Outcrossing and coexistence of genetically modified with (genetically) unmodified crops: a case study of the situation in the Netherlands

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Abstract

With the introduction of genetically modified (GM) crops the EU has demanded that individual member states enact measures to prevent inadvertent admixture – through outcrossing – of genetically modified organisms (GMOs) with products from conventional and organic farming. A literature review on outcrossing was prepared for the Coexistence Committee installed in the Netherlands in 2004. For sugar beet and potato, isolation distances do not appear to be of overriding importance, as true seeds are not part of the harvested product. The only route for admixture is through persistence of GM hybrid volunteers, and these should already be subject to strict control in good agricultural practice. Data on maize indicate that a distance larger than 25 m is needed to keep admixture below the EU labelling threshold of 0.9%, and larger than 250 m to remain below the 0.1% threshold as favoured by organic farming organizations. Oilseed rape is more complex because apart from pollen flow also persistence of volunteers in and outside arable fields, and hybridization with wild relatives play a role. At the present state of knowledge, isolation distances of 100–200 m and rotation intervals of 6–8 years might be warranted for the 0.9% threshold. It is as yet not clear whether a threshold of 0.1% is achievable in practice. The conclusions are compared with the measures recommended by the Dutch Coexistence Committee.

Additional keywords: gene flow, isolation measures, maize, oilseed rape, sugar beet, potato, *Zea mays, Brassica napus, Beta vulgaris, Solanum tuberosum*

Introduction

The development of genetically modified (GM) crops has led to a lot of debate about their value for sustainable agriculture. Particularly in Europe there is a strong reluctance to the introduction of genetically modified organisms (GMOs). In the summer of 2003, the EU decided that forms of agriculture using GM crops should be able to coexist alongside forms that adhere to avoiding the use of GM crops. Farms willingly avoiding the use of GM crops can be of a conventional or organic nature. In practice, EU decision 2003/556/EC (Anon., 2004d) demands that measures should be enacted to avoid inadvertent admixture of GMOs with products from organic or conventional farming. The EU has transferred the decision on specific measures guaranteeing coexistence to the individual member states. In 2004, the Dutch Minister of Agriculture, Nature and Food Quality installed a Coexistence Committee comprising representatives from the most relevant parties in the primary agricultural production sector. This committee also included conventional and organic farmers and representatives from the breeding industry.

An important potential source of admixture is outcrossing between neighbouring agricultural fields. So in order to support the decision process in the Coexistence Committee, a review was prepared of the scientific literature on outcrossing in maize (*Zea mays*), oilseed rape (*Brassical napus*), sugar beet (*Beta vulgaris*) and potato (*Solanum tuberosum*). These crops were thought to be the most relevant ones because of the impending introduction of transgenic varieties and/or their sensitivity to outcrossing under normal farming conditions.

Among important recent studies on the subject is the report on outcrossing from the European Environmental Agency (EEA; Eastham & Sweet, 2002). Specifically for coexistence, the JRC–IPTS (Joint Research Centre – Institute for Prospective Technological Studies) in 2002 produced a scenario study edited by Bock *et al.* (2002). This included examples of seed production in oilseed rape and the cultivation of forage maize and ware potatoes. A year later, the Danish Working Group on the coexistence of genetically modified crops with conventional and organic crops presented a report on all relevant crops (Tolstrup *et al.*, 2003). Both reports are based on published data and modelling. The programme GENESYS was used for modelling oilseed rape (Colbach *et al.*, 2001a, b) and MAPOD for maize (Angevin *et al.*, 2001). Furthermore, in November 2003, the first European conference on the coexistence of genetically modified crops with conventional and organic crops (GMCC-03) was held in Denmark. The proceedings (Boelt, 2003) mainly contain studies on oilseed rape and maize, and to a lesser extent on sugar beet.

In spring 2004, the European project SIGMEA (Sustainable Introduction of Genetically Modified crops into European Agriculture) was started. SIGMEA (Anon., 2004e) is mainly studying oilseed rape, maize and sugar beet, and comprises 45 institutions from virtually all countries of the EU. One of the aims of SIGMEA is bringing together as many outcrossing data sets as possible in a structured manner. Where necessary, additional studies are performed. The project furthermore validates and extends the GENESYS and MAPOD models for application to sugar beet.

This review describes the most recent knowledge on outcrossing based on the studies mentioned above, especially on original publications and preliminary reports including literature that has been published since. Hardly anything has been published on outcrossing in the Dutch situation, although the oldest reference found on the subject is on a maize experiment performed in the Netherlands (Meijers, 1937). Only very recently, outcrossing tests were performed in field trials of the transgenic amylopectin potato of AVEBE in the northern part of the Netherlands (Anon., 2004g). So much of the following is based on studies from elsewhere in Europe and from Canada, USA and Australia.

In the Netherlands there is very little commercial seed production of the crops reviewed. There is only small-scale commercial production of seeds of vegetable forms of beet, mainly red beetroot, a substantial part of which is for organic farming. An important acreage of potato seed tuber production is present, but potato tubers are by definition not a product of outcrossing. Also, the Dutch Coexistence Committee only addressed the outcrossing issue in crop cultivation. Therefore, commercial seed production will not be elaborated upon in this review and neither will other ways of admixture that may occur further on in the post-harvest processing chain.

In the following, an overview of the most relevant basic principles of outcrossing will be described. Subsequently, specific information on each crop will be discussed. Finally, conclusions on separation measures needed will be discussed and compared with the measures agreed upon by the Dutch Coexistence Committee and published on 1 November 2004 (Anon., 2004f).

Aspects of outcrossing

The basic pattern of outcrossing is described by the leptokurtic pollen dispersal curve (e.g. Eastham & Sweet, 2002). The essentials of this curve are that most of the outcrossing occurs close to the pollen source with a strong exponential decrease with distance. Outcrossing may continue at a low level over longer distances. The tail of the curve is more difficult to quantify because of the low hybridization rate found, which may vary substantially depending on environmental conditions. The amount of outcrossing is influenced by various factors, like cultivar, compatibility, flowering synchronization, availability of pollinators (insects) and weather conditions (wind).

In agricultural practice, field size is an important factor mainly because of the competition between incoming pollen and pollen produced by the field itself. Thus, a relatively small field next to a large field will show a higher level of outcrossing than a field of equal size, due to the smaller amount of competing pollen from the smaller field. Also, the longer the field that borders a source field the more outcrossing it will have. In the older literature, outcrossing has often been measured in small-scale experiments with individual plants or small plots as acceptors and the results are difficult to extrapolate to large agricultural fields.

The threshold of admixture with GM material above which a product should be labelled as GM has been set by the EU at 0.9%. However, the organic farming organizations aim at production that is essentially free from GM material. So they are in favour of a threshold of 0.1%, which at present is the most practical detection level for checking whether or not harvests are mixed with GM material. Both thresholds will be discussed below.

Admixture in harvests will be measured by using a real-time polymerase chain reaction (PCR) quantification method (for reviews see Holst-Jensen *et al.*, 2003 and Miraglia *et al.*, 2004). Results will be expressed as the ratio between the number of copies of the transgene and the number of the crop's haploid genomes present (EC's Recommendation 2004/787/EC; Anon., 2004b). This ratio may depend on the number of copies of the transgene that become inserted in the GM crop's genome during transformation, and on the relative amounts of embryo, endosperm and maternal tissue in the seeds. The endosperm in most cases is derived from a fusion of two maternal nuclei and one sperm nucleus, and therefore contains two maternal genomes for each paternal genome. So Papazova *et al.* (2005) found the relative amounts of the paternally derived genome versus the maternally derived genome in maize grains to occur in a ratio of 1.1 to 2.1. With hybrid maize as pollen source, the transgene will generally be present in a heterozygous (hemizygous) state. As a result, only half of the incoming pollen will contain the transgene. So outcrossing results will differ substantially from those obtained using a variety with a homozygous dominant marker trait as pollen source. Using the real-time PCR method with the tetraploid potato, outcrossing results will be even more favourable provided that only one copy of the transgene is inserted during transformation. Quantification methods are undergoing evaluation within the European Network of GMO Laboratories – ENGL (Anon., 2004a).

The patterns of admixture through pollen flow discussed in this section mainly apply to oilseed rape and maize, in which the harvested product consists of or, at least, contains seeds. In potato and sugar beet, the pollen flow can only exert an indirect influence, since only vegetative parts are harvested. Although bolters may occur, the biennial sugar beet normally does not even flower before harvesting. Details for each of the four crops will be discussed below.

Maize

In maize, the grains are part of the harvested product. Under north-western European circumstances, there are no volunteers from grains spilled during harvest, and compatible wild relatives are totally absent. Although basically self-compatible, in practice the crop shows 95% outcrossing because the maize plant has separate male and female inflorescences that differ in time of flowering. Asynchrony in flowering occurs both between plants within the crop and between male and female flowers within the same plant. In the Netherlands protogyny is quite common, whereas more to the south protandry is usual due to higher temperatures. Moreover, the second ear on a plant is silking much later, so the level of admixture will depend on the number of ears successfully developing on the plants during the growing season (Struik *et al.*, 1986; Struik & Makonnen, 1992). Pollination mainly occurs by wind. Maize pollen is comparatively heavy in comparison with what is usual for a grass species and therefore settles relatively quickly.

Large-scale field experiments have recently been carried out within the framework of the Farm Scale Evaluations (FSE) (Firbank *et al.*, 2003) in the UK and in Spain, the only EU country with an important acreage of transgenic (Bt) maize (Alcalde, 2003). The FSE results for the UK have been published in a report by Henry *et al.* (2003) whereas those for Spain have only been published preliminarily (Melé *et al.*, 2004). The FSE looked at a total of 55 combinations of GM (transgenic herbicide tolerance) and non-GM fields of 3.3 ha each over two different years. There was little variation between years, but considerable variation between sites, mainly depending on wind conditions, but also on flowering synchronization and field form (i.e., length of the border between GM and non-GM field). Non-linear regression analyses gave the following indications for isolation distances. In the UK experiments an admixture of less than 0.9% was attained at 24.4 m, less than 0.3% at 80 m and less than 0.1% at 257.7 m. The Spanish experiments, using Bt maize, indicated a lower distance at which 0.9% admixture was reached: in the order of 10–12 m. Apart from differences in experimental set up, this may be related to differences in climatic conditions: the colder and more humid conditions in the UK (and also in the Netherlands) favour a longer pollen viability and also influence the pattern of asynchrony in flowering between male and female inflorescences as described above.

The above results refer to adjoining fields. In case of separated fields the heavy maize pollen would settle quite rapidly: e.g. Raynor *et al.* (1972) showed maize pollen deposition at 60 m to be only 0.2% of that at 1 m from a source. Studies on combinations of fields in a normal agricultural setting in southern France by POECB (Programme Operationnel d'Evaluation des Cultures issues des Biotechnologies, Benetrix, 2004) (Foueillassar & Fabié, 2004) and Spanish studies also showed admixture dropping below 0.9% at a distance of 25 m. Part of the French studies used conventional grains in waxy maize recipient ears as hybridization marker instead of the Bt marker in the Spanish studies, but the results were re-calculated to make them comparable with a situation with a transgene in a heterozygous state.

More recently, these figures were corroborated by studies from InnoPlanta at 30 sites in seven states in Germany (Weber & Bringezu, 2005). At distances of over 20 m from Bt sources GM admixture was generally below 0.9%. Also the most recent scientific publication of three years of field experiments in Canada (Ma *et al.*, 2004) gave an outcrossing rate below 1% at 28 m downwind (10 m upwind).

All these values obtained in experiments representative of agricultural conditions may deviate considerably from the results from the older scientific literature. This can be explained by experimental differences. In the first place, there is the type of marker used. Most of the recent studies used a real-time PCR quantification method, whereas previous studies used a morphological (colour, xenia) grain marker. Moreover, these morphological markers will often have been present in a homozygous state in the source. In contrast, the GM hybrids mostly have the transgene in a heterozygous state. In the second place, there is the configuration of fields. For instance, when 9-m^2 plots were used next to a 3-ha source, Jones & Brooks (1950) found relatively high outcrossing values that ranged from 0.3% at 50 m up to 0.7% at 300 m. Under such circumstances, competition from pollen in the receptor plots will be weak relative to the large amount of pollen produced by the source field. Salamov (1940), who is regularly cited in coexistence discussions, found a record value of 0.79% at 600 m in an agriculturally more representative set up of 10 ha white hybrid maize next to a 2 ha yellow source. However, consultation of the original publication in Russian (translation by D. Finaev) learned that Salamov's results were incompletely represented by Jones & Brooks (1950). The white hybrid maize used as receptor turned out to contain an admixture with yellow grains and yellow grains were used as marker to determine the amount of hybridization. For this reason Salamov himself did not regard his values as fully representative of the amount of hybridization at longer distances.

Oilseed rape

Oilseed rape represents a far more complicated situation than maize. The species is self-compatible, but outcrossing ranges from 5 to 55% (Timmons *et al.*, 1995). Seeds shattered before or spilled during harvest may enter into secondary dormancy and survive in the seed bank for at least 10 years. These seeds can give rise to volunteers in subsequent years and could be either a source or a recipient of GM outcrossing events. Such volunteer populations may also develop outside agricultural fields at roadsides and in other ruderal areas. Finally, there is the possibility of hybridization with wild relatives growing in the vicinity of oilseed rape fields.

A large number of studies have been published on oilseed rape. Gene flow has been studied from the level of individual plants (Lavigne *et al.*, 1998) to representative agricultural landscapes, such as Selommes (Burgundy) in France (Champolivier *et al.*, 1999) and Tayside (Scotland) in the UK (Ramsay *et al.*, 2003).

Gene flow studies within the FSE (Farm Scale Evaluations) have not been published yet so that real-time PCR quantification methods applied to large-scale outcrossing experiments have not been publicized either. Most studies have used transgenic herbicide tolerance (trHT), such as LibertyLink and RoundupReady, as a highly efficient marker for the detection of outcrossing by spraying seedlings with the appropriate herbicide. Damgaard & Kjellsson (2003) have made a meta-analysis of a number of studies from the UK, France, Denmark, Sweden, USA, Canada and Australia. Like in maize, relative field size was shown to be important. With a 200 m field depth, that is, field dimension in the direction of the pollen source, admixture remained below 0.1% at an isolation distance of 100 m, whereas with a 50 m field depth, admixture was about 0.3% up to a distance of 200 m. The figures referred to the upper level of the confidence interval. The results on field size are highly relevant to the Dutch situation, since the bulk of oilseed rape cultivation, which is centred in the northern province of Groningen, is taking place on oblong fields that are parallel to each other, and that sometimes may be very long. In agreement with the meta-analysis by Damgaard & Kjellsson (2003), modelling in the Tayside landscape of Scotland with more regular fields showed an isolation distance of 100 m to be sufficient to remain below an admixture level of 0.1%. In the Australian situation, with larger fields (25–100 ha), Rieger *et al.* (2002) found outcrossing in 63% of the fields, with a maximum admixture level of 0.197%. Only 7 out of the 63 fields tested showed an admixture level above 0.03%.

The general conclusion from these studies is that at an isolation distance of 50 m, admixture due to pollen flow will remain below the EU threshold of 0.9%. Important exceptions are the varietal associations, which are extremely susceptible to foreign pollination because of the low amount of competing pollen that they produce themselves. For instance, the varietal association Synergy, which consists of 80% male-sterile and 20% normal fertile plants, showed a much higher percentage of foreign pollination than normal fertile varieties (for instance, 18–45 times higher in a comparison with cv. Apex; Eastham & Sweet, 2002). On the other hand, their low pollen production makes varietal associations as a transgenic source population far more advantageous for non-GM neighbouring fields than genetically engineered conventional varieties. However, in the long run, apart from pollen flow, there are several complicating factors:

1. Outcrossing, seed bank and volunteers. In a modelling approach, Squire *et al.* (2003) indicated 1% volunteers five years after a crop with good agricultural practice in a 1:2 rotation with wheat. With a treatment far more restrictive than practised nowadays, 0.12% admixture could be attained in 3 years. On the other hand, without any treatment, the 1% level would only be reached after 16 years. Admixture from neighbouring GM fields will also partly end up in the seed bank. In their Burgundy (France) field site, Champolivier *et al.* (1999) established the numbers of double trHT volunteers, i.e., volunteers that were the result of hybridization from an adjacent field with a different trHT oilseed rape crop in the previous growing season. At 1, 20 and 65 m they found 2, 0.2 and less than 0.01% volunteers, respectively. Combining a relatively unfavourable average admixture level of 0.2% with the simple but realistic calculation by Lutman (2003) of 2 volunteers per m^2 after 5 years (5000 seeds lost per m^2, 2% survival over 5 years, 2% yearly germination), would result in about 40 transgenic plants per ha. This does not take into account additional admixture in later seasons. Moreover, in the long run all will depend on the extent to which the transgene provides a fitness advantage under the cultivation conditions practised.
2. Feral populations from seeds lost, for instance during transportation of harvests. To these populations, *mutatis mutandis*, the same applies as under 1. That is, much depends on any fitness advantage that the transgene may provide. Although feral populations are often of an ephemeral nature, Pessel *et al.* (2001) showed population survival for at least 8 years at a site in France, and Ramsay *et al.* (2003) for at least 12 years in Tayside, Scotland. In the Tayside study area, about a quarter of the feral populations survived for longer than 3 years. The feral populations may hybridize with surrounding oilseed rape crops too, e.g. 4% foreign pollination into a roadside population in Tayside (Ramsay *et al.*, 2003). In their turn, non-transgenic roadside populations could offer some protection to non-GM cultivation by producing pollen competing with any incoming transgenic pollen.
3. Hybridization with wild relatives. In ecological impact studies, a number of species have been addressed in sequence of decreasing likelihood of successful hybridization: *Brassica rapa, B. juncea, Raphanus raphanistrum, Hirschfeldia incana (*syn. *B. adpressa), B. oleracea, B. nigra, Erucastrum gallicum* and *Sinapis arvensis*. For the majority of these species, only low percentages of hybridization have been found under agricultural conditions: e.g. for *R. raphanistrum* none or practically none (Australia: Rieger *et al.*, 2001; Switzerland: Thalmann *et al.*, 2001; UK: Eastham & Sweet, 2002; Canada: Warwick *et al.*, 2003) or 10^{-7} to 2×10^{-3} (France: Darmency *et al.*, 1998; Chèvre *et al.*, 2000). Persistence is expected to be low due to problems with backcrossing during further introgression and chances of loss of the transgene during the process (for a recent review see Jenczewski *et al.*, 2003). Once again, the ultimate result depends on any advan-

tage provided by the specific transgene. The most relevant species will be *B. rapa*. By combining GIS data and field studies Wilkinson *et al.* (2003) estimated the number of hybrids generated each year for the UK as a whole at 32,000 in natural populations along waterways and 17,000 in feral populations of arable areas, numbers that are low compared with the amount of oilseed rape grown. However, locally situations may be substantially different. In the most extreme case reported up to now, Hansen *et al.* (2001) found that 44 out of 102 volunteers tested from a field that had been cultivated organically for 11 years showed molecular-genetic markers indicating ongoing introgression from oilseed rape (*B. napus*), i.e., the greater part of them looked most like BC2-generation plants. Hybridization with wild relatives is part of monitoring obligations in the EU directive 2001/18/EC (Anon., 2004c).

The most comprehensive approach to reaching an advice on isolation measures has been the modelling in the programme GENESYS by Colbach *et al.* (2001a, b). Using this modelling programme, Colbach *et al.* (2004) reported the need for an isolation distance of 200 m to keep admixture below 0.9% in an area of intensive cultivation in France. However, the GENESYS model is still being validated for the reliability of its predictions. The model uses explicit spatial models of the study area so that results cannot be extrapolated immediately to other areas. In addition, GENESYS systematically underestimates outcrossing levels, for which compensation is necessary afterwards.

Potato

In potato, true seeds are not part of the harvest. So outcrossing is only of indirect relevance. Seeds that are the result of hybridization from neighbouring fields would need to germinate and produce tubers capable of surviving until a next potato crop in order to lead to any admixture in the harvest. There is a lot of variation in the amount of flowering and fertility between varieties. Outcrossing is mainly by insects at a rate of 0–20%. True seeds may survive for at least 10 years in the seed bank, but volunteers arising from true seed are poor competitors compared with plants grown from tubers (Askew, 1993). After mild winters, large numbers of groundkeepers (tubers staying behind after harvest) can survive and volunteers arising from them can cause serious weed problems in the next crop in the rotation. However, for phytosanitary reasons, strict control of potato volunteers is already part of good agricultural practice. There are no compatible wild relatives with which outcrossing may occur. Eijlander & Stiekema (1994) showed that the species *Solanum nigrum* and *S. dulcamara*, both quite common in the Netherlands, did not produce any viable offspring in crossing experiments with potato.

The most recent outcrossing tests by AVEBE at Valthermond in the Netherlands have only been preliminarily published (Anon., 2004g). The results of 7.3% at 0 m, going down to 0.7% at 1.5 m and 0% at 5 m are in line with results from the UK and New Zealand published previously by Tynan *et al.* (1990), McPartlan & Dale (1994) and Conner & Dale (1996). For the UK, the largest outcrossing distance found was 10 m (0.017%). The only exception is found in a publication from Sweden by Skogsmyr (1994), referred to in several recent reports (e.g. Eastham & Sweet, 2002). It mentions 72% at 0–1 m and 31% at 1000 m, which are unlikely high figures. Conner & Dale (1996) ascribed these values to a PCR artefact. Skogsmyr (1994) had to rely solely on the PCR tests, because direct tests on herbicide tolerance conferred by the transgene appeared to have failed. Conner & Dale (1996) managed to obtain additional information allowing to make re-calculations with the help of an alternative marker (the skin colour of source variety Désirée): 1.3% at less than 1 m, 0.5% at less than 3 m and 0% at 1000 m. By spraying the growth regulator MCPA it is possible to suppress berry formation and thus avoid ending up with admixed seeds in the soil, but this could influence tuber yield (Veerman & Van Loon, 1998).

At harvesting as many as 300,000 tubers per ha may remain in the field, which is more than originally planted. Tubers will not survive for more than one year because of physiological ageing, unless they have the opportunity to grow out and produce fresh tubers. Therefore, with strict volunteer control according to good agricultural practice, groundkeepers will not survive until the next potato crop in the rotation, which may be after 3 (conventional) to 6 (organic) years. The use of a 1:2 rotation in the starch-potato growing area in the north-eastern part of the Netherlands is rapidly diminishing (H. Bonthuis, personal communication).

Sugar beet

Like in potato, seeds are not part of the harvest in the biennial sugar beet. In principle, the beet is harvested before the onset of flowering. However, occasional bolters do occur, depending on variety and environmental conditions, such as cold during early growth. In addition, annual weed beets occur, which most probably arose through hybridization with wild beets in the seed production areas in southern Europe (Desplanque *et al.*, 2002). To prevent weed beets, sugar beet seeds are thoroughly tested for their occurrence (maximum allowed 0.05%). Admixture will only be possible when seeds produced by outcrossing survive and form harvestable beets in a next beet crop. In good agricultural practice, bolters as well as weed beets need to be carefully controlled. The compatible wild relative of sugar beet, sea beet (*Beta maritima*), occurs in small numbers mainly along the south-western coast of the Netherlands.

Outcrossing from transgenic bolters with weed beets was found to occur in the range of 0.07% to 0.8% of the offspring from weed beets tested in the vicinity of a transgenic crop (Champolivier *et al.*, 1999, Vigouroux *et al.*, 1999, Bartsch *et al.*, 2003). Outcrossing levels will depend on weed beet density. Mücher *et al.* (2000) found an average of 9 bolters per ha in a survey of 250 km^2 in Rhineland, Germany. Numbers varied considerably between fields: one exceptional field held 80,000 bolters per ha. Kempenaar *et al.* (2003) reported a comparable average of 10 bolters per ha for the Dutch situation. Based on these numbers, the following rough calculation can be made. About 1% GM hybridization for weed beets occurring at 10 per ha and each beet producing about 1500 seeds, would lead to about 15 seeds per ha capable of producing new bolters, taking into account a survival rate during winter of 10% as reported by Jørgensen *et al.* (2002) for crop/wild hybrids under Danish conditions. These are low numbers, but Bartsch *et al.* (2003) showed that a worst case scenario starting with 7 surviving seeds per ha and each weed beet producing a bolter could lead to 70,000 GM plants per ha in 12 years, i.e., in case no control of bolters is taking place. Moreover, a recent study by Arnaud *et al.* (2003) showed the additonal possibility of dispersal through seeds. They showed this to have occurred in a coastal area in France by tracing a maternally inherited crop marker in wild beets. Therefore, the key issue for coexistence is bolter control before anthesis and subsequent seed set. If successful, such a control measure will also serve as protection of the vegetable seed production that is occurring on a small scale in the Netherlands, also for organic purposes. A further improvement could be the use of triploid varieties, which has two advantages: (1) bolters in triploid varieties are less fertile, and (2) when using tetraploids as transgenic pollinator lines there would be a minimum chance of creating new transgenic weed beets (Desplanque *et al.*, 2002). However, in modern sugar beet breeding there is a tendency to return to diploid varieties.

Additional measures

Differentiation in timing and extent of flowering period

The above described Spanish field experiments with maize showed the potential of using different flowering periods. If neighbouring fields differed more than 2 weeks in sowing date the average hybridization level in the outer rows was more than 20 times lower than in a situation with a difference of less than 2 weeks (Alcalde, 2003). However, under northern European conditions, the short growing season for maize is severely limiting this approach. For the Dutch situation, Lotz & Groeneveld (2001) showed a very quick drop in yield with any postponement of sowing in early spring. An alternative might be the use of varieties differing in their maturing rate. However, as experienced by Ingram (2000) in UK variety testing, the current maize varieties appear to show little difference in their time of flowering.

Oilseed rape has an extended flowering period. Therefore, separation in time probably will only be effective by combining spring and winter varieties (Ingram, 2000). However, in the Netherlands, almost only winter varieties are used because of their higher yield (Kempenaar *et al.*, 2003).

Barriers

The best physical barrier to outcrossing is provided by the crop itself, since not only the outer rows of a crop provide a physical barrier, also the crop as a whole produces competing pollen interfering with fertilization by foreign pollen. For instance, in the Spanish maize field tests described above, discarding the first 4 to 8 rows sufficed to obtain an admixture level for the whole field of less than 0.9%. At larger distances between fields (where outcrossing levels are in the 0.1% range) the protective effect of the outer rows is less discernible: Rieger *et al.*, 2002 found no statistically significant differences between borders and central parts of the large oilseed rape fields studied in the Australian situation.

The much-cited classic by Jones & Brooks (1952) describes the effect of a row of trees combined with a brushwood undergrowth. This reduced hybridization levels, but only for the first 50 to 90 m. In seed multiplication hemp barriers are being used. Saeglitz *et al.* (2000) showed a dense 5-m hemp barrier not to be very effective in preventing outcrossing from a small (0.04 ha) transgenic sugar beet field with male-sterile beets. Unfortunately, no comparison was made with the situation without a hemp barrier. Both tree and hemp barriers appeared to be less effective than the crop itself. This will undoubtedly be related to pollen competition. Moreover, there is an additional risk in using barriers: the barriers may influence local wind conditions thereby even increasing admixture levels in certain parts of the fields. For instance, in the FSE, outcrossing 'hotspots' were regularly observed at distances of 100–150 m and were associated with the presence of tree areas around the field (Henry *et al.*, 2003). Admixture levels at such 'hotspots' were below 0.9%, but not necessarily below 0.1%.

Other measures

A whole range of biotechnological adaptations has been proposed for limiting gene flow, such as chloroplast transformation, apomixis and 'terminator' technology, generally referred to as GURT (Genetic Use Restriction Technologies) (for a review see Daniell, 2002). In maize the method most likely to be feasible in the near future is the one proposed by Feil *et al.* (2003): growing mixtures of male-sterile transgenic maize plants with non-transgenic pollinators. With well chosen combinations, yields will be at least as high as with normal hybrids, but the method will obviously only work if not every single plant needs to be transgenic to obtain the desired cropping conditions (Feil *et al.*, 2003). This is the case with Bt maize where a certain amount of non-transgenic crop is even deemed necessary to avoid overcoming the resistance by the insects in the so-called high-dose/refuge strategy.

Discussion

The data on gene flow in sugar beet and potato imply that isolation distances are not of overriding importance for coexistence. Sugar beet usually does not flower before harvesting. So there is mainly a need for a very strict control of occasional bolters and weed beets to enable coexistence. In potato, the crop usually flowers before harvest, although fertility varies widely among varieties. However, pollen flow was shown not to extend very far: the largest distance found was 10 m with an admixture level of 0.017%, which is well below the threshold of 0.1%. Such pollen flows can only lead to admixture if there is an opportunity for the resulting seeds to grow out into a state of producing tubers in a subsequent potato crop. This should hardly be possible under current good agricultural practice in which volunteers already have to be strictly controlled for phytosanitary reasons. So there is mainly a need for a safety distance between GM and non-GM fields that is sufficient to keep harvests of sugar beet or potato separate.

For maize there is a clear need for isolation to enable coexistence. There are two complications with the assessment of isolation distances:

1. Uncertainty about the implementation of labelling thresholds depending on the interpretation of the EU rule of a maximum 'adventitious presence' of 0.9% of transgenes in a harvest. Adventitious presence was defined as the unintentional, incidental and technically unavoidable co-mingling of produce with trace amounts of transgenic origin. Can adventitious presence be taken as allowing any measurable presence of transgenes below 0.9%? In practice this would mean that a harvest admixture, averaged over a whole field, that remains below 0.9% does not necessitate labelling. Or does 'adventitious presence', that is, technically unavoidable admixture, need to be taken as essentially aiming at no admixture to be expected based on the isolation measures enacted, which would practically speaking imply the use of a maximum threshold of 0.1%? Or would it be taken, in a milder version, that an admixture higher than 0.9% would not be allowed anywhere in a field? In the more strict interpretation, the 0.9% threshold would only serve for completely unexpected disasters. This interpretation may conflict with another objective of the EU regulation, i.e., practicality. The strict interpretation of 'adventitious presence' would lead on the one hand to a need for large isolation distances, which might economically not be feasible, and on the other hand to difficulties in enforcement because of the problem of establishing the causes of admixtures in a given lot or product.
2. The standardization of the quantification method within the EU. The real-time PCR method will be used and according to EC recommendation 2004/787/EC (Anon., 2004b) results will be expressed as numbers of transgenes per number of haploid genomes, which is the most practical way (for reviews see Holst-Jensen *et al.*, 2003 and Miraglia *et al.*, 2004). Exact figures will depend on the way results are calibrated on the basis of a crop- and transformation event-specific standard sample, which is being evaluated within the ENGL network (Anon., 2004a). With maize an additional aspect has to be taken into account, i.e., most of the maize in the Netherlands is grown for silage. In maize silage the grains account for up to half of the weight of the end product (Ingram, 2000). In practical testing it is hard to judge beforehand to how much less admixture this may lead, as this also depends on the efficiency with which the transgene is PCR-quantified in a mixture of grains and other plant material compared with quantification in grains alone. At the present state of knowledge, the data on maize imply that admixture in individual ears remains below 0.9% from 25 m onwards and below 0.1% from 260 m onwards. A need for larger isolation distances may be invoked to create safety margins, but these have yet to be determined.

Also for oilseed rape there is a need for isolation distances in coexistence, but the situation is far more complex than with maize. Based on pollen flow data alone, an isolation distance of 50 m may be enough to remain below a threshold of 0.9%, but this most probably will not be enough in view of additional gene flow by seed dispersal and seed bank formation. The best approach available at present is the French GENESYS model, the most recent results of which imply an isolation distance of 200 m for the threshold of 0.9% in an intensively cultivated crop in France (Colbach *et al.*, 2004). However, these results cannot be extrapolated to other growing areas and also need further validation (Colbach *et al.*, 2004).

In Denmark, coexistence legislation was enacted in 2004. Crop-specific details still need to be filled in, but for each crop there are already recommendations from the Danish Coexistence Working Group in the comprehensive report by Tolstrup *et al.* (2003). For sugar beet, in a scenario of 50% GM crops, these directives are as follows: an isolation distance of 50 m and a rotation interval of 3 years for conventional farming (0.9% threshold) and 100 m and 5 years, respectively, for organic farming (0.1% threshold). Figures for potato are: conventional farming 20 m with a 3-year-rotation interval and organic farming 20 m with 4 years. Particularly for sugar beet it could be argued that isolation distances are unnecessarily large. Such large distances take into account a risk of insufficient control of bolters, in which case outcrossing over larger distances is possible with wind-pollinated beet. The Dutch Coexistence Committee agreed on an isolation distance for sugar beet of 1.5 m for a conventionally grown crop and 3 m for an organically grown one. For potato these distances are 3 and 10 m, respectively. For potato and sugar beet these distances are adequate only in combination with strict regulations on volunteer and beet bolter control. Such distances appear to be sufficient for keeping harvests separated between fields. An isolation distance of 10 m for potato still seems to be rather large because of the small likelihood of admixture through pollen flow. Apparently, the Coexistence Committee preferred solid safety margins, which might be warranted by the possibility that admixed seeds, once formed, could survive in the soil for a considerable period of time (at least 10 years) and could give rise to admixed tubers whenever volunteer control would be alleviated. From a practical point of view, implementation would be feasible by feed back of cultivation plans and rotations between neighbouring farms. Announcement of GM cultivation plans before 1 February of each year is therefore part of the regulation plan in the Netherlands. Potato most likely is also the first transgenic crop to be introduced in the Netherlands (the AVEBE amylopectin potato). Monitoring is part of the recommendations of the Dutch Coexistence Committee, which could for instance be important for assessing whether strict bolter control in beet is effective. This monitoring will point out whether the proposed measures either work out well, have to be tighter or could even be alleviated.

The Danish recommendations on isolation distances for maize was 200 m for conventional and 300 m for organic farming. Based on recent data, the 200-m distance appears to be considerable for a threshold of 0.9% GM admixture. The 200-m distance was primarily derived from the IPTS report (Bock *et al.*, 2002), in which an area of intensive farming in France was modelled, using MAPOD (Angevin *et al.*, 2001). The larger distance may in part be explained by applying a 0.3% admixture threshold for the certified starting seeds and by the use of a source variety with the transgene in a homozygous state. Like with GENESYS, the modelling results cannot be simply extrapolated to other areas and need additional validation. The Dutch Coexistence Committee agreed on 25 m for conventional and 250 m for organic farming. These distances may be rather short in view of the FSE calculation of 24.4 m for attaining the 0.9% threshold and 257.7 m for attaining the 0.1% threshold (Henry *et al.*, 2003). Indeed, these distances were calculated for admixture levels in the grains and could be shorter in case of silage maize. However, to what extent admixture levels will differ between grain and silage maize has not yet been ascertained by the real-time PCR test. So the Dutch Coexistence Committee also advised additional research on isolation distances under Dutch cultivation circumstances and to have the introduction of the GM maize accompanied by monitoring.

For oilseed rape, the Danish Working Group formulated recommendations only for conventional farming: a 100-m isolation distance with an 8-year rotation interval. This does not appear unreasonable in view of the complexities described above. The Danish Working Group did not advise on organic farming and on the case of varietal associations because of the large uncertainties about the feasibility of attaining the thresholds. Mostly because of such uncertainties, the Dutch Coexistence Committee completely refrained from advice on oilseed rape. Moreover, oilseed rape is not a very important crop in the Netherlands and introduction of transgenic varieties is not expected in the short run.

In conclusion, the research results published so far reasonably allowed to draw up measures to prevent undesired outcrossing for sustaining coexistence of GM with non-GM agriculture, at least for sugar beet, potato and maize. Nevertheless, particularly in maize, it is advisable to do additional research on isolation measures under Dutch circumstances and to monitor the efficacy of such measures during introduction of GM varieties. The prime exception is oilseed rape. The large uncertainties about the quantification of the various gene flow routes stress the need for continued research in this crop.

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