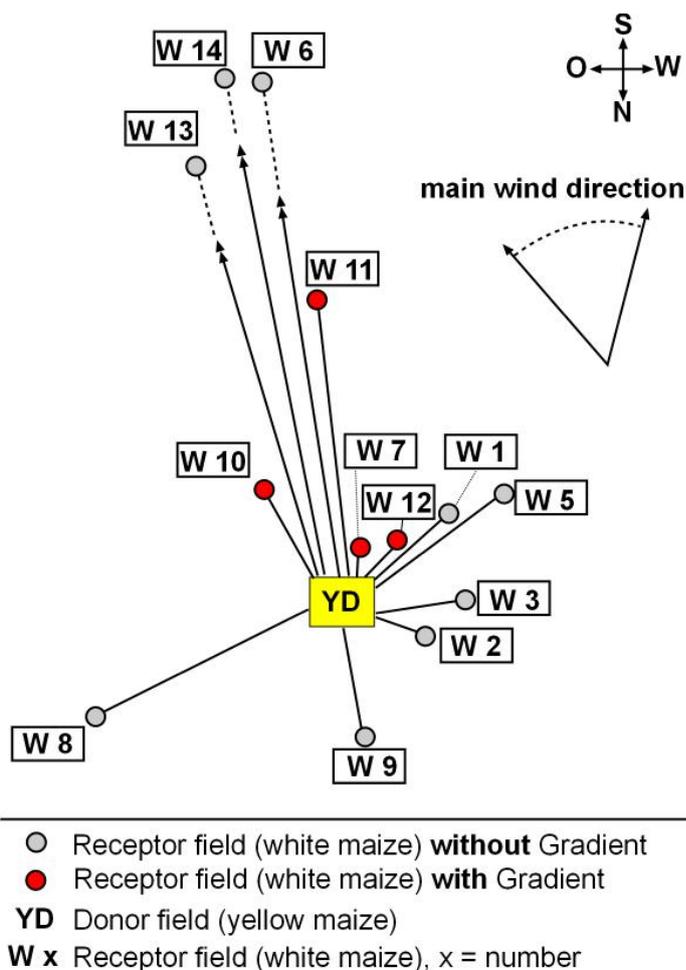
Only in four fields (out of 13) spatial gradients of varying cross-pollination could be found. These gradients were characterized by a higher cross-pollination rate on the border row with a rapid decrease in direction inside the field (Example in Figure 3.3).



**Figure 3.3: Example of cross-pollination with marked gradients (field W7); maximum values of 1.2%.**

A simplified overview on the different cross-pollination distribution types of the fields is presented in Figure 3.4 in the following way: the center represents the next yellow grain donor field; around the yellow field all the white grain fields of the two experimental years are displayed in direction and distance, the fields with and without a cross-pollination gradient are marked differently.

The four fields with marked gradients had higher cross-pollinations on the border rows orientated in the main wind direction to the next yellow grain pollen donor field. Seemingly higher cross-pollinations were due to horizontal wind forces only in main wind directions at distances between 52 m and 371 m. All the other cases with randomly distributed cross-pollinations within the field were either not in the main wind direction or farther away.

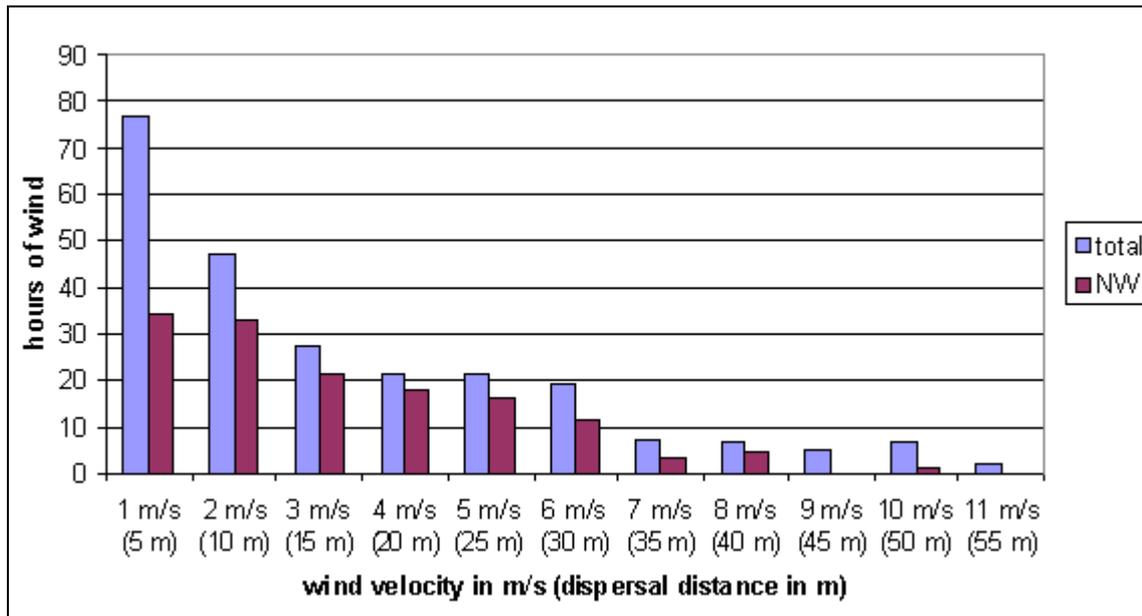


**Fig.3.4: Cross-pollination in white maize fields with and without a marked gradients in relation to distance and direction of the next yellow grain donor field (See Table 1 for field details)**

### 3.4.3 Horizontal pollen dispersal distance and wind velocity

Tassels were at a height of around 2.5 m and silks at a height of around 1 m. In consequence, pollen had a settling distance of around 1.5 m to reach the silks. According to *Aylor (2002)* the settling speed of maize pollen is between  $21 \text{ cm s}^{-1}$  to  $32 \text{ cm s}^{-1}$  depending on hydration status. In the best case for dispersal there is an available timespan of around 5 s between the settling from the height of the tassels to the silks, if there are no vertical forces counteracting against gravity and settling speed.

According to these deliberations a possible dispersal distance was classified for the duration time of actual wind speeds of both years, which were similar (Figure 3.5).



**Figure 3.5: Possible dispersal distance of pollen as influenced by the duration of wind speed classes in total and in the main wind direction North-West (NW) in year 2003;**

Most of the wind was of low velocity, especially when it was not in the main direction (North-West) (Fig. 3.5).

### 3.5 DISCUSSION

#### 3.5.1 The white grain marker hybrid for cross-pollination measurements

In control experiments on short distance cross-pollinations of up to 80% were found for single late developed plants of the white grain hybrid with long anthesis silking intervals and reduced pollen competition. This maximum cross-pollination potential of our white hybrid is in similar range of varieties from other studies (*Ma et al., 2004; Jones and Brooks, 1950; Stevens et al., 2004*). Though modern hybrids can have reduced male flowering by reduced tassel branches and pollen shedding (*Duvick and Cassman, 1999*), the white grain hybrid had normal tassel size and was a good pollinator.

Usually 100% of yellow grain pollen will show cross-pollination because present varieties are homozygous for yellow color (except variety Goldenso). However, many commercial GM - maize hybrids are at present hemizygous for the transgenic trait with only 50% of pollen containing the transgene in contrast to the yellow color gene. Therefore cross-pollination rates were divided by factor two in order to make results comparable to cross-pollination of GM-hybrids at present.

Without a tedious preselection of the seeds for the absence of yellow seeds a few cases of seed contamination occur. Within 22 800 sampled ears there was one case of an ear with completely yellow grain, i.e. a seed contamination of 0.004%. Some background contamination with yellow grain seeds was reported in other studies as well (*Stevens et al., 2004; Salamov A.B., 1940*). *Rieger et al. (2002)* monitored effective pollen dispersal from herbicide resistant canola. A remarkable feature of these data was the high variability in the level of outcrossing at long distances. *Aylor et al. (2003a)* concluded that this variability is a hallmark of long-distance dispersal in the atmosphere due to the long-extending tail that results from the properties of atmospheric turbulence. *Henry et al. (2003)* also noted in several of their 55 maize fields that the levels of gene flow showed a marked increase at distances of 100 – 150 m from the GM source; they concluded that effects of the landscape and of air movement over the crop could explain this phenomenon. Such spots of increased yellow grains in the white maize fields occurred here also. In some cases a time-consuming check of surrounding plants of such spots was done and an ear with completely yellow grains as contamination plant detected. But such spots were rare events (maximum 2 spots on a field) as the measured contamination rate of white grain seeds was 0.004%.

### **3.5.2 Rate and frequency of cross-pollinations**

In comparison to other studies on long distance pollen dispersal of maize with very varying rates, the results here are close to the lowest rates as found by *Matsuo et al. (2004)* with 0.23% in 100 m and 0.06% in 200 m distance and by *Byrne and Fromherz (2003)* with 0.05% in 183 m. However, it is not quite clear from some other reports if these really are related to a single-sample point or a defined area, where the cross-pollination rate of a single sample gets diluted by the frequency of totally occurring cross-pollination events.

### **3.5.3 Pollen dispersal and pollen competition**

As mature pollen is released even by a slight breeze or vibration most pollen will be shed on low wind speeds with very short dispersal range (*Aylor, 2003b*). But there are also a few hours with strong winds in turbulent weather conditions (e.g. thunderstorms) that may be responsible for dispersal of a small portion of pollen in different directions over long distance, as indicated by the randomly distributed low cross-pollination events over all fields. Without any support by vertical wind movement, e.g. thermic and outstanding gusts, pollen would disperse only to a maximum of 55 m, with the most dispersed on shorter distances, which

could partly explain low cross-pollination rates beyond this distance and cross-pollination gradients only in four fields that were orientated in main wind direction.

Even though some very long distance dispersals for pollen of trees with similar size to maize pollen have been recorded in range up to 500 km (*Rousseau et al., 2003*), it is improbable to get maize cross-pollinations over this distances. Pollen grains marking long distance transport are generally in very low number (*Campbell et al., 1999*); they will be confronted by intense pollen competition within a receptor field. About 6 million (*Jarosz et al., 2005*) and 25 million (*Poehlman and Sleper, 1995*) pollen are produced by a maize plant, depending on the variety, thus there will be over 25 000 pollen for each single grain set. In conclusion a successful cross-pollination is very unlikely at long distances.

The effect of pollen competition, and also the spatial variation of pollen dispersal and deposition, was tested by a small plot of about 500 detasseled white grain maize plants located between yellow and white grain fields. In the main wind direction and at a distance of around 625 m to next maize fields still 20% of these detasseled plants had at least one grain per ear instead of an average of 1.8% of samples with cross-pollination in male fertile white grain fields. So pollen competition may be a strong protection against cross-pollination. In all these test plots the ratio of yellow to white grains was 1:1 which was in good relation to their planted areas.

#### **3.5.4 Vertical pollen dispersal**

Though horizontal winds allow just for a low pollen dispersal range, some pollen grains are able to fertilize successfully over long distances. The parameters for successful long distance dispersal must be the effects of vertical forces, like thermic and atmospheric mixing turbulence, that prevent the pollen from settling down and thus prolong dispersal distance. For an approximation of the pollen take-off by vertical movement a scaffold was installed with horizontal cords of adhesive pollen traps (*Fonseca et al., 2002*) at heights up to 5m from 0 to 12 m into a maize field in 2004. On days with good thermic mixing conditions 250 pollen  $\text{cm}^{-2}$  were found at the height of the tassels between the rows whereas at 4m height only 2 pollen  $\text{cm}^{-2}$  were deposited; this indicates that only a very small (0.8%) portion of the shed pollen could take off and obtain better conditions for long distance dispersal. Most take-off was found at the border row, where wind meets the plant wall and in consequence turbulences can lift up the pollen more easily (*Zeng and Takahashi, 2000*); such pollen grains can then be caught by sucking airflows. *Irwin and Thresh (1988)* reported convective thermals with

around  $1 \text{ m s}^{-1}$  vertical velocity that are sufficient to distribute biota within the lowest 1000 m of the atmosphere within 15 min. *Jarosz et al. (2003)* found a somewhat less dramatic decrease of pollen density with distance but the measurements were done differently. At the experiment site here the speeds and duration of wind were generally higher than in the Swiss flatlands, this may have favoured horizontal pollen movement but may have reduced the generation of thermic convective cells that are dependent on conditions of high heat fluxes during low wind speed (*Gryning and Batchvarova, 1999*). But more representative data are still needed in order to clarify these preliminary data about pollen take-off as a potential factor for long distance dispersal.

### **3.6 CONCLUSION**

Although there are many factors influencing the pollen dispersal most pollen will be shed over short distance and probably will not interact with most of these factors. Orientation to prevailing horizontal wind speed is important, with an effect of up to 350 m. Due to low cross-pollination effects even a very low seed contamination made it impossible to judge potential longer distance transport caused by specific meteorological events with vertical movements though the results did not exclude this totally. But the risk could be excluded that commodity maize would be contaminated with regard to threshold level of 0.9%, and even to technical detection limit of PCR analysis (0.1%), at distances above 100 m under the present experimental conditions. The diffusely distributed cross-pollination events at longer distances still could require more detailed studies in cases where any cross-pollination has to be strictly avoided.

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## **4 Variation in cross-pollination of maize in short distance**

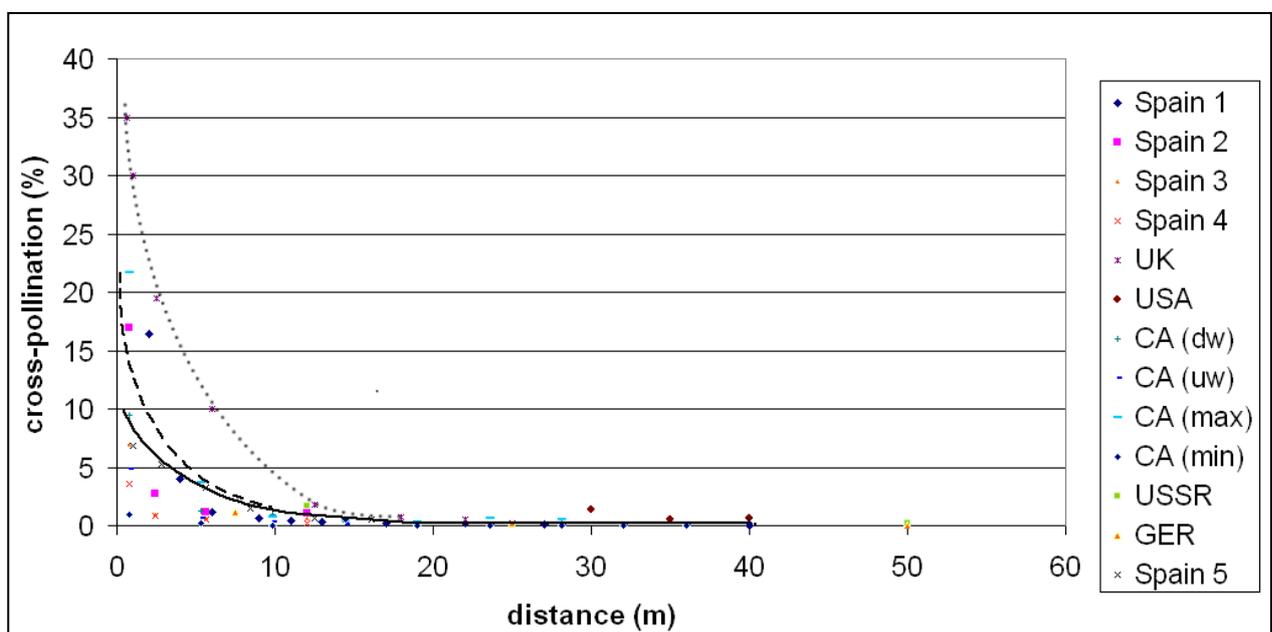
### **4.1 ABSTRACT**

Numerous field experiments have been carried out on cross-pollination but so far the results differ widely, probably to some extent due to the different experimental designs and settings, making comparisons and generalizations complicated. Six experiments were carried out at the field level in the colline zones of Switzerland in the years 2003 and 2004, checking in direct neighbourhood the pollination of fields planted with white grain colour marker maize by adjacent fields with conventional, colour dominant yellow grain maize. The main factors of influence tested were wind direction, flower synchrony and the size of donor and receptor field parts. As the visual assessment of single yellow grains on white grain ears allows for high sampling densities, distinct ways of distribution could be followed. Field size ratios varying from 3.6:1 to 1:8 had nearly no influence on the cross-pollination rate. The synchrony between donor pollen shedding and receptor silking was an important component for cross-pollinations; the time slot for good synchrony and high cross-pollinations is quite narrow, for a pollen donor from about five days earlier up to three days later pollen shed related to silk emergence of the receptor. Furthermore the homogeneity of plant development within a receptor field showed weak plants that were silking when internal pollen dispersal was exhausted. Just in a well wind exposed location a clear effect of to the main wind direction was found, otherwise the wind effect was probably minimized by the varied topography. Clearly recognizable variations existed generally at shorter distances up to 15 m, where pollen concentration is usually high enough to interact with factors to a measurable extent but in distances beyond 15 m all experiments had a similar low cross-pollination rate.

### **4.2 INTRODUCTION**

GM-maize (genetically modified maize) is widely distributed over the world nowadays and cultivation areas increase every year; still the cultivation is limited to a few countries. Quite a number of countries, especially countries from the EU, prepare to introduce GM-maize varieties at present and in the near future. As maize is a wind-pollinating crop and can cross-pollinate in neighbouring fields, for a coexistence of both types of cultivation, conventional and GM crops, countries have defined or had to define certain thresholds of intolerable GM content in a conventional crop product and guidelines how to handle the situation have to be elaborated. An important part of these guidelines is the definition of appropriate isolation distances to limit out-crossing of transgenic maize to the defined thresholds. Although there

are several studies on cross-pollination so far (see Figure 4.1) a clear decision about appropriate separation distances is still difficult because experimental settings are very heterogeneous and accordingly the results are varying within an appreciable range. Data exist from visual grain colour markers, simulating transgenic maize, and from true GM material. As the latter is heterozygous for the transgene, contrary to homozygous yellow grain colour, all data derived from visual grain markers are divided by factor two for a correct comparison here. Existing studies worldwide with exact data tables of cross-pollination at sample points of certain distance were used to sum up in a diagram and to show maximal and commonly found cross-pollination rates.



**Figure 4.1: Maximum cross-pollination rate found by several studies**

Dotted line: Old study of 1947; Dashed line: the maximum cross-pollination rates of new studies; Bold line: Maximum range of most new studies;

Data sources: Spain 1 (Molina, 2004); Spain 2 (Brookes et al., 2004); Spain 3 (Brookes et al., 2004); Spain 4(Brookes et al., 2004); UK (Bateman, 1947); USA (Jones and Brookes, 1950); CA (Ma et al., 2004): dw = downwind, uw = upwind, max = maximum, min = minimum; USSR (Salamov, 1940); GER (Wilhelm, personal communication); Spain 5 (Molina, 2004).

In Figure 4.1 it is observable, that most cross-pollination data are in a similar range, but there also exist some extraordinary higher cross-pollination rates shown as dotted line (highest rate of old studies) and dashed line (highest rate of new studies). So far the data of all relevant experiments have in common that the cross-pollination rate rapidly decreases within the initial 30 m distance but sometimes a long tail with low cross-pollination rates exists over a hundred meters or more. The initial cross-pollination rate in the first adjacent rows (distance 0.75 – 1 m) varies between different experiments from highest rates of about 35 % in old studies

(Bateman, 1947) to values between about 22 % in recent studies with modern hybrids (Byrne and Fromherz, 2003; Ma et al., 2004) to lowest initial cross-pollination rates of 2 % (Ma et al., 2004). Variation in the first row close to the pollen donor, the maximum cross-pollination rate, may be a general indicator, how good conditions (e.g. weather, flower synchrony) were for cross-pollination. Beyond the initial 30 m cross-pollination values are in most cases in a similar way below 0.9% (Henry et al., 2003; Bénétrix, 2004; Bénétrix and Bloc, 2003; Melé, 2004; Weber et al. 2005a; Weber et al. 2005b); there are also examples of higher rates, that may be the result of special climatic and topographic conditions or maize varieties, that are not comparable to pollination biology and seed purity of modern hybrids today. A certain constant „background noise“ extending over longer distances and the occurrence of „hot spots“ with single extraordinary high cross-pollinations may often be a result of seed contaminations. 100% seed purity is usually not guaranteed as assumed or observed by several authors (Stevens et al., 2004; Salamov A.B., 1940). Jemison and Vayda (2001) found a seed contamination by 0.16% of GM variety in the control of their maize variety.

Quite a number of further studies unluckily do not provide detailed data for comparisons. But results apparently fit within variation range of cross-pollination rates shown in Figure 4.1. Henry et al. (2003) did 55 field experiments with a source field next to a receptor field. By a regression equation they fitted the cross-pollination rates: 0.9% at 24.4 m and 0.3% at 80 m. Bénétrix (2004) found in France in a source field surrounded by a recipient field at the initial 10 m distance cross-pollinations of 1-2%, in distances greater than 10 m values were below 0.9%. Bénétrix and Bloc (2003) found a clear wind effect in France in a recipient field next to a source, with values below 1% in distances of 10-12 m downwind and 5-7 m upwind and 25 m downwind of a site with very strong winds. In field designs in Spain with a source surrounded by recipients values were below 0.9% in 10 m downwind or 2 m upwind (Melé, 2004). Data from 27 field experiments in France corroborated the decrease of cross-pollinations below 0.9% at distances above 25m to a source field (Fabié, 2004). In some experiments (Weber et al., 2005a; Weber et al., 2005b) the researchers did not take sample points at specified distances but took a mixed sample directly from the harvest machine. These mixing samples are representative for a certain distance area where dilution effects can be expected. This may be a realistic approach according to analytical control of coexistence in agricultural practice. But this data of mixing samples do not reveal distinct distribution patterns like the approaches with sample points; therefore they were not considered for further deliberations, although there data seem to fit in the same cross-pollination range.

In conclusion, the data of already existing experiments show variation of cross-pollination rates within a certain maximum – minimum range. Some assumptions about the reasons of this variation have been drawn but in most cases there are no clear explanations available yet. Therefore in this study experimental settings were chosen in representative farming regions with different constellations of factors of influence on cross-pollination rate like there are wind, size ratios of pollen donor and receptor fields as well as the synchrony of donor and receptor plant stands. Focus was on short distance as it would be the case of adjacent transgenic maize and conventional maize fields.

### **4.3 MATERIAL AND METHODS**

#### **4.3.1 Site descriptions**

Six field experiments were conducted in Switzerland in the Kanton Zurich (latitude 8° 40' 60" N, longitude 47° 25' 60", altitude 516 m) and in the Kanton Zug (latitude 8° 25' 0" N, longitude 47° 10' 0", altitude 445 m) in the years 2003 and 2004. The climate is temperate with an annual precipitation of about 1100 mm (with a maximum of 140 mm in June and July) and an average temperature of 7.9°C with highest values in July (16.7°C). Both locations are typical for the Swiss Midlands with a mixture of woods, settlements and intensive agricultural areas with wheat, maize and grassland in a hilly topography. All soils were Eutric Cambisols with a sandy loam texture.

#### **4.3.2 Maize varieties**

Xenia effects were used for the detection of cross-pollination by choosing maize varieties with different grain colour inheritance. Yellow grain colour is dominant over white grain colour. If pollen of a yellow grain variety fertilizes in a white grain variety field there will arise a yellow grain on the white grain ear at every successful cross-pollination event. The cross-pollination rate is then measured by counting the number of yellow kernels on white ears. For pollen receptor fields the white grain experimental hybrid DSP-WH (DSP17007, Delley Seeds and Plants Company Switzerland) was used, which has comparable agronomic traits to modern hybrids. Yellow grain varieties were Monopol, Romario and PR39G12. All four hybrids are in early maturity group, pretested in 2001 and 2002. The seed contamination of DSP-WH by yellow kernels was very low, none were directly detected here; from extensive other trials the contamination rate was calculated to be around 0.004%.

### 4.3.3 Field experiment design

Field experiments were designed to focus on certain factors of influence on cross-pollination.

**Table 4.1: Overview of field experiment designs**

Flower asynchrony in days was calculated as the difference in days between the date of mid-yellow grain maize pollen shedding to the date of mid-white grain maize silking, e.g. + 1 days means that the yellow maize pollen shedding was two days later than the white maize mid silking.

<b>Name</b>	<b>Design See Figure:</b>	<b>Year</b>	<b>Size white grain (ha)</b>	<b>Size yellow grain (ha)</b>	<b>Field size ratio yellow : white</b>	<b>Sowing date white grain</b>	<b>Flower asynchrony (d)</b>
A03	4.2-A	2003	0.96	1.00	1: 1	25.04.03	+ 1
B03-A	4.2-B	2003	0.91	0.38	1: 2.5	05.05.03	+ 3
B03-B	4.2-B	2003	0.28	1.02	3.6: 1	05.05.03	+ 3
C04	4.2-C	2004	1.04	0.13	1: 8	18.05.04	0
D04	4.2-D	2004	2.12	1.46	1: 1.5	07.05.04	+ 5
E04	4.2-E	2004	1.08	0.95	1: 1	27.04.04	+ 7

The white grain test hybrid had an anthesis silking interval of around 2-4 days. The sowing dates of the yellow grain varieties varied, therefore in Table 4.1 the more relevant flower asynchrony is recorded.

Fig. 4.2 - A (field A03)

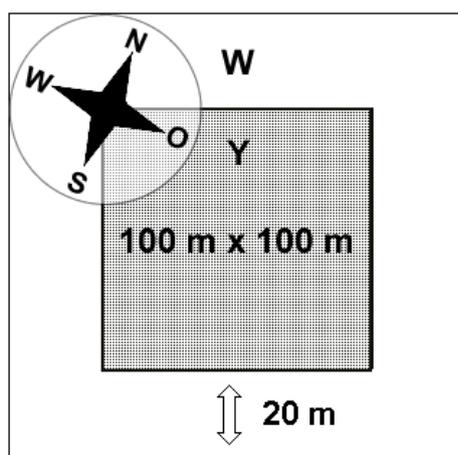


Fig. 4.2 – B (field B03-A and B03-B)

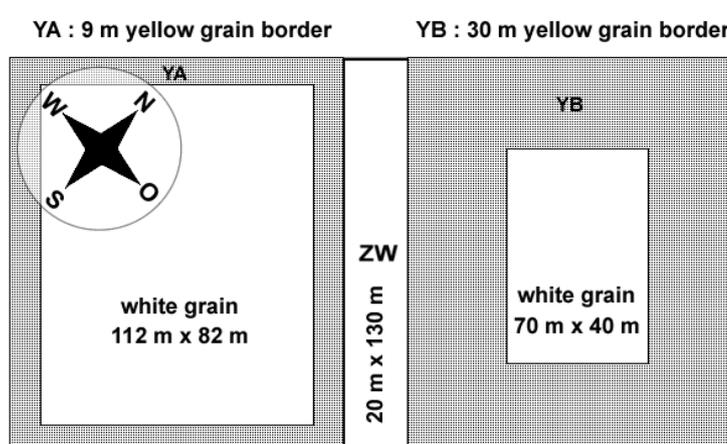


Fig. 4.2 – C (field C04)

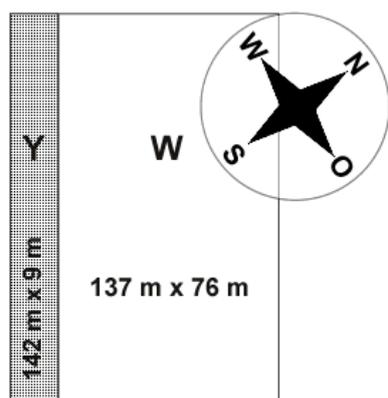


Fig. 4.2 – D (field D04)

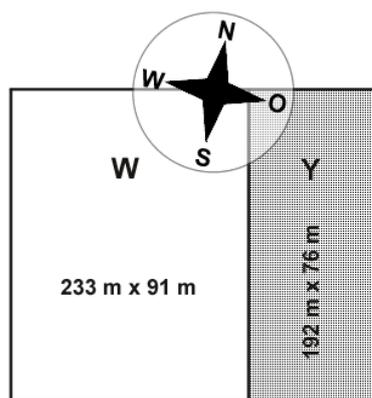


Fig. 4.2 – E (field E04)

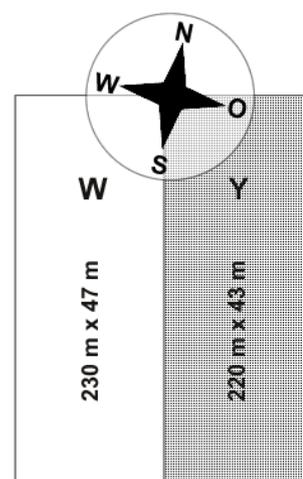


Figure 4.2.: Field layouts of test fields

Letters “Y” (yellow grain) and “W” (white grain) mark the positions of yellow grain donor and white grain receptor field parts in relation to the geographic direction; for further information see Table 4.1

#### 4.3.4 Cultural Procedures and Crop Management

Sowing of the white grain maize was done in close collaboration with one local contractor in order to guarantee that before sowing a new kernel colour type the machine was carefully cleaned of remaining yellow kernel seeds by using a pneumatic jet of water. The row distance was 80 cm and the final plant density about 9 plants per m<sup>2</sup>. Cultivation e.g. herbicide treatment and fertilizer application, was done according to recommended agricultural practice for the grain yield goal of 9 t per ha.

#### **4.3.5 Synchronisation of flowering**

The flower synchronisation of all fields was checked by regular counting at four field positions every third day, taking into account the start of the begin of pollen shed in white and yellow grain maize and the time to mid-silking in white kernel maize only. Main fertilization took place in all fields within about ten days but in some fields there was variation of plant development of single plants extending complete fertilization time of some fields to around 20 days. Difference in flowering synchronisation of male yellow grain pollen shed and female white grain silking was calculated as number of days delayed (+) or earlier (-) in relation to the white grain pollen receptor field.

#### **4.3.6 Meteorological Measurements**

Hourly measurements of wind direction, wind speed, air moisture, air temperature and global radiation was received from a nearby meteorological station of MeteoSwiss. In both years it took around 22 days from the earliest fertilization to the latest fertilisation of all test fields. For this pollination period those hours were eliminated as not valid for pollination that had a vapour pressure deficit lower than 0.2 kPa (*Jarosz et al., 2005*). The daily hours taken into account for pollen shedding were from 6:00 a.m. to 6:00 p.m., as this is the period where nearly all pollen shedding occurs (*Jarosz et al., 2003*).

#### **4.3.7 Sampling**

The experiment fields were in most cases in the shape of a long rectangle. Every 4th row was a sample row (2.4 m distance) and within these sample rows every 10 m a sampling point was marked where five ear samples were checked (about 1200 sample points and 6000 ear samples per ha). For determination of average ear kernel number per ear of a field 30 ears were randomly chosen and all kernels counted. This basis was taken for the percentage calculation of outcrossings as the five ears at the sample points were counted for the occurrence of yellow grains only.

#### **4.3.8 Data Analysis**

The cross-pollination rate (CP%) of a sample point is calculated as the number of the yellow grains of the five ears divided through the average total grain number of five ears.

$$\text{CP\%} = [ (\text{SPE1} + \text{SPE2} + \text{SPE3} + \text{SPE4} + \text{SPE5}) / (5 * \text{AVK}) ]$$

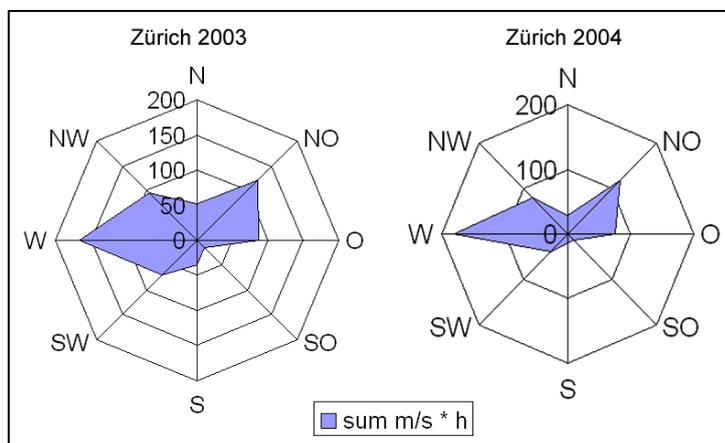
- CP% Cross-pollination of a sample point
- SPE<sub>x</sub> Ear number x at sample point (5 sample ears per sample point)
- AVK Average kernel number of ears of a field

As the yellow grain colour is homozygous (100% of pollen will contain yellow grain colour gene) but transgenic varieties at present are hemizygous, with only 50% of pollen containing the transgene, our data were divided by two to make them comparable to results with transgenic maize. To compare the cross-pollination rates with other data with visual markers, the values here have to be multiplied by factor two again, accordingly.

## 4.4 RESULTS

### 4.4.1 Impact of wind on cross-pollination

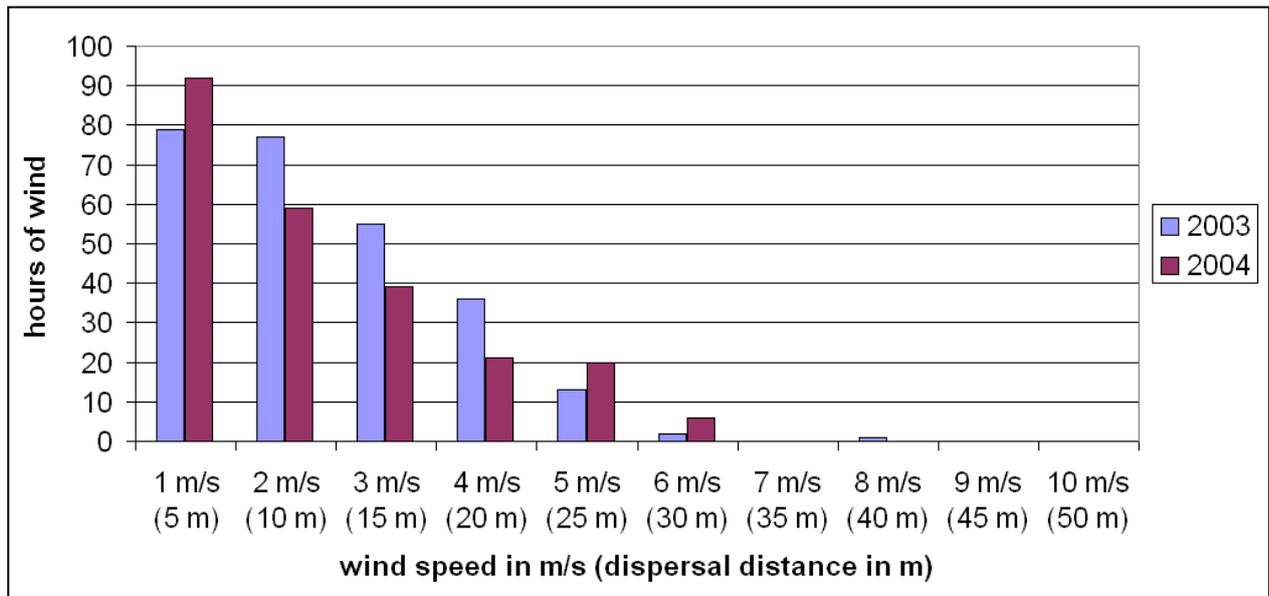
Two fields, see Fig. 4.2-A and 4.2-B, were chosen for the assessment of the wind impact on pollen dispersal, the first one with an inner pollinator core and the latter with a pollinator mantle. Wind measurements showed a similar wind situation during the main maize flowering period for both years; the orientation was mainly from the directions West-Northwest and East-Northeast, with much lower impacts from other directions (Fig. 4.3).



**Figure 4.3.: Hours of wind summed up as m/s\*h per direction in year 2003 (left) and 2004 (right)**

Due to a very diverse micro-shattered topography with hills and woods, typical for the Swiss Midlands, the actual impact of winds at a certain time at a certain field point can have deviated from these average data in some cases. Pollen shedding of the tassels occurred at a height of about 2.5 m and the silks were in a height of around 1 m in the early maturing hybrids used here. In consequence, pollen had a settling distance of about 1.5 m. Aylor (2002)

reported settling speed of maize pollen between  $21 \text{ cm s}^{-1}$  to  $32 \text{ cm s}^{-1}$  depending on the hydration status. In the „worst-case“ of a light pollen there would result a timespan of around 5 s for pollen settling down from the tassel to the silk, if there are no other vertical forces acting against gravity. According to these deliberations the wind speed could be assigned to corresponding dispersal distances (Fig. 4.4).

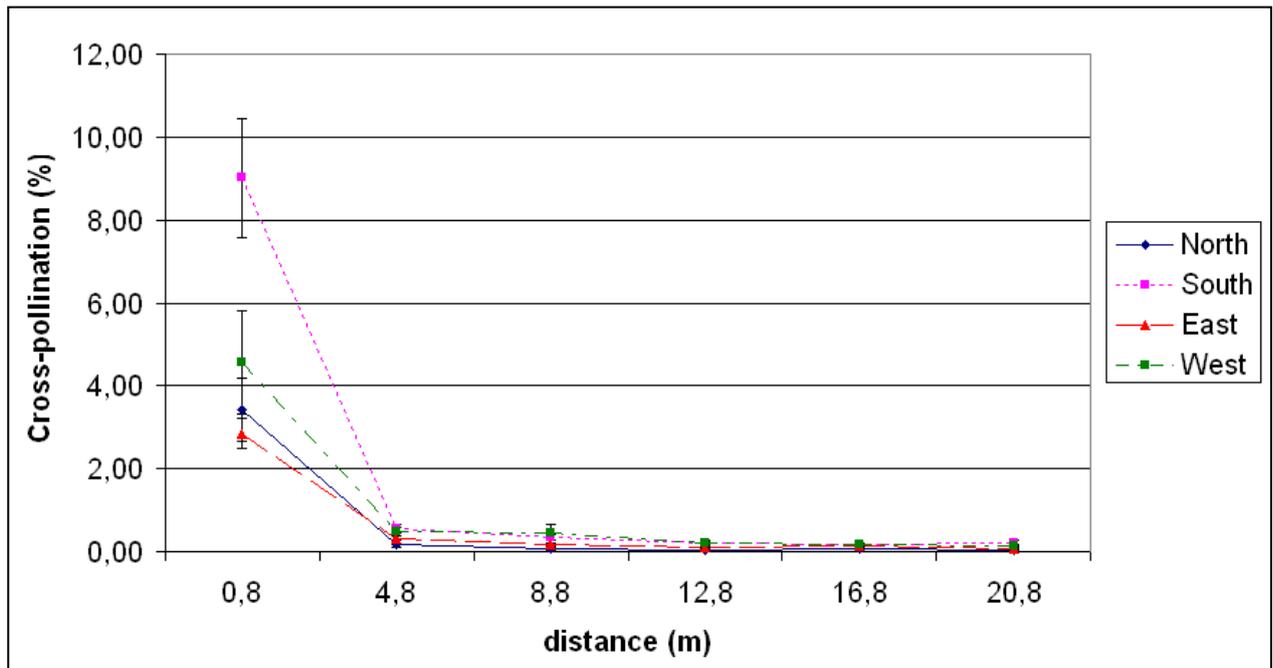


**Figure 4.4.: Wind speed and corresponding dispersal distances with an assumed timespan of around 5 s for pollen settling down from the tassel to the silk in the years 2003 and 2004**

Figure 4.4 shows for both years a similar potential dispersal distance by horizontal wind of up to 30 m, with most of the pollen probably settled over shorter distances. As mature pollen gets shed even by a slight breeze or vibrations (*Aylor 2003*) the prevailing low wind speeds will release the highest quantity of pollen in conditions with only a short horizontal dispersal. Of course wind is a complex phenomenon with gusts, turbulence and thermal processes making it always possible for a certain amount of pollen to reach much greater distances.

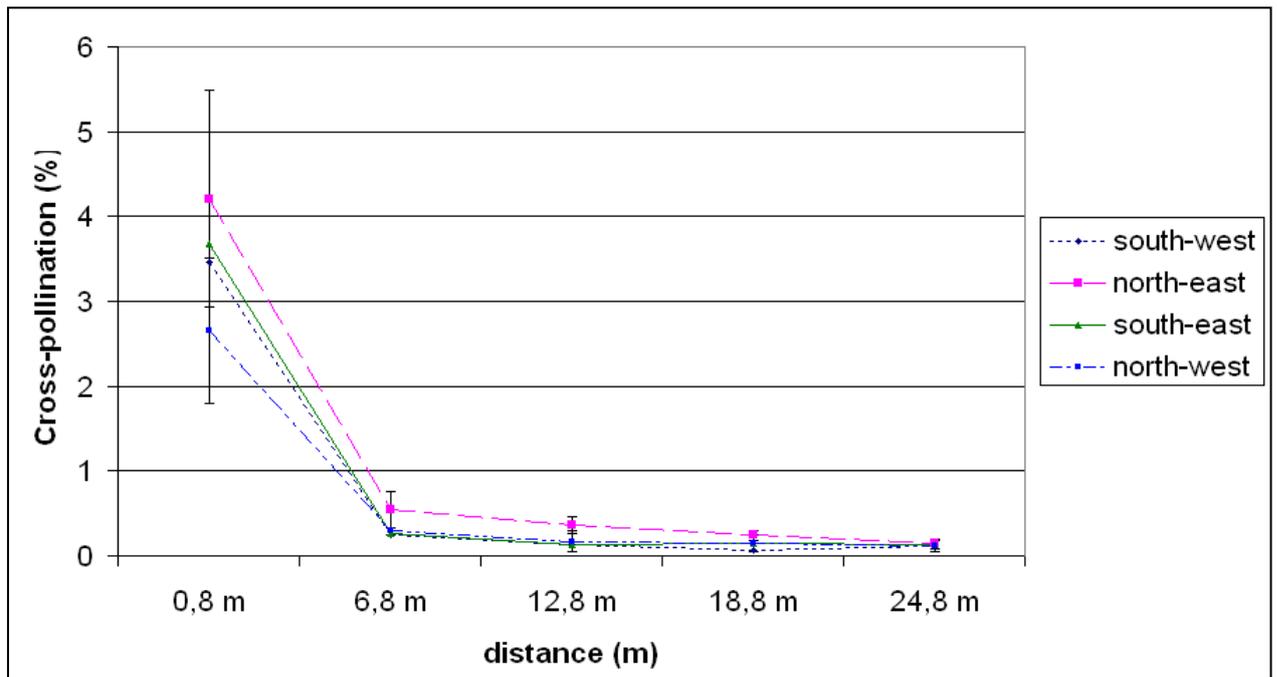
The field site “A03” (Figure 4.2-A) was chosen due to the fact that it was exposed to the prevailing wind directions and embedded in a landscape with wheat fields and grassland that have only low wind friction and distraction effect. As seen in the meteo data the prevailing wind direction was from the north; in consequence the highest cross-pollination rates were in the south direction (Fig. 4.5), in the initial distance (0.8 m) nearly twice higher than in the other directions. Due to the fact that most wind was of low speed, the cross-pollination rate

decreased rapidly over distance. In average of all directions, cross-pollination rates started with a rate of around 5% at the border line to yellow grain maize and dropped down below 0.9% at 13 m distance. Observations on single plants showed maximum rates of up to 16% and minimum rates of 1.5% yellow grains at the border line, indicating a considerable plant to plant variability.



**Figure 4.5.: Field “A03”: cross-pollination rates in different wind directions from the central pollen source core.**

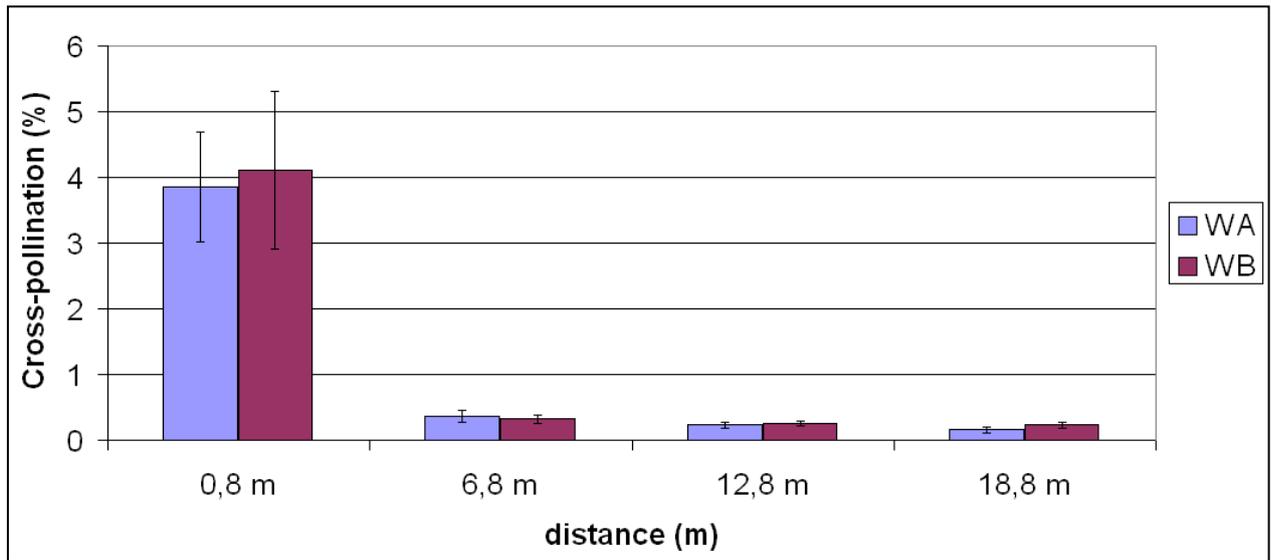
The Field layout of experiment „B03-A“(Figure 4.2-B) was planned for study the influence of wind direction and of field size ratios on cross-pollination rate. A core of white grain maize was surrounded by a nine meter yellow grain mantle to check how far and strong cross-pollination will reach in the core field. In representation of the varied landscape configurations, this site was shielded against wind by settlements, hills, and woods in contrary to the previous case (Field “A03”). Although some deviation of values was observed at 0.8m distance from the pollen source, these differences were not significant (Fig. 4.6). Cross-pollination rates in all directions varied close to the yellow grain pollen source around 3 - 4 %, similar to the variation of around 3 – 5 % of the non wind affected directions in experiment “A03” (Fig. 4.5), which showed in main wind direction a clear wind effect by an initial cross-pollination of around 9%.



**Figure 4.6.: Field “B03-A”:** different lines show the difference in cross-pollination rates in different directions from the pollen source core.

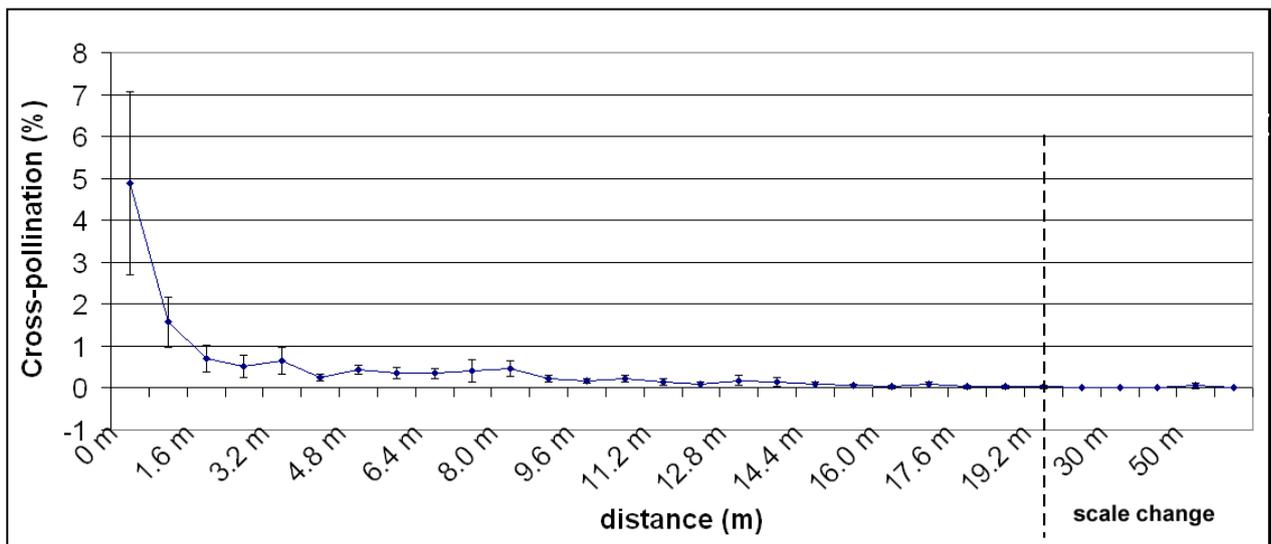
#### 4.4.2 Impact of pollen donor / receptor ratios

The second main purpose of the experiment “B03-A” and “B03-B” (Figure 4.2-B) was to study the impact of different field ratios of the pollen donor and receptor. White grain maize was surrounded in one field part (WA, Fig. 4.2-B) by a 9 m yellow grain variety (YA) and in the second field part (WB, Fig. 4.2-B) by a 30 m yellow grain variety (YB). The white grain stripe ZW with 20 m width served as buffer space. In the case of „WA“ a much higher yellow grain „pollen pressure“ could have resulted in higher cross-pollination rates; however, the values in both cases were nearly the same, independent of the distance to the pollen donor (Fig. 4.7).



**Figure 4.7.:** Cross-pollination rates of the two white grain parts WA and WB of Field “B03” with different pollen donor / receptor ratios of 1: 2.5 and 3.6: 1, respectively; data are the average of all four wind directions; vertical bars indicate the standard deviation.

In the case of Field „C04“ (Figure 4.2-C) a very low pollen donor / receptor ratio of 1:8 was tested that could have diminished the initial rate of outcrossings at close distance as well as the values at larger distances, but this was not the case (Fig. 4.8).



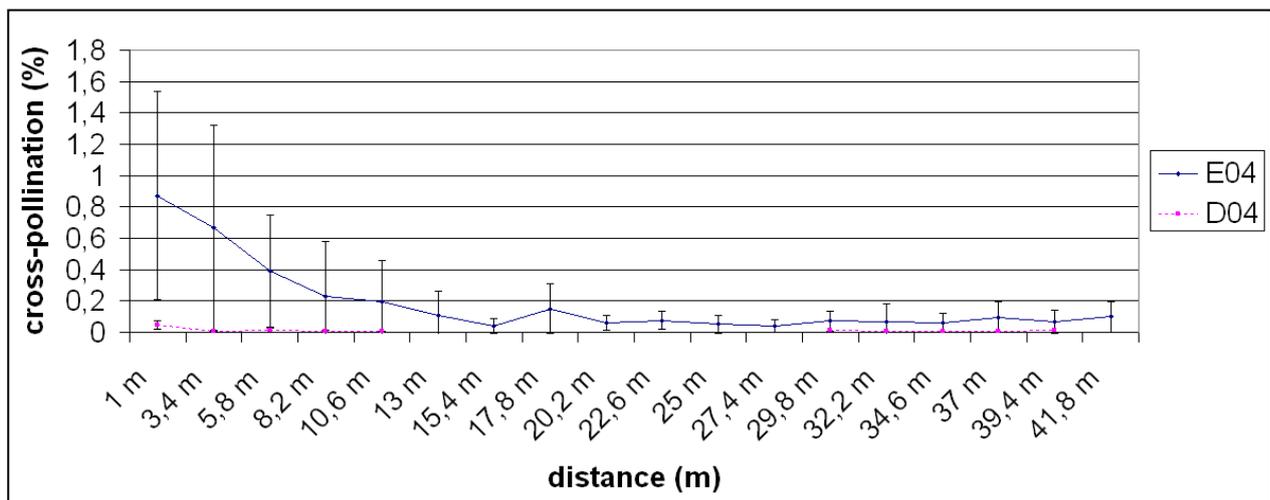
**Figure 4.8.:** Field size ratio and cross-pollination rate (Experiment “C04”). Pollen donor / receptor ratio of 1:8; vertical bars indicate the standard deviation.

This experiment was carried out in the following year with quite different meteorological conditions and sampling was carried out at longer distances up to twice the range that has

been tested in the other experiments ; as can be seen clearly, there was no deviation from the results described before (c.f. Fig. 4.7).

#### 4.4.3 Impact of flower asynchrony

All experiments presented above had a close flower synchrony of mid-yellow grain maize pollen shedding to mid-white grain maize silking. In a wider sense this could be regarded as the internationally expression Anthesis Silking Interval (ASI) though this is usually used for isogenic internal field situations. Furthermore, for the purpose of studying cross-pollination, the average field ASI is just one factor, as described below, the divergent developmental patterns at the single plant level can have a much larger impact. In two fields, experiment „D04“ (Fig. 4.2-D) and „E04“ (Fig. 4.2-E), a greater flower asynchrony was induced by delayed sowing of the pollen donor.



**Fig. 4.9: Impact of flowering asynchrony on cross-pollination rates with a retardation of mid-pollen shedding of the donor to mid silking of the receptor by + 7d (Experiment “E04”) and +5 d (Experiment “D04”); vertical bars indicate the standard deviation.**

In the experiment „D04“ (Fig. 4.2-D), yellow grain pollen shedding was retarded by five days to the mid-silking of the white grain maize. The resulting cross-pollination rate was very low in comparison to the three previously described experiments; those had a good synchrony and started with average cross-pollination values of about 4% at 0.8 m distance whereas here the average value in „D04“ was 0.05% in the first adjacent row (Fig. 4.9). The low cross-pollination rate was due to the fact, that the silks were mostly fertilized within a few days after emergence (observable by wilted silks five to six days after their appearance) depending on weather conditions.

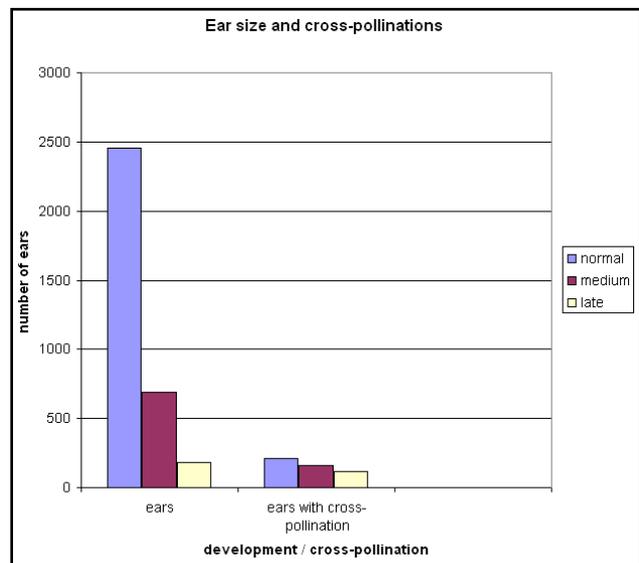
In experiment “E04”, surprisingly, inspite of the greater flower asynchrony of 7 days, cross-pollinations commenced with around 1.15% average in the first adjacent row, with a high variation (Fig. 4.9).

A detailed analysis at the single ear level showed that very numerous cross-pollinations had occurred on single plants, but mainly on those with small ears (Fig. 4.10. and Fig. 4.11.). This was particular to this field, indicating a heterogenous early development of white grain plants and accordingly probably a considerable number of weak plants that were in the silking stage after most neighbouring plants already run out of pollen shedding.

**Fig. 4.10**



**Fig. 4.11**



**Fig. 4.10.: Normal (> 16 cm), medium (12 - 16 cm) and small ears (< 12 cm), probably corresponding to normal, medium and late plant in this experiment field**

**Fig. 4.11.: Ear size and cross-pollination: numbers of sampled normal, medium and small ears, left-hand bars; number of ears per class with cross-pollinations, right-hand bars**

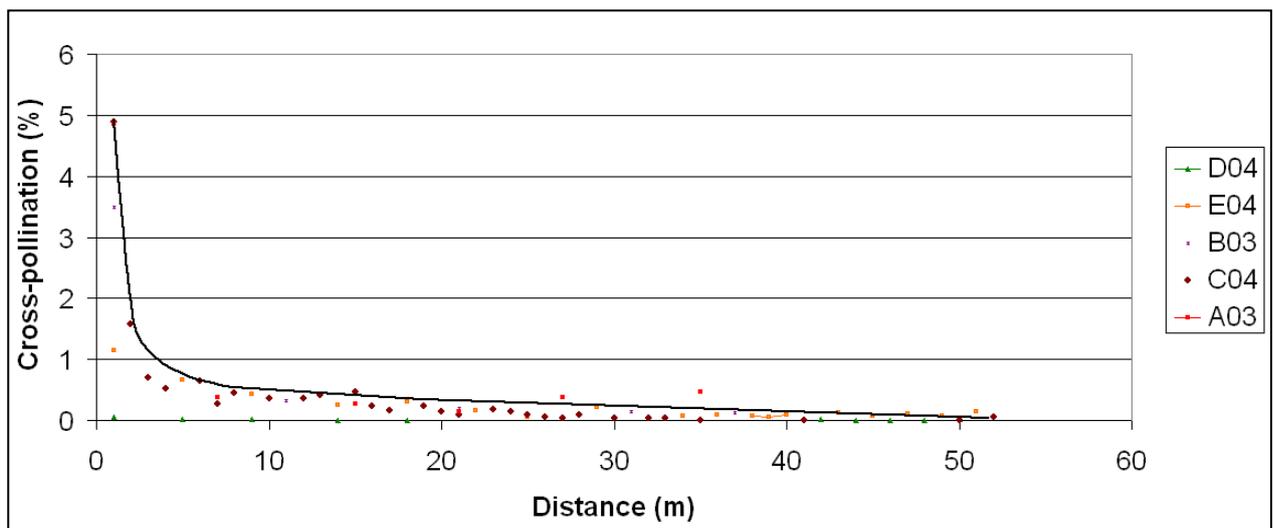
Ears were classified according to length, by checking first the range of ear sizes, and second by defining the maximum and minimum size classes. Due to different yield and ear size levels of fields every field has to be classified by an own field specific ear length scale. The normally developed plants had probably finished fertilization already when the yellow variety started pollen shedding, resulting in a small rate and frequency of cross-pollinations. But small ears, probably from late developed plants, started silking within pollen shedding period

of the donor field and exhibited high rates of cross-pollinations, accordingly values were high (up to 80%). As these ears were small with a low number of grains, a high cross-pollination rate on them had a much lower impact on the whole field average. Nevertheless, they contributed to 47% of the total cross-pollination of the field, although they comprised only 5% of all ears. This heterogeneity of the white grain field development would explain the discrepancy that with a seven days instead of a five days difference the former still showed a considerably higher cross-pollination.

## 4.5 DISCUSSION

### 4.5.1 Rapid decrease with distance

All experiments showed a rapid decrease of cross-pollination with distance to the pollen source, except those two experiments with a flower asynchrony of five and seven days later pollen shedding of the donor that already started with quite small values. Cross-pollination rate of all experiments are summed up in a diagram (Fig. 4.12).



**Figure 4.12.: Variation in cross-pollination across all experiments (Swiss experiments 2003 / 2004); the bold line follows the maximum cross-pollination rates (“worst-case”)**

On longer distance (beyond 15 m) cross-pollinations are in most experiments similarly lower than 0.9% except some rare peaks that could have resulted from seed stock contamination or some wind turbulence events. The variation of the cross-pollination rates was in a similar range as most other experiments from various parts of the world (Fig. 4.1); a rather large

standard deviation within and between the experiments here, or considerable maximum values at close distance to the pollen source have to be accepted as facts that cannot be easily explained without an extremely high investment in monitoring that would be at the cost of the case number to be evaluated. Generally lower values here might be due to the special climatic conditions and small structured landscape of Switzerland and the pollination biology of the white grain test hybrid, as its ASI of about three days would ensure a good internal pollen supply at the start of silking, which is also a feature of modern hybrids with a short flowering time.

The rapid decrease of cross-pollination rate can be explained by the interaction of pollen settling speed and the horizontal winds (see Fig. 4.4). As the wind was mostly of low speed, most of the pollen was released with a low potential dispersal range. Due to complex atmospheric processes like gusts and thermal upwinds some pollen may reach greater distances. But this portion of pollen will be confronted to a tough pollen competition by pollen shedding within the receptor maize field with high pollen concentrations just around the plant; therefore the chance of successful cross-fertilization gets low.

#### **4.5.2 Highest variation in the initial distance**

Highest variation of cross-pollination occurred in the initial 10 m distance. This phenomenon can be explained by the fact, that pollen concentrations are highest at this distance and factors of influence in cross-pollination will have there the greatest effect. Comparison of the data of the first adjacent row show most clearly how conditions have been for cross-pollination.

In the first adjacent row (0.8 m distance) the cross-pollinations in most cases were around 3-6 % (experiment A03, B03, C04). Similar rates were also found in experiments of other authors (*Ma et al., 2004; Brookes et al., 2004; Molina, 2004*; see Fig. 4.1). The experimental fields were embedded in a typical landscape of the Swiss Midlands with a mixture of settlements, hills and woods. So in many cases the fields were only accessible to slowed down or distracted winds, which could explain the similar cross-pollination rates without a clear wind direction effect on some locations.

In a wind exposed location the cross-pollination rate did rise up to 9% in the first row (0.8 m) downwind of the donor field (see Fig. 4.5 in comparison to Fig. 4.6). *Ma et al. (2004)* found within eight field experiments variations of cross-pollination rates in the row adjacent to the donor between 1 - 22% in downwind direction. Cross-pollination rates decreased in case of flower asynchrony of five days (experiment D04) to 0.05 % and in case of flower asynchrony of seven days (experiment E04) to 1.14 % in the first adjacent row (0.8 m).

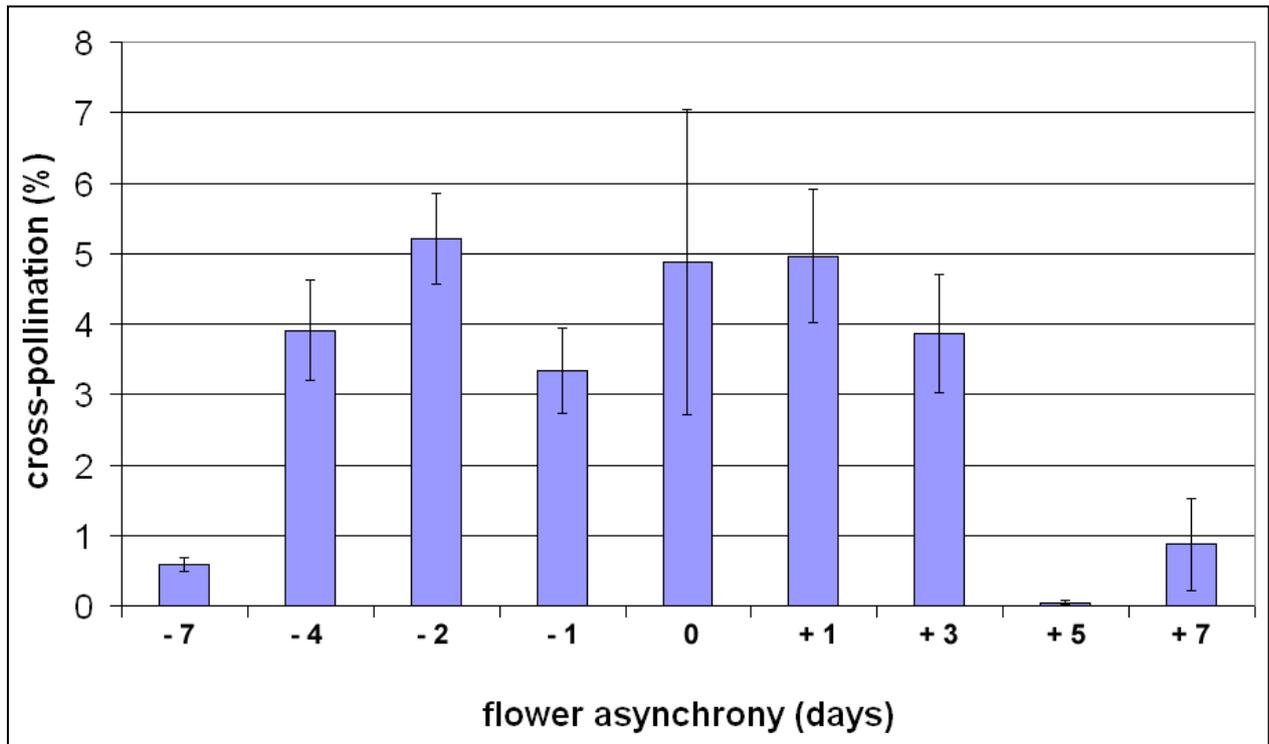
Though many other factors can modify the wind effect on cross-pollination, flower synchrony probably is the most effective modification factor. Single plants in experiments here had cross-pollination rates up to 16%. These plants may have been in best synchrony for external pollen sources. There could be a considerable variety impact on cross-pollination with respect to the amount of total pollen released and the dynamics of pollen release throughout the daytime and throughout the days of pollen shedding.

#### **4.5.3 Impact of pollen donor and receptor field sizes**

It is generally assumed that the size ratio of pollen donor and pollen receptor fields must be of high importance on short and long distance outcrossing values; but no respective values are present in literature. However, there was no difference in cross-pollination rates at source receptor ratios as different as 1: 8, 1: 2.5 and 3.6: 1 (Figures 4.7 and 4.8; 9 m and 30 m wide pollen sources). This was probably due to the fact that most cross-pollination occurred within the initial 10 m close to the donor, as seen in other experiments before. If pollen donors are further apart than 10 m most of this pollen just will not reach inside the receptor field because of the short dispersal distance. It could be assumed, that a field-size ratio effect may be higher at a wind exposed location. But it was obvious (Fig. 4.5) that the wind affected mainly cross-pollination rates at close distances. Therefore it can be assumed, that a field size-ratio effect will be similarly low at more wind exposed locations. If these findings apply to the large field sizes and to long distance transports as well has still to be seen.

#### **4.5.4 Flowering synchrony**

To investigate the impact of flower synchrony on cross-pollination a sum-up of all experiments has been done (Fig. 4.13). Flower synchrony is represented in relation of the mid male flower of the pollen donor field to the mid silk emergence of the pollen receptor field. For example, - 4 days means the start of pollen shedding of the donor was 4 days earlier than silk emergence of the receptor and + 2 days means the pollen shedding of the donor was 2 days later than the receptor silks. It is observable that other factors like e.g. wind direction and exposition modifies the flower synchrony effect, as cross-pollination rate at the end is always the result of all factors of influence. But flower synchrony is a prerequisite for cross-pollination and that factors of influence have the chance to effect.



**Fig. 4.13: Summary of Cross-pollination rates across all experiments in relation to flower synchrony.** Flower synchrony is presented as the difference in days between time of mid-yellow grain maize pollen shedding to the date of mid-white grain maize silking, see Table 4.1. Cross-pollination rate is related to the first adjacent row (0.8 m distance).

In general, apart from some variation, highest cross-pollination rates occurred at synchrony levels of - 4 to + 3 days, with a clear decrease outside of this range. The decrease in cross-pollination by a seven days earlier start of flowering of the donor field, accompanied by an anthesis silking interval of 2-4 days of the receptor field, may be the effect of beginning reduction of pollen shedding of the donor field after 9 days. On the other hand, the very low cross-pollination rate of a five days delayed pollen shedding of the donor field may indicate that most silks of the receptor field were already completely fertilized at this date, as it was observable that at the field level silks were wilted (fertilized) after 5 to 6 days. In conclusion the time slot for good synchrony and high cross-pollinations may be very narrow and could be modified, of course, by weather conditions, as too wet weather will prevent pollen shedding and warm optimum conditions could accelerate the pollen shedding and fertilization processes. Furthermore, this time slot will be affected of the state of the field, how equally the field is developed. An inhomogenously developed field could prolong the flower and

possibility of cross-pollinations (c.f. Figures 4.11 and Fig. 4.13). Although yellow grain pollen shedding started seven days later, the cross-pollination rate rose due to frequent cross-pollination on small ears, that probably correspond to late developed plants. In such cases even an extremely late pollen donor can be successful as the receptor field would already have passed the main phase of pollen shedding. This is a clear indication that a homogenous development of the receptor field is an important additional factor to keep cross-pollination rates low. This may be a problem in cases of low-input agriculture where control measures like intensive seed bed preparations or reduced input of fertilizers are prominent and in fields with areas of natural soil heterogeneity.

#### **4.6 CONCLUSION**

Some supposed major factors of influence were studied for their effect, none was effective beyond the initial 15 m of a receptor field; this was due to a generally limited pollen dispersal distance. With respect to the legal threshold values for GMO-free product this initial variation will be diluted with field size. As some important factors on cross-pollination are not predictable (e.g. meteorological conditions) discussions about cross-pollination should be orientated to „worst-case“ scenario with maximum cross-pollination rates. Such „worst-case“ will get more probable, if the receptor field is downwind of a GM field in a wind exposed landscape and when flower synchrony is favourable. All studies worldwide, including those presented here, prove that variation will always be quite high and will never be fully explained at the practical field level; nevertheless, some factors like the specificity of varietal flowering biology and of topographic patterns should be evaluated better to close some knowledge gaps. But it is difficult to separate the effect of certain influence factors as cross-pollination rate at the end is always the sum of several effects. Some of them, like meteorological conditions, are not possible to plan ahead.

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## 5 General Conclusions and Outlook

### 5.1 The grain colour system

The grain colour-based system has been widely approved for the detection of cross-pollination since many decades all over the world, mostly by using dominantly yellow and recessively white grain maize varieties (*Salamov, 1940; Jones and Brooks, 1950; Hutchcroft, 1958; Raynor et al., 1972; Paterniani and Stort, 1973; Garcia et al., 1998; Ma et al., 2004; Stevens et al., 2004*). Our own results are surely one of the largest data set at all generated by this system and these fully corroborate the results from literature mentioned before. Therefore we can conclude that the system of detection of cross-pollination by yellow and white grain maize varieties is a very reliable and efficient system for studying also issues of cross-pollination in context to coexistence. Advantages are the low costs and therefore the potential of intensive sampling and having numerous repetitions. Together with the simplicity of the method, that reduces the error susceptibility in experiments, this method enables to generate very detailed and robust data about cross-pollination.

### 5.2 Status quo of knowledge about cross-pollination in maize

When all available data of cross-pollination in maize are summed up some regularities become apparent, although there are always some variations that must be accepted within a certain range. Cross-pollination rate rapidly decreases within the initial distance of 15 m of a pollen source, a phenomenon that has been found in similar extent by many other research groups all over the world (*Ma et al., 2004; Molina, 2004; Brookes et al., 2004; Henry et al., 2003; Bateman, 1947*). In the experiments here this rapid decrease could be related to pollen settling speed with a potential dispersal distance in this range, modified by the prevailing wind speeds (see Chapter 4 and Fig. 4.4).

Different factors of influence affect cross-pollination mainly on the initial distance, where concentration of pollen is high, but only to low effect on greater distances beyond 30 m. In wind exposed locations cross-pollination rate of the first adjacent row to the pollen source can double in prevailing wind direction, but this wind effect had only a great impact on the initial 5 m; beyond 15 m the cross-pollination rate was similar in all directions (See Chapter 4 and Fig. 4.5). Thus it can be concluded that even in alpine regions at a small field dimension this additional wind effect gets diluted to a low effect on cross-pollination rate of whole field. Flower synchrony has a great impact on cross-pollination rate. As silks get fertilized and start senescence quickly on the single plant level there may be only a small time slot of a few days

for potential cross-pollination. Thus a well synchronized development of plants would surely minimize the possible time slot for potential cross-pollination at the field level. From various experiments here the main time slot for possible cross-pollinations had a range between seven days earlier and 4 days later start of pollen shedding by the donor field stand, in relation to the main silking date of the pollen receptor stand. The decrease in cross-pollination by even earlier flowering is depending on how long and massive pollen shedding of the pollen donor field is. Maximum pollen amounts are shed when tassel branches start to shed pollen, a few days after the start of anthesis. A reduction in cross-pollination is observable, when pollen shedding of the donor starts seven days earlier than the receptor; this indicates that pollen shedding decreased in this weather conditions at least nine days thereafter, as the anthesis silking interval of the receptor plants was around three days. Highest cross-pollination rates are expected with a synchronous start of male flowering in the pollen donor and female flowering in the pollen receptor field, where around two days later most of the silks start to emerge (depending on weather and variety) and the pollen donor field reaches to maximum pollen shedding by flowering of the tassel branches. Such a case of complete synchrony did not occur here. Even a seven days later pollen shedding of the donor could give rise to unexpected higher cross-pollination rate but this could be largely explained by considerable numbers of late developed plants in the receptor field; this favoured to cross-pollination due to low pollen competition.

Widely differing field size ratios of pollen donor and receptor plants did not show an effect on cross-pollination rate at the investigated distances; this is probably an effect of the short dispersal distance of maize pollen. Beyond 10 m only a small portion reaches in the receptor field but due to high quantitative pollen competition in the receptor field this donor pollen has only a low chance for fertilization.

On long distances, greater than 100 m, the cross-pollination rate was very low and did in no case exceed 0.02 % in total on 0.5 to 1.5 ha fields. Only in fields orientated in the prevailing wind direction distinct albeit small cross-pollination rates on the field borders were observable in distances of 50 m to 371 m. These findings are in conformity with the measurement of horizontal wind speeds that showed a potential dispersal range of up to 55 m, with most pollen settling within much lower distances. In addition, an experiment about pollen take-off showed that only a very small portion of pollen moves into higher air layers with potential longer dispersal distances. The very small amount of long distance dispersed pollen will meet a tough pollen competition in the receptor field and chance of cross-

pollination is probably very low. Rare “hot-spots” of abnormally high cross-pollinations in some fields were related to contamination plants whenever checked for in detail. In conclusion, such “hot-spots”, also observed by other authors (*Henry et al., 2003; Ma et al., 2004*) may be more often the results of seed contaminations than of extreme weather events.

Obviously, cross-pollination at short distance follows different mechanisms than at long-distance. At short distance the most important effects will be by flower synchrony, wind exposition and wind velocity. Furthermore canopy architecture, canopy wind turbulence, and pollen retention on plant surfaces may be important factors like described by *Aylor (2005)*.

On long distance dispersal additional factors of influence get important. Certain meteorological mechanisms like wind gusts or thermals are necessary to lift pollen into higher air layers and initiate dispersal over long distance. The properties of the surrounding landscape may interact in a complex way with atmospheric dispersal mechanisms. Physiological parameters of the pollen like viability may change during the dispersal and impact on cross-pollination probability, too (*Aylor, 2004*).

### **5.3 Challenges for cross-pollination research**

Although the knowledge about cross-pollination in maize has progressed, there are still some important gaps; on a quantitative scale right now it is not clear how much of understanding will be improved by filling them.

The type of landscape and its corresponding meteorological conditions like development of thermals or secondary wind systems are difficult to understand and measure. Atmospheric motion systems are generally positively correlated in space and time scales. Motions which occur in small volumes of air have short life spans and motions involving extensive volumes have substantially longer time periods. For example, wind blowing across a rough surface generates tiny eddies that persist for a few seconds. Isolation on a dry desert surface can cause thermal convective circulations (dust devils) which may be several meters in horizontal diameter, 100 m high, and persist for several minutes (*Russel and Wilson, 1996*). In the experimental locations investigated here the landscape was small structured and mixed, which is typical for many parts of Switzerland. Under these conditions strong atmospheric circulations may less evolve than in much more homogenous landscapes where stronger effects may be found. Also, the question remains, if there could be special weather events that would result in “hot-spots” of higher cross-pollination beyond the expected distances.

However, the extensive results of the long distance experiments here indicate that “hot spots” would be more probably due to seed contamination than to special meteorological events.

So far, experiments were mostly done with only one receptor maize variety. It is not fully understood to what extent varieties may differ in the susceptibility to cross-pollination; this could happen due to their flower biology like anthesis silking interval or physiological parameters like pollen competition during germination and tube elongation on the silks. The search for pollen incompatibility factors (*Frei, 2005*) have shown that there exist differences in competition during pollen tube growing on the silks. Some researchers have observed ratios of grain colours that deviated from the expected values after selfing maize that was heterozygous for grain colour; this may indicate different pollen competition ability depending on pollen genotype (*personal communication X. Foueillassar and U. Grossniklaus*). Different cross-pollination susceptibility and ability may modify the found cross-pollination rates, depending on the genotype combinations of receptor and donor maize. And the physiological differences may be affected in a different way by environmental conditions. This could lead to deviating cross-pollinations under similar conditions but different genotypes.

As a number of crucial environmental impacts cannot be controlled in field experiments, only long-term monitoring would allow the detection of extreme events that may occur at large temporal scales with unexpected consequences. As experimental factors can be varied only to a limited extent, due to the related costs, it will scarcely be possible to understand in all details, which factor was responsible for an effect, as the extent of cross-pollination is always the sum up effect of many different factors. Furthermore, it would require a truly multidisciplinary approach including the participation of scientists with agricultural, meteorological and geographical expertise to analyze the combination of different impact factors. But the results of all experiments here are nevertheless a powerful proof that the most decisive patterns of cross-pollination can be reasonably well explained with the existing knowledge about pollen dispersal can be understood; thus the missing pollen dispersal issues may only contribute to a slight improvement of cross-pollination knowledge in relation to the high input of labour and money to get this knowledge; thus there may never be 100% percent prediction, similar to other complex systems e.g. weather forecast.

#### **5.4 Limits of predictions and modelling**

Cross-pollination rates are dependent upon local conditions, such as topography and weather. Such site-specific parameters are difficult to measure and limit our ability to generalise results

and predict pollen dispersal. To improve predictions and investigations of cross-pollination modelling can deliver additional support. Rapid technological developments in computing power have markedly improved the utility and availability of atmospheric transport and dispersion models. Many models were developed for general applications in atmospheric science, and have been adapted for specific applications (*Westbrook and Isard, 1999*).

An already existing model for maize pollen cross-pollination is MAPOD (*Angevin et al., 2003*). By assuming “worst-case” scenarios it seems to be possible to draw general guidelines for handling coexistence that can be adapted to more precision by further experiments in future. But there are numerous parameters and the variability sums up as well as the errors. If the model demands input of too many parameters its use will be limited to intensive scientific conditions and less suitable to agricultural practice.

## **5.5 Conclusions for agricultural practice**

Several particular features have to be regarded for the use of cross-pollination knowledge in managing coexistence of agriculture.

### **5.6.1 Different types of cultivation**

Different types of maize products exist, ranging from silage maize (complete plant) to exclusively grains; this has consequences for the resulting transgene content in relation to cross-pollination rate, depending on the harvest product. Only transgenically cross-pollinated grains of conventional maize can deliver transgenic DNA content to the harvest product. If the harvest product contains also conventional material like silage maize, there is a dilution of transgenic DNA by “conventional” DNA. The results of 28 silage maize fields were compared with 8 fields of maize grain harvest in the German “Erprobungsanbau”; but the calculated cross-pollination rate was similar, although it was expected that grain maize should have much higher values than silage maize due to the aforementioned dilution effects (*Weber et al., 2005a; Weber et al., 2005b*). This feature is probably due to instability of DNA that gets digested rapidly in harvested leaf material by internal and external nucleases. In conclusion at the end it is only the DNA protected within the grain that will be accounted for by PCR analysis and the variation in cross-pollination rate by product type may be low.

### **5.6.2 Organic farming**

Organic farmers usually market their products under protected labels that require GM-freedom; therefore they want a threshold close to the possibilities of technical analysis

(around 0.1%). This is very difficult to handle, as cross-pollination happens mainly on close distance; but over long distance there can also occur some cross-pollination at very low rates. According to the results of the alpine region in Urner Reusstal (see Chapter 3), organic farming would not be affected by GM-maize itself in distances above 50 m to 100 m, disregarding other additional sources of contamination during seed, harvest and transport. In organic farming no herbicides are allowed and the nutrient input is usually lower; this may result in a higher weed pressure affecting a wider anthesis-silking interval (like observed in some field parts here), making organic maize fields possibly more susceptible to cross-pollination. This should mainly affect field borders because of the short dispersal range of pollen.

### **5.6.3 Mixing of the transgene content at harvest**

In most experiments on cross-pollination the samples were harvested from well defined and well distributed sample points. But in agricultural as well as in future control practices with defined thresholds, samples usually are taken of a large mixed harvest portion. In comparison to the science-based single-spot observations there will be usually dilution by harvest due to mixing of areas with high and low cross-pollination in agricultural practice. This effect was checked in the German “Erprobungsanbau” (*Weber et al., 2005a; Weber et al., 2005b*); mixed samples were taken from larger harvest areas, with distances to the pollen source of 0-10 m, 20-30m up to 50-60 m. At distances up to 20-30 m and 50-60 m transgene contents were always below 0.9%, although values up to 1.8 and 3,7% occurred at 0-10 m in grain and silage maize, respectively (*Weber et al., 2005a; 2005b*).

The mixing effect on the transgene content not only depends on the harvest process but also on the direction of harvest procedure. If it takes place across the gradient of cross-pollination mixing is much more effective than along the cross-pollination gradient.

### **5.6.4 Maize seed production**

The maintenance of genetic purity in cross-pollinated plants is the most important issue for breeders and seed production companies (*Jones and Brooks, 1950; Burris, 2001*). Fields for the production of hybrid seed usually contain rows of pollen producing (male) plants alternated with rows of sterile or detasseled (female) plants acting as pollen receptors. Depending on the planting pattern, as much as 80% of the plants in a field are detasseled female parents and do not produce pollen. As a consequence, they are highly receptive to both the pollen from the male parent but also to adventitious pollen carried in from neighbouring fields by the wind. Due to inbred depression and a general trend to reduced pollen production,

the male parent plants usually produce less pollen than commercial maize cultivars. In order to ensure a high degree of purity of the hybrid seed (usually 99.5%), strict cultivation conditions are respected (*Brookes et al., 2004*). Thus the cross-pollination susceptibility is much higher during the breeding process and during seed multiplication; special guidelines are necessary and already implemented independently of the GM issue. Minimum separation distance in the EU is 200 m for all categories of seed production and this is reckoned sufficient to maintain inbred lines at 99.9% purity (*MAFF, 2000*).

#### **5.6.5 Specialty seeds: Topcross high oil maize**

Some more special types of cultivation, i.e. production of Topcross high oil maize by mixing a cytoplasmatic male sterile hybrid with a high oil maize pollinator exist in rare cases. This cultivation type has similarities to the hybrid seed production discussed above. Depending on the ratio of maize plants with and without pollen production, there could be an increased susceptibility to foreign pollen, because of lower pollen production and competition within the field (*Lambert et al., 1998*). This would be also the case in the Plus-Hybrid system where up to 80% of the plants should be male-sterile in order to ensure xenia effects by cross-pollination with a non-related hybrid (*Weingartner et al. 2002*).

#### **5.6.6 Pharmaceutical GM maize plants**

In the production of pharmaceuticals by GM-plants, sometimes molecules are produced that are not allowed to get in the food chain. Both, the US Department of Agriculture (USDA) and the Food and Drug Administration (FDA) have started to make deliberations how to handle the production of pharmaceuticals in crops (*Mascia and Flavell, 2004*). Such plants that are engineered for the use as “green factories” have the potential to become important sources of plant-made industrial compounds and plant-made pharmaceuticals (e.g. blood plasma proteins, human hepatitis B vaccine) because of several advantages comparing to production in modified mammalian cell cultures or in microbial bioreactors. A spread of these transgenes could be associated with great actual risks and intensive care is needed to avoid cross-pollination. The USDA in USA regulates this production with some guidelines. In 2003, regulations for growing open-pollinating maize of this type require at least 1.6 km spatial isolation from other maize fields (*Stevens et al., 2004*). In future, new methods of gene containment could solve this problem.

### **5.7 Cross-pollination of next generation transgenic maize**

The present first generation of transgenic crops carries only a few simple transgenic traits, such as herbicide resistance or the BT-Protein for certain insect resistance, based on the possibilities of the early tools of plant genetic engineering (*Maliga and Graham, 2004*). In future there could be much more diverse applications and varieties available. Like in classical breeding, it can be assumed that different transgenic lines with special abilities will be combined. In this case, if every parental line of a hybrid contains a transgene, the GM plant will be transgenic homozygous in comparison to the transgenic heterozygous varieties at present. In consequence, these transgenic homozygous plants will shed pollen that contains up to 100% transgene and not 50% transgene like at present. To establish guidelines that also fit in the future this has to be considered. Results of experiments, done by transgenic heterozygous material, have to be doubled and results done by cross-pollination with homozygous color grain may show the maximum cross-pollination by transgenic varieties of the future.

Beyond pharmaceuticals, modern plant biotechnology has opened up further options for producing large quantities of commercially important industrial products in plants. These technologies have made it possible to use well-developed systems of agricultural systems to produce large quantities efficiently and cheaply in comparison to expensive small scale production in labs by bioreactors. The literature is full of examples of products like speciality oils, enzymes, plastics, vaccines and antibodies (*Raskin et al. 2002; Ma et al. 2003*). As can be deduced from numerous cross-pollination experiments now, it is possible to handle coexistence to a certain threshold but zero tolerance seems to be impossible. Therefore commercial interest exists in developing gene containment technologies to handle coexistence with a zero cross-pollination tolerance for a second generation of transgenic plants that are intended for molecular farming and production of biopharmaceuticals. Several different types of gene containments are in development and it is at present not clear which technology will be applicable to which case and point of time.

One containment technology is focused on Apomixis, which is the process of asexual reproduction through seed, in the absence of meiosis and fertilization, generating clonal progeny of maternal origin. Apomixis might be achieved by de novo engineering in crops and be combined with male sterility. The mechanism is very complex and poorly understood so

far. Therefore apomixis technology will not be available in the near future (*Spillane et al., 2004; Koltunow and Grossniklaus, 2003*).

The RBF (Recoverable block of function) technology blocks gene flow from transgenic plants at the molecular level. It prevents hybridization of transgenic plants with sexually compatible plants. Besides the known barnase and similar “suicidal” genes, other blocking constructs can be used, which can act at the RNA, protein, metabolic or hormone level (*Kuvshinov et al., 2001*).

Another approach uses gametophytic cross-incompatibility genes of maize to establish a fertilization barrier against GM contamination (*Frei, 2005*). In a similar way, the manipulation of DNA methylation may also offer effective approaches for creating hybridization barriers and to control gene flow between species (*Bushell et al., 2003*).

The transformation of the foreign genes into the chloroplast genome is also a useful route because, in many plant species, chloroplast DNA is usually not transferred from pollen to egg. A chloroplast-based expression system that is suitable for the commercial production of recombinant proteins in tobacco leaves has been developed recently (*Maliga 2002*).

A solution, ready to use for the near future is the Plus hybrid system that combines male sterile hybrids grown in mixtures with male-fertile non-isogenic pollinator hybrids (*Feil et al., 2003*). The transgenic variety could be male sterile, by introduction of CMS (cytoplasmic male sterility) like usual in hybrid breeding. Except the exclusion of transgenic pollen dispersal this system also promises grain yield advantages brought about by CMS and xenia. This system is suitable for cases, where it is not necessary to have a 100% transgenic field, e.g. BT-resistance; in this case an admixture of “conventional” plants is even desirable for resistance conservation management. If there is a need of a 100% transgene field like in cases of herbicide resistance, it may just be suitable for a reduction of the total amount of transgenic pollen that can be released. However this system would be of great advantage as a further safety measurement in the production of pharmaceuticals as the pollen flow from the transgenic plants could be blocked or reduced.

In conclusion, there are some possibilities for gene containment. The introduction of such containment systems may change the debate and handling of coexistence as there should be no more cross-pollination. But transgenic breeding is also time and cost intensive, and after successful development of these containment technologies it will also need some time to introduce them in new transgenic varieties, and finally introduce them on the market. Also the old transgenic varieties will be used as long as possible for economic reasons. New developments might be unattractive for companies because of patent rights and license fees.

Time will show if these new technologies will find their way in agriculture, or if coexistence will be handled and accepted with regulations and other methods like separation distances as discussed so far. But these developments should be carefully considered with regard to further research requirements to understand in detail all possible impact factors on this process. The time needed to solve the last cross-pollination riddles may be so long that new gene containment technologies will be ready for use meanwhile, making it futile to invest more research on cross-pollination and pollen dispersal.

So there arises the final question: Is cross-pollination of maize just a topic of temporary interest now?

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