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Outcrossing frequency in selfing and apomictic plant species subject to containment measures in GMO development regulation

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Table of contents

Preface

This report was commissioned by the COGEM in order to reassess the knowledge basis on outcrossing in the plant species presently categorised as basically having a selfing breeding system in the so-called “Lijst van inhullingsverplichtingen” (“ list of inflorescence bagging regulations”), formerly called “Appendix C”. Many thanks are due to the supervisory committee, consisting of W. van Delden (Prof. Em. Groningen University/COGEM), T. de Jong (Leiden University), H. den Nijs (Amsterdam University/COGEM), C. van der Vlugt (BGGO) and C. van Rijn/P. van Beurden (COGEM), for critical guidance and support. Thanks are also due to the experts who kindly advised me about their crops/species: J. Bergervoet/K. Boutillier/O. Dolstra/H. Helsper/O. Scholten (Plant Research International), M. Aarts/H. de Jong (Wageningen University), N. Bas/I. Boukema/R. van Treuren (CGN), D. Klein Geltink/A.J. van Wijk (NAK), B. de Boer (Free University Amsterdam), the Dutch breeders’ organisation Plantum through its representative A. van den Hurk, P. van Dijk (Keygene), P. Metz (Seminis), K. Boot/A.-J. Bouwman/H. de Jong (Innoseeds), B. Schuiling/I. Westerdijk (Wiersum), P. Keijzer (Fobek), B. Uijtewaal (Nunhems/COGEM). The contents of this report, however, remain the responsibility of the author.

Executive summary

One of the traditionally important elements in assessing the safety of the introduction of transgenic plants in agriculture is the possibility of transgenes moving into wild populations by the natural phenomenon of gene flow. Measures apply to keep gene flow to other plants of cross-fertile species contained in the research phase of newly developed transgenic plants, at which stage knowledge about the effects of the transgene on wild populations still will be limited. In The Netherlands, containment measures are described in the so-called “Lijst van inhullingsverplichtingen” (“ list of inflorescence bagging regulations”), formerly called “Appendix C”, where all species are listed in which transformation research is performed, together with containment actions deemed necessary per species. Containment measures depend on the type of breeding system of the relevant plant species, and the likelihood of contact with cross-fertile relatives in the Dutch flora.

Originally, selfing and apomictic species were regarded as having a negligible likelihood of gene flow by pollen outside of research facilities. Apomictic species produce seeds in which the embryo is not a product of fertilisation following pollination and so the plant arising from the seed is genetically identical to the mother plant, like with selfing. However, with an increase in awareness that selfing plant species rarely are a hundred percent selfing under natural conditions, nor are apomicts a hundred percent apomictic, the Dutch advisory committee on biosafety issues of GMOs (genetically modified organisms), COGEM, wished to re-evaluate the species list with containment rules (“Lijst van inhullingsverplichtingen” or Appendix C). Therefore, the present study describes an attempt to provide the best available overview of the present knowledge of outcrossing frequencies in a selection of the most relevant selfing or apomictic species on the “Lijst van inhullingsverplichtingen”: the Brassicaceae *Arabidopsis thaliana*, *Boechera* (*Arabis*) *holboellii, B. stricta* (*A. drummondii*), *Thlaspi caerulescens* and *Thellungiella halophila*, the Fabaceae *Phaseolus vulgaris*, *Pisum sativum* and *Vicia sativa, Linum usitatissimum* of the Linaceae, *Lactuca sativa/serriola* of the Asteraceae and the Poaceae *Poa pratensis*, *Triticum aestivum* and *Hordeum vulgare*.

In a number of studies, low amounts, 0-2%, of cross-fertilisation have been established in *A. thaliana*. Higher amounts were found in a genotype with a lowered male fertility, i.e. up till 10%. The occurrence of cross-fertilisation is corroborated by extensive population-genetic studies on natural populations, from which, however, no specific outcrossing rates can be deduced. The species is common in The Netherlands. Among the wild relatives reported to be more or less cross-fertile, only *A. arenosa* is rarely found in cities and the southern part of the province of Limburg.

B. holboellii and *B. stricta*, and the hybrid between the two species, *B. xdivaricarpa*, represent a complex case, in that first a distinction must be made between apomictic and sexual reproduction, and subsequently within sexual types, between selfing and outcrossing. This is already not simple in experimental set-ups, but even harder within natural populations using (molecular) markers. Thus, with the few studies found, too little data are available to give any indication of possible outcrossing rates. However, both basic species are native to North America, and do not occur in the wild in Europe.

Available data support the inference that *T. caerulescens* is basically a selfing species. However, there were also indications for fairly high outcrossing rates to occur in wild populations, but the data are too sparse to name any figures. Together with its occurrence in the southern part of the province of Limburg and more recently also in cities (Rijnmond) in The Netherlands, this may warrant additional experimental research on the breeding system of *T. caerulescens*.

Thellungiella halophila has gained some popularity in research as an extreme salt- and cold-tolerance model plant that again is related to the most popular model plant *A. thaliana*. There was very little published about gene flow so far, and also experts could add only very little oral information on the subject. The species is native to USA, Canada and China and is found only under extremely high salt conditions. No cross-compatible species are known in The Netherlands.

In common bean, *Phaseolus vulgaris*, the outcrossing rate appears to be quite variable depending on specific circumstances. It varies mostly from 0-2%, to as high as an average of 30%, which may even be called high for a species regarded as selfer. Breeders' experiences in The Netherlands are outcrossing rates of 0-0.1%, with a maximum of 0.5%. No feral occurrence is known in The Netherlands (the species is native to South America), but both *P. vulgaris* and the cross-compatible *P. coccineus* are grown in vegetable gardens. Seed production is limited to the province of Limburg.

Also Pea, *Pisum sativum*, is not an exclusive selfer, but outcrossing rates are low and no rates exceeding 5% were reported. Breeders' experiences in The Netherlands are negligible outcrossing rates. No feral occurrence of pea or cross-compatible wild relatives is known in The Netherlands (the species is probably only native to SE Europe and Asia), but a diversity of varieties is commonly grown by horticulturalists and in vegetable gardens.

Judging by its high outcrossing rates, *Vicia faba* cannot really be enlisted as a basically selfing species. Instead, it is having a mixed breeding system meaning that, considerable levels of outcrossing can occur depending on circumstances, but no self-incompatibility factors are involved, so that selfing also remains entirely possible. No wild occurrence of *Vicia faba* is known in The Netherlands (the species probably originates from Asia), but it is commonly grown by horticulturalists and in vegetable gardens.

Flax, *Linum usitatissimum*, is not a completely selfing species, but outcrossing figures are generally not higher than 5%. Breeders' experiences in The Netherlands are that outcrossing is low for lack of insects at seed multiplication sites. Flax is sometimes found as escape from cultivation in disturbed road sides in The Netherlands. The largest amount of cultivation is found in the region of Zeeuws-Vlaanderen in the extreme SW of the country. No cross-compatible wild relative is known in The Netherlands. Flax is cross-compatible with a series of wild species that share a chromosome complement of 2n=30. However, European species among them, such as *L. bienne* and *L. tenue*, have a more southern occurrence and have not been recorded in The Netherlands.

As a facultative apomict, *Poa pratensis* may show sexual reproduction also in cultivars selected for apomixis, normally at a rate of 1 to at most, 10%. Scanty evidence indicated that sexual reproduction mostly follows an outbreeding mode. In this species, highly apomictic plants still produce considerable amounts of pollen, since fertilisation of the endosperm is conditional to proper seed development. A recent publication showed such pollen to be capable of siring up to 15% of the seeds on a nearby non-apomictic individual of the same species. However, this has only been tested on the progeny of relatively few plants per accession (18 each from 6 accessions, including the closely related *P. angustifolia*). *Poa pratensis* is very common in The Netherlands in grasslands and road sides. The distribution of one other species recently recognised within the complex, *P. angustifolia*, is not yet completely known. Using a transgenic marker in the afore mentioned recent study, hybridisation was found with *P. angustifolia*, but not with other species of the Dutch flora, *P. annua*, *P. trivialis*, *P. compressa*, *P. nemoralis*, although the latter species was reported previously to hybridise with *P. pratensis* in the USA.

Wheat, *Triticum aestivum*, usually shows a low outcrossing rate of 1% or below. However, varieties with significantly higher rates occur, of up till around 10%, depending on inflorescence characteristics. Breeders' experiences are that there are usually no isolation distances necessary for seed production, or maximally 3-10 m for pedigreed seed. There is practically no feral occurrence of wheat in The Netherlands and also no cross-fertile wild relatives occur, such as members of the genus *Aegilops* do elsewhere in Europe.

Barley, *Hordeum vulgare*, shows contrasting outcrossing values between spring and winter type. Whereas the spring type has low rates and will show low likelihoods as pollen donor with its mostly cleistogamous flowers, the winter type shows higher outcrossing rates of up till 12.5%. Thus, according to breeders' experience, spring barley does not need any specific isolation measure in seed production, but winter barley does so with a recommended distance of 250 m. There is no feral occurrence of barley in The Netherlands, and also no cross-compatible wild relative, as no fertile progeny could be produced by crossing with the most closely related *H. murinum, H. marinum, H. secalinum* or *H. jubatum.*

Relatively few studies have appeared on outbreeding in lettuce. These indicate that outcrossing levels of 1-5% are normal for *Lactuca sativa*. Breeders' experiences are that outcrossing in isolated seed production areas in California and Australia only take place when insects are present and generally remains below 1%, but there are no publications about this.

Lettuce's close wild relative *L. serriola* (prickly lettuce, NL Kompassla) is very common in The Netherlands and most of Europe, and to all practical purposes could be considered conspecific with *L. sativa*, as it is freely crossable with the cultivated form.

A quick literature scan indicated that sunflower is basically an outbreeder. The wild progenitor is self-incompatible, which has only been partially overcome during the development of crop variants. Modern inbred material shows a selfing rate of between 80 and 100%. In The Netherlands, sunflower only occasionally emerges from seed spillage, e.g. at river beaches or on sparsely vegetated dunes. There are no cross-compatible wild relatives in The Netherlands, but the species is in horticultural use.

In conclusion, according to expectations, all purportedly selfing species discussed in this report were shown to be perfectly capable of outcrossing. This has a certain evolutionary logic, since a low rate of outcrossing would sufficiently contribute to creating the variability that would be advantageous for survival in a changing environment. Outcrossing rates vary with genotype, experimental design, pollinating agent, and other environmental conditions. Particularly, stress conditions, such as elevated temperatures or drought, may strongly influence outcrossing by affecting pollen viability or increasing flower opening, e.g. in legumes. Generally, outcrossing rates are higher in the wild conspecific taxa than in crop varieties and in the regions of origin of a crop species than elsewhere, for instance, because of lack of optimal pollinating insect species outside of regions of origin.

A few possible limitations with extrapolating the results on outcrossing to implementation of containment measures are discussed. First, often per crop species, only a few genotypes having efficient markers for detecting outcrossing were used as pollen donors. Therefore, variation in siring capabilities between genotypes has been less extensively studied and this is an important aspect with regard to assessment of possibilities for gene flow from a transgenic crop plant to wild relatives. At the other end, the outcrossing potential of the wild relative outside of the research facilities has also been less extensively studied. The second point concerns the actual practice in research situations with newly developed transgenic plants, when one needs to consider that this will take place in enclosures like greenhouses. Under such conditions outcrossing will be less likely for lack of insects and wind to effect pollen transfer, as explicitly stated even for *Phaseolus vulgaris* in the review by Bliss (1980). Thus, these species are treated as selfers in standing multiplication practice in greenhouses. In some cases, like for multiplication of lettuce for germplasm collections, already additional measures may apply to keep free from insects, in this case, aphids because of the need to avoid transfer of viruses (I. Boukema and L. de Groot of CGN, pers. comm.). However, little further evidence on outcrossing rates under greenhouse conditions could be retrieved from the literature and this may need attention in future research.

Introduction

One of the traditionally important elements in assessing the safety of the introduction of transgenic plants in agriculture is the possibility of transgenes moving into wild populations by the natural phenomenon of gene flow. Since it would be difficult to control this process, once a transgene would have introgressed into wild populations, measures apply to prevent gene flow to plants of cross-fertile species in the research phase of newly developed transgenic plants, at which stage knowledge about the effects of the transgene on wild populations still will be limited. In The Netherlands, containment measures are described in the so-called “Lijst van inhullingsverplichtingen” (“inflorescence bagging regulations”), formerly called “Appendix C” , where all species are listed in which transformation research is performed, together with containment actions deemed necessary per species. Containment measures depend on the type of breeding system of the relevant plant species, and the likelihood of contact with cross-fertile relatives in the Dutch flora.

Breeding systems vary widely among Angiosperms. At the one extreme, there are obligate outcrossers, such as cabbage (*Brassica oleracea*), in which selfing is prevented by self-incompatibility factors inhibiting pollen tube growth into stigmas by own pollen. Molecular mechanisms of these so-called S factors have been elucidated for Brassicaceae (cf. review by Kachroo et al. 2002). At the other extreme, there are selfing species, which are the main subject of this report. They are fully self-compatible and moreover have mechanisms promoting predominant self-fertilisation, one of the most effective being cleistogamy, in which pollination occurs within closed flowers. More details about such mechanisms are mentioned under the individual species sections below. Between both extremes, there is range of mixed breeding systems, in which species are self-compatible, but show varying rates of cross-pollination. Cross-pollination rates also depend on environmental factors. In this regard, an important aspect is the pollination vector involved. In the species discussed in this report, these are wind, e.g. for the grasses and cereals, and insects, e.g. for the legumes. Another breeding system influencing the likelihood of gene flow is apomixis, which is present in plant species from diverse families, such as discussed here from Brassicaceae and Gramineae. In apomixis, embryos are developing from unfertilised and mostly unreduced egg cells by several mechanisms that depend on the species involved, thus eliminating a need for pollination to occur, except sometimes for a proper development of the endosperm. More details are discussed under the relevant species sections below.

In the first instance, selfing and apomictic species were regarded as having a negligible likelihood of gene flow by pollen moving out of research facilities. However, with an increasing awareness that selfing plant species rarely are a hundred percent selfing under natural conditions, nor are apomicts likewise a hundred percent apomictic, the Dutch advisory committee on biosafety issues of GMOs (genetically modified organisms)

COGEM started to re-evaluate the species on the containment obligations list (“Lijst van inhullingsverplichtingen” or Appendix C) in 2003. This proved not a simple task, for it was not easy to obtain a complete overview of the literature in this area, which is often spread over many old publications that are not easily accessible. Moreover, there are differences in the amount of information available per species. In addition, plant breeders also often were not able to provide a complete and scientifically validated overview of their experiences with their crop species.

Therefore, the present study describes an attempt to provide the best available overview of the present knowledge of outcrossing frequencies in selfing species on the containment list. The study is based on an exhaustive search of the literature and information requests with experts from the scientific and breeding community, including genebank experts. In the following, the exact approach and some general comments on outcrossing are first detailed, followed by a description of the results per plant species, each grouped per taxonomic plant family. Species discussed are the following: the Brassicaceae *Arabidopsis thaliana*, *Boechera* (*Arabis*) *holboellii, B. stricta* (*A. drummondii*), *Thlaspi caerulescens* and *Thellungiella halophila*, the Fabaceae *Phaseolus vulgaris*, *Pisum sativum* and *Vicia sativa, Linum usitatissimum* of the Linaceae, *Lactuca sativa/serriola* of the Asteraceae and the Poaceae *Poa pratensis*, *Triticum aestivum* and *Hordeum vulgare*. Among these, *Boechera* (*Arabis*) *holboellii* and *Poa pratensis* are known as apomicts, the rest of the species as basically selfers.

Approach

The scientific literature was searched using the following databases:

- Biological Abstracts available for the years 1971-2006
- CAB available for the years 1910-2006
- Current Contents for the year 2006 as most up to date addition to the above

Keywords used were the relevant species names, outcrossing, cross-fertilis(z)ation, breeding system, hybridis(z)ation, allogamy(ous), autogamy(ous), gene flow, (cross-)pollination, sexual reproduction (in case of apomicts).

Additional information from scientifically unrefereed, so-called “grey” literature, was searched in the Wageningen Unversity Library catalogue and the internet search machine Google using the keywords above.

In parallel, experts were consulted as follows:

- Experts at the author’s research institute, Plant Research International, were queried for i) their own knowledge on outcrossing in their species of expertise, ii) names of researchers/breeders outside of PRI that are deemed experts in the relevant species.
- Experts outside of the author’s institute, PRI, who were queried as above, were identified as follows:
    - A request in writing was sent to the Dutch organisation of plant breeders, Plantum NL. Upon this request, information on a number of crop species was obtained by e-mail and a number of experts’ names were provided.
    - Experts’ names were provided by colleagues at PRI as mentioned above.
    - Particularly for non-crop species, additional experts in academia in The Netherlands were traced using the Google search machine on the internet.

Experts were asked to share i) all experience on outcrossing they had with the relevant species, ii) whenever available, unpublished reports or data on outcrossing for the relevant species, and iii) any literature references or other researchers they could advise. These references were matched against what was found by the literature search described above and any additional lead from experts was further followed by tracing additional references from e.g. manuals mentioned or looking for additional publications from researchers named.

General remarks about assessing outcrossing rates

For reasons of clarity, two obvious but sometimes terminologically confused aspects of cross-fertilisation need to be distinguished: firstly, the outcrossing rate occurring in the genotypes receiving the pollen from other plants, and secondly, the effectiveness by which the genotype used as traceable pollen donor fertilises the receptor plant. The second aspect could be circumscribed as “siring capability” of the plant. In the context of this report, the latter aspect, the siring capability, is obviously relevant to plant genotypes used for transformation. The outcrossing rate itself then is naturally most relevant to wild genotypes having a likelihood of receiving pollen from transgenic plants at research facilities. However, often such outcrossing rates have been tested only on cultivated plant varieties, as described under the species sections below.

Especially in the older literature, outcrossing is mainly assessed using a donor variety showing an easily scorable morphological character, such as flower colour, that distinguishes it from the receptor variety and that inherits in a dominant fashion. This means that the character will be shown by all hybrid progeny (F1) that resulted from outcrossing in the receptor variety. To avoid misinterpretation as a consequence of seed admixture of the receptor with the donor, segregation of the progeny from the putative hybrids (F2) is often tested: only those showing the expected ratio of 3:1 of the dominant versus the recessive state of the character (e.g. coloured vs. white flowers) are counted as true products of outcrossing. More recently, also molecular and even transgenic (DNA) markers are used that have the advantage that they can relatively easily be assessed already at the seed or seedling stage of the progeny of the receptor (see Schlötterer 2005 for a recent review). The most efficient marker available is transgenic herbicide tolerance, which can be applied rather simply by spraying thousands of progeny seedlings with the appropriate herbicide and subsequent testing of the survivors on presence of the transgene using a specific PCR reaction.

With all these experimental methods to establish outcrossing rates, one has to take into account that they basically only measure outcrossing of the receptor variety or genotype with a specific donor genotype. This does not necessarily represent the full siring potential of the donor variety, for outcrossing could also occur between plants of the donor variety, but this cannot be assessed for lack of markers. Likewise, siring of plants of the receptor variety by other individuals of the same variety cannot be established, as they are usually similar in genotype and therefore, also lack distinguishing markers. Sometimes, studies assume outcrossing rates within the receptor variety to be similar to those with the donor variety and thus simply double the figure found with the marker from the donor genotype in the receptor variety in order to obtain the total outcrossing rate. However, this needs not be the case, since outcrossing rates were often shown to vary with genotype. For instance, with regard to the flower colour regularly used as marker for outcrossing, insects have been shown to often have a preference for specific flower types and so outcrossing within a variety could be significantly higher than to the receptor variety chosen for the experiment. In a rare example in flax, outcrossing was studied in both directions, that is, also the "donor" variety was tested for outcrossing by looking at a series of F2 progenies for a 1 : 3 occurrence of the recessive flower colour of the "receptor" variety. The outcome was that outcrossing was significantly higher in the "donor" variety (Robinson 1937). So, unless there would be markers distinguishing all plants in the experimental set-up, it rarely will be possible to assess the full outcrossing rate of a specific individual. However, in the practical context of this report, the likelihood of outcrossing with a transgenic pollen donor is most relevant and that is the same situation as studying outcrossing with any other detectable marker originating from a line used as pollen donor in an experimental set-up.

With the advent of molecular markers, there has appeared quite a body of literature on genetic diversity in crop as well as wild populations. These data also contain indirect information on the breeding system of the populations studied. This could be helpful in species where little experimental information is available and may also shed some light on outcrossing capabilities of a species in general, that is, for a larger set of genotypes than often tested in the experimental approach. For extracting indications of breeding system, a few population-genetic parameters generally calculated in these studies are most relevant (for an extensive overview of population-genetic aspects, see Nei 1987):
The observed vs. the expected heterozygosity. The expected heterozygosity is calculated from allelic variation found within a population and represents the relative amount of heterozygotes expected when there would be complete outbreeding between all plants of a population (panmictic population). The actually observed heterozygosity will be the lower the more selfing the plants are, for even the rare heterozygotic products of outcrossing will subsequently disappear by repeated cycles of selfing leading to increased homozygosity in the offspring.
Fst (Wright's statistics) or *Gst* (the latter often used in the analysis of dominant markers, that is, markers in which the heterozygotic state of the character cannot be established directly, as opposed to codominant markers where this is possible in principle): This is a measure of the relative amount of variation in the data that is explained by the variation between populations as compared to the total variation, which includes the variation within the populations. In an outbreeding species, a considerable amount of gene flow will be possible between populations, except when there are serious barriers between the populations. Thus, little difference will exist between populations and so Fst or Gst will be low, mostly in the order of 0-0.2. With selfing populations, likelihood of gene flow between populations is correspondingly low and so, the chances for variability between populations are considerably higher. Therefore, higher values of Fst or Gst are found, generally in the order of 0.5-0.6.

In the following, results will be discussed per plant family and species, respectively. The basic data extracted from the literature are summarised in the table that will be made available separately in an electronic form. All references used for the table are at the end of this report, but will not all be cited in the text below.

Results on outcrossing per family and plant species

Brassicaceae

Arabidopsis thaliana (DC.) Heynh.: thale cress (NL Zandraket)

Arabidopsis thaliana is the most popular Angiosperm model species, both in academia and in crop improvement. Flower development shows protogyny, that is, the female parts mature at an earlier stage than the male parts. Thus, during development, the stigma protrudes through a ring of anthers at which time however no pollen shedding occurs. Later on, anthers extrude and release pollen upon stigma. Therefore, there is a window for outcrossing, which is the period between the stigma extruding from the petals and the moment of dehiscence of the anthers after emergence from the corolla. Flowers were shown to emit terpenes which could function as cues for insect pollinators (Chen et al. 2003). The species is self-compatible, which was associated by Kusaba et al. (2001) with mutational inactivation of self-incompatibility genes (S loci). In line with this supposition, Nasrallah et al. (2004) were able to create self-incompatible *A. thaliana* by transformation with self-incompatibility (S) genes from the close relative *A. lyrata*.

Outcrossing rates vary mainly from 0-2% (Robbelen 1971, Snape & Lawrence 1971), but also significantly higher values were found, up till 10% (Trnena et al. 1987). The latter relatively high outcrossing values in the order of 5-10% were shown to be due to low male fertility in one of the lines used by Trnena et al. (1987). Only one study reported on the relationship of outcrossing with distance from the pollen source: a maximum distance of 0.5 m in Tan et al. (2005).

A most intriguing observation was first published by Bergelson et al. (1998a) and subsequently extended by Bergelson & Purrington (2002): four lines representing separate transformation events, but made from the same ecotype and containing the same construct conferring herbicide tolerance, showed widely varying siring rates on wild type plants from the same ecotype that was used for producing the transgenic lines, i.e. 1.9%, 1.3% (0.8%in second year), 8.6% (10.7% in second year) and 12.4% outcrossing. A control genotype having a herbicide tolerance based on mutation induction produced a lower level of 0.34% on the same receptor. The value of 12.4% outcrossing is the highest reported among the studies scoring outcrossing levels using a donor marker on the progeny.

The involvement of insect pollinators was also reported: most frequently solitary bees, flies and thrips (Hoffmann et al. 2003a). Syrphids were also observed by Bergelson & Purrington (2002) and were experimentally shown to be effective pollinators using a male-sterile line by Drescher & Kranz 1987.

The above outcrossing figures were practically all assessed using inbred laboratory lines, except partly for Tan (2005) who used plants from local natural populations as receptor together with a transgenic Columbia laboratory line as donor. However, with *A. thaliana*, also a lot of studies have appeared on population genetics of accessions or natural populations using various types of molecular markers or gene sequencing. Results from these studies are consistent with a predominant selfing nature of the species: a low observed heterozygosity and about half of the variability is accounted for by inter-population variation (F_{st} or G_{st} in the order of 0.5-0.7). Gene sequence variability (e.g. in *Adh*, alcohol dehydrogenase) is generally lower than in the related outcrossing species *A. lyrata*. In a recent detailed study using 876 sequences across the whole genome on populations from all over the world, Nordborg et al. (2005) showed a population structure in line with a certain amount of outcrossing and gene flow occurring. Generally, it is not possible to infer more exact outcrossing figures from these studies, except for the one by Abbott & Gomes (1989), who deduced a maximum of 0.3% outcrossing in seven natural populations in the UK using a polymorphic isozyme locus.

The species is common in The Netherlands. Among the wild relatives reported to be more or less cross-fertile, such as *A. lyrata*, *A. pumila* and *A. petraea*, only *A. arenosa*, is rarely found in cities and the southern part of the province of Limburg. The only evidence of hybridisation with the latter was found in Mitchell-Olds 2001: in that review, *A. suecica* is being regarded as an allopolyploid from a cross between *A. thaliana* and *A. arenosa*.

In conclusion, in a number of studies low amounts of cross-fertilisation have been established in *A. thaliana*. Higher amounts were found in a genotype with a lowered male fertility, i.e. up till 10%. The occurrence of cross-fertilisation is corroborated by extensive population-genetic studies on natural populations, from which, however, no specific outcrossing rates can be deduced. Most striking with regard to GM biosafety assessments was the observation that lines varying only in the transgenic event, but not in the herbicide tolerance construct that was used in their production, differed in their siring capabilities, leading to more or less higher outcrossing rates in the ecotype tested, up to 12% in field tests. At the time, this observation triggered some discussion on the generality of these small-scale studies (cf. Gray 1998, Arriola 1998), but there was apparently no follow-up research and, as far as could be ascertained, nothing the like was reported for crop species up till now. Therefore, it is hard to draw any firm conclusions on possible causes, whether it concerns any pleiotropic effect from the specific transgenic event or may be a consequence of somaclonal variation induced during the tranformation and regeneration process. It might be useful to study whether this was an isolated observation or that it could be a more generally occurring phenomenon.

Boechera (*Arabis*) *holboellii* (Hornem.) Á. Löve & D. Löve: Holboells rockcress and *B. stricta* (Graham) Al-Shehbaz (*A. drummondii* A. Gray): Drummond's rockcress

Both species have some popularity in research as apomixis models related to the popular model species *A. thaliana*. There has been very little published about gene flow yet, and also experts could add only very little information on the subject. *B. holboellii* is known as a facultative apomict with predominant pseudogamy (Roy & Rieseberg 1989). Among two triploid and one diploid populations, Naumova et al. (2001) found only apomictic seed set, but the diploid population also produced meiotic embryosacs. Using isozymes on three relatively isolated Californian populations, Roy (1993) found indirect evidence for sexual reproduction: a few plants showed higher homozygosity rates than a typical fixed heterozygotic apomict. Moreover, two heterozygotic plants from one of the populations combined isozyme patterns of several of the clonal types from the population. In addition, also diploids were found in another population, and diploids were expected to reproduce sexually. Sexual reproduction is also expected to be the basis of the recurrent origin of apomictic polyploid *B. holboellii.* This was shown indirectly by the combined patterns of variation in chloroplastid DNA that is exclusively transmitted through the female line, and nuclear DNA using SSRs(= microsatellites) (Sharbel & Mitchell-Olds 2001, Dobeš et al. 2004). Further to this, the pseudogamous system implicates the maintenance of pollen production. Thus, pollen could successfully be transported also from strictly apomictic plants to (partially) sexual individuals (cf. under the apomictic *Poa pratensis* below).

B. holboellii is interfertile with *B. stricta*, with the latter more probably being the paternal species in view of the better pollen viability. Their hybridisation product is the species hybrid *B. xdivaricarpa*. The origin of *B. divaricarpa* from repeated hybridisation of the two parental species was corroborated by ITS analysis by Koch et al. (2003) and SSR analysis by Dobeš et al. (2004). *B. stricta* is possibly also apomictic but generally diploid with fertile pollen as opposed to *B. holboellii*. Isozyme and SSR patterns of natural populations are consistent with selfing and rare outcrossing: the proportion of heterozygous loci is only 0.6-3%. In addition, four progenies were shown to be similar to the maternal genotype but manual outcrossing remained completely possible leading to heterozygotic progeny (Roy 1995). Among 15 populations from the Rocky Mountains, an F_{st} of 0.56 was found (Song et al. 2006).

Both species are native to North America and were recently shown to be only distantly related to European *Arabis* species by phylogenetic analysis (cf. Koch et al. 2003). For that reason, they were re-assigned from the genus *Arabis* to the genus *Boechera*.

In conclusion, *B. holboellii* and *B. stricta*, and the hybrid between the two species, *B. xdivaricarpa*, represent a complex case, in that first a distinction must be made between apomictic and sexual reproduction, and subsequently within sexual types, between selfing and outcrossing. This is already not simple in experimental set-ups, but even harder within natural populations using (molecular) markers. Thus, with the few studies discussed, too little data are available to give any indication of possible outcrossing rates. However, both parental species are native to North America, and do not occur in the wild in Europe.

Thlaspi caerulescens J. & C. Presl: alpine pennycress (NL Zinkboerenkers)

Thlaspi caerulescens has gained some popularity in research as heavy metal (Zn, Cd & Ni) tolerance/accumulation model plant that is related to the most popular model plant *A. thaliana* (Assunção et al. 2003). There has been little published about gene flow yet, and also experts could add only very little information on the subject.

The basically selfing nature of the species is corroborated by population-genetic studies on natural populations: Molitor et al. (2005) found higher levels of genetic variation in heavy metal accumulation between populations than within, Koch et al. (1998) also found highest levels of variation between populations across Europe (G_{st} 0.55-0.76). However, the latter authors also found populations with an estimated unexpectedly high level of outcrossing: up to 0.88 in a UK population. Also, Dubois et al. (2003) found a lower value for F_{st} (0.36) among populations from Belgium, Luxembourg and France, than usual for a selfing species, with those from France showing the highest levels of heterozygosity.

Thlaspi caerulescens is found in the southern part of the province of Limburg along the river Geul and more recently also in cities (Rijnmond). Nothing was found in the literature about cross-compatibility with other *Thlaspi* species occurring in The Netherlands.

In conclusion, available data support the inference that *T. caerulescens* is basically a selfing species. However, the data also indicate a possibly reasonably high outcrossing rate occurring in wild populations, but the data are too sparse to name any figures. Together with its occurrence in The Netherlands, this may warrant additional experimental research on the breeding system of *T. caerulescens* and its possible cross-compatibility with wild relatives.

Thellungiella halophila

Thellungiella halophila has gained some popularity in research as an extreme salt- and cold-tolerance model plant, again with the advantage of being related to the most popular model plant *A. thaliana* (Inan et al. 2004). There is very little published about gene flow yet, and also experts could add only very little information on the subject.

The species is native to USA, Canada and China and is found only under extremely high salt conditions. No cross-compatible species are known in The Netherlands.

Fabaceae

Phaseolus vulgaris L.: common bean (NL Boon – bruine boon, snijboon, spercieboon etc.)

The flowers are having stigmas receptive for two days. Pollen is released the evening before flower opening, and as Knuth (1898) already mentioned, pollen becomes first deposited on the style. The pollen is only transferred to the stigma when the flower is agitated by an insect. Otherwise, stigma contact with pollen occurs a short time after opening of the flower by retraction of the stigma into the keel. Thus, there is a window during which outcrossing may take place.

The earliest report on unquantified but high amounts of outcrossing in the species is reported by Müller (1873), mentioning experiments performed by Darwin. There are further quite a number of reports on outcrossing in common bean, most of them based on studies in the USA and Latin America. Outcrossing rates varied widely, from 0-28.5%, but in the majority of cases figures remained below 2%.

Higher figures were mostly attributed to the presence of a higher number of insects, which in particular cases could be illustrated by outcrossing figures varying between different seasons in line with the presence of insects (Brunner & Beaver 1989: eg 16 vs. 40%, Wells et al. 1988: eg 19 vs. 47%). Stoetzer (1984) showed in his experiments that a particular group of insects, carpenter bees (*Xylocopa* spp.) were far more effective than the common honeybee: in an enclosed site, the honeybee brought about only 0.1% outcrossing, in contrast to an average of 4.8% with the local carpenter bees. Carpenter bees were also mentioned in relation to high rates of outcrossing of 15-20% in Puerto Rico (Bliss 1980). Antunes et al. (1973) attributed the relatively high percentages of 6.2-10.6% found in Brazil to thrips (Thysanoptera). Another factor, however, must have been the specific experimental plan: Antunes et al. (1973) and also Wells et al. (1988) had a preponderance of the donor variety as compared to the receptor variety in their set-ups. The most extreme value found, 66.8% by Wells et al. (1988), was found in one line (FM53) that was also aberrant in an extreme variability of outcrossing rates between plants (0-78%). Apparently, one must be aware of the rare occurrence of lines showing exceptional outcrossing behaviour.

The occurrence of male sterility is also reported in common bean, e.g. in cv. “Swedish Brown” (Mutschler & Bliss 1980) and in the induced mutant *do* (dwarf outcrossing), the latter having outcrossing rates of 10-56% (Nagata & Bassett 1984, Brunner & Beaver 1989).

Not always could an explanation be provided for higher outcrossing rates: Barrons (1939) only mentioned that it is apparently higher under his conditions in Alabama (USA). This study also showed the variation with distance: a decline from 8.26% at 1m to 2.63% at 8 m. Alan & Moh (1966) observed 0.20 and 0.19% at 0.50 and 1.00 m, resp., but only 0.05% at 0.25 m, which was attributed to reduced insect visits as a consequence of the very close planting in the latter case.

Molecular marker patterns found in natural populations are generally consistent with a predominant selfing nature (e.g. González et al. 2005). However, clear indications for low levels of introgression from crop forms to wild populations occurring in the species' area of origin in South America were reported by Papa & Gepts (2003) and Hoc et al. (2006).

No wild occurrence is known in The Netherlands (the species and its wild relatives are native to South America), but both *P. vulgaris* and the cross-compatible *P. coccineus* are grown in vegetable gardens. Seed production is limited to the province of Limburg.

In conclusion, the outcrossing rate appears to be quite variable in common bean and may even be high for a species regarded as selfer, depending on specific circumstances. Breeders' experiences in The Netherlands are outcrossing rates of 0-0.1%, max. 0.5%. Stress may lead to higher outcrossing rates by stimulating flowers to open more widely. In seed production, a separation distance of 50 m is applied, but even then sometimes hybridisation proves possible with other types of beans, such as haricots, from vegetable gardens, which can be recognised by aberrant seed colour. As remedy against undesirable cross-pollinations, breeders also mention possibilities of deterring bumblebees with a volatile or decoying them by more attractive *Phaseolus* species. The latter possibility may be a risky one, for the presence of an attractive variation of other flowers may also achieve exactly the opposite: an increase in visits on all plants by facilitation (De Jong & Klinkhamer 2005, cf. Ghazoul 2006).

Pisum sativum L.: pea (Erwt – doperwt, capucijner etc.)

At the start of anthesis, the anthers release pollen inside the still closed flower (Knuth, 1898). Flowers are opening 24 hrs after pollen release, potentially offering a window of opportunity for cross-pollination. Normally, however, fertilization is effected at the flower bud stage and the zygote is dividing or already turned to a two-celled embryo at the time of full flower expansion (Cooper 1938). Insects are not necessary for stimulating seed set by selfing.

In line with the initially cleistogamous behaviour of the flowers, outcrossing rates are usually low and vary mainly between 0-2%. Some variation was found between cultivars. For instance, the white-flowering fodder pea variety 'Edit' showed higher outcrossing rates in the proximity of violet-flowering fodder peas (Dostálová et al. 2005) and fasciated forms tend to have more flowers open at a time, so were more prone to outcrossing, e.g. 2.4% vs. 1.2% on a normal type, as reported in Germany by Loennig (1983).

Like with the common bean, an important environmental factor is the presence of insect pollinators: mainly bumble bees (*Bombus* sp.). For instance, at two locations in Germany differing in pollinator presence of *B. agrorum* and *B. terrestris*, Loennig (1984) found 1.9% with high numbers of bumblebees vs. 1.2% with lower numbers. According to Dostálová et al. (2005), also a damaging pea weevil like *Bruchus pisorum* may cause outcrossing. The only significantly higher outcrossing rates of up till 3.9% found in Peru were ascribed to tropical conditions with the presence of carpenter bees (*Xylocopa* spp.) by Harland (1948), the same type of bees that were reported as important for common bean in Ethiopia by Stoetzer (1984) (see previous section about *P. vulgaris*). Stress like extremely high temperatures may induce outcrossing by precocious flower opening and also pesticide treatment around flowering may do so (Dostálová et al. 2005). Giordano et al. (1991) tested outcrossing with distance increasing inwards from an outer circle of donor plants: 0.24% at 1m, 0.14% at 2 m and 0.12% at 3 m from the donor plants.

No wild occurrence of pea or cross-compatible wild relatives is known in The Netherlands (probably only native to SE Europe and Asia), but it is commonly grown by horticulturalists and in vegetable gardens.

In conclusion, pea is not an exclusive selfer, but outcrossing rates are low and no rates exceeding 5% were reported. Breeders' experiences in The Netherlands are negligible outcrossing rates. Thus there is actually no separation distance applied in seed production, except for a practical 1 m ensuring harvest separation.

Vicia faba L.: faba bean (NL Tuinboon)

In the absence of insect visits or other tactile flower movements, hardly any auto-deposition of pollen occurs (Knuth, 1898). The flowers have longer corolla tubes than is usual in the papilionaceous flower structure. The stigma is receptive before anther dehiscence, but shortly before anther dehiscence, the style is elongating faster than the anther filaments in a way that stylar hairs below the stigma prevent pollen to reach the stigma directly in some genotypes. This separation of female and male function in time and space is known as protogyny in combination with herkogamy (spatial separation of female and male flower function). This flower system promotes outcrossing. Thus, the species shows a variation in the flower structure and mechanics that determines the degree of separation of stigma and pollen released in the keel upon insect tripping. This leads to a variation from complete auto-deposition to actually having a mechanical barrier to autogamy. Autofertile types are producing a stigma tip exudate before opening, other types only after opening or even after having been induced by insect tripping, which will promote allogamy. There is little evidence for the existence of self-incompatibility factors (Bond & Poulsen 1983).

Outcrossing rates are reported to vary between 4-84% (on average around 35%). There is quite some variability between genotypes: for instance, outcrossing ranged from 1-55% among 108 cvs tested at Wageningen, The Netherlands (Metz et al. 1993). Despite the large variability in outcrossing rate between receptors, no influence of the pollen donor on these rates was detected. In a later study, Metz et al. (1994) did also not find any influence from flower colour. In a regression analysis on breeding material, higher outcrossing rates were found to be associated with higher production of inflorescences with fewer flowers. In addition, in *major* cultivars such a relationship was found with few-ovule flowers of smaller size and with a style-ovary angle smaller than 90^0 (Suso et al. 2003).

Like with the other Fabaceae discussed above, an important factor is the presence of insect pollinators: for instance, a comparison between Brittany (France) and Andalucia (Spain) showed a 26 times abundance of the solitary bee *Eucera numida* in the latter area leading to 32 times more positively visited flowers than in France. With positive visits, insects entering the flower from the front was meant, which would be most effective for cross-pollination in contrast to “robbing” where nectar is approached through a hole at the side made by the “robbing” insect. By doing so, no cross-pollination is achieved. In France, the bumblebees and honeybees were relatively scarce and often behaved as robbers. As a result, outcrossing reached a level of 65% in Spain as opposed to 33% in France. Only in France, outcrossing was correlated with the number of insect visits. In another study by Link et al. (1994) on environmental variability, the outcrossing means among European locations varied between 43-74% with a standard deviation among locations of 6%. The authors looked at meteorological effects, but the only variable with a significant effect found was average temperature: the cooler the higher the outcrossing rate.

Nadal et al. (1999 and 2006) tested outcrossing with distance from the pollen source: 16.8% at 1 m, down to 3.0% at 60 m, and down to 0.59% at 184 m in another case, both experiments using small target plots.

Breeders have not been able to find cross-compatible wild relatives and no wild occurrence of *Vicia faba* is known in The Netherlands (it probably originates from Asia), but it is commonly grown by horticulturalists and in vegetable gardens.

In view of the high outcrossing rates, *Vicia faba* cannot be enlisted as a basically selfing species. On the contrary, it is showing a mixed breeding system like oilseed rape (*Brassica napus*), that is, both selfing and outcrossing may occur at varying rates, depending on circumstances, but no self-incompatibility factors are involved. In line with the high level of outcrossing, 200 m isolation distance is recommended in seed production. Breeders’ experiences in The Netherlands are that abiding by such isolation measures, there are no problems with outcrossing.

Linaceae

Linum usitatissimum L.: flax, linseed (NL Vlas)

At flower opening, stamens bend inwards discharging pollen on the stigmas, but there is a brief period when there is a possibility of cross-pollination due to room present between anthers and stigma. In a few varieties, the stigma tip extends above anthers for a while increasing the chance of cross-pollination. Flowers are usually pollinated in the first day of opening and soon afterwards shed the petals thus diminishing attractivity for pollinators; under poor weather conditions, flowers may last an extra day, which may also promote outcrossing (Dillman 1938, Gill 1987).

Outcrossing rates reported vary mainly between 0-3%, occasionally up to 5%. The highest rate of 4.9% was found with plants mixed closely together in the same row by Gürbüz (1999). Outcrossing was shown to depend on flower type: large disc flowers have higher rates than the funnel-shaped types and the more rarely occurring tubular forms have the lowest rates (e.g. 0.3% instead of 1.9% for the disc flower type, Dillman 1938). Disc flowers are also the better pollen providers when used with a male-sterile line (Keijzer & Metz 1993). There are also differences between blue and white flowered cultivars. In an experiment with blue-flowered and white-flowered lines, Robinson (1937) tested outcrossing in the progeny from both types: average outcrossing in blue-flowered types from white-flowered ones was 2.5% and the other way round 3.6%. Male-sterile lines have also been described (Gill 1987, Keijzer & Metz 1993).

Insects are important in pollination of flax. Wind is an unlikely vector: pollen is heavy and very few were caught in experiments with sticky glass slides (Gubin 1945). Outcrossing is reported by bees, bumblebees, flies and butterflies; involvement of thrips is controversial, e.g. Henry & Tu (1928) proposed a role for thrips whereas Dillman (1938) very much doubted this (for further discussion refer to the concluding remarks at the end of this report). Seed set is sometimes reduced when insects are excluded by caging (e.g. from 35 to 4 % in Russian experiments), which may be due to insect movement of pollen making up for poor pollen vitality under hot conditions, for the effect is not found under temperate conditions in Germany and the USA (Williams 1988). Hot weather leading to a decrease in pollen vitality could also account for regional differences found between Oregon and Michigan. Higher outcrossing rates were attributed to the cooler and more humid climate of Michigan by Robinson (1937).

Henry & Tu (1928) also tested outcrossing with distance: 1.26% at 0.3 m (1 ft) diminishing to 0.33% at 1.5 m (5 ft).

High levels of heterozygosity reported in Swedish flax using isozymes raised questions about the selfing nature of flax (Månsby et al. 2000). However, these findings were challenged by attribution to misinterpretation of isozyme gels: apparently alleles from various loci were scored as deriving from one and the same locus (Van Treuren, CGN, pers. comm.)

Flax is sometimes found as escape from cultivation in disturbed road sides in The Netherlands. The largest amount of cultivation is found in the region of Zeeuws-Vlaanderen in the extreme SW of the country. No cross-compatible wild relative is known in The Netherlands. Flax is cross-compatible with a series of wild species that also have a chromosome complement of 2n=30. However, European species among them, such as *L. bienne* and *L. tenue*, have a more southern occurrence.

In conclusion, *L. usitatissimum* is not a completely selfing species, but outcrossing figures are generally not higher than 5%. Breeders' experiences in The Netherlands are that outcrossing is low for lack of insects at seed multiplication sites.

Poaceae

Poa pratensis L.: meadow grass (UK) or Kentucky bluegrass (USA) (NL Veldbeemdgras)

The species is known as a facultative apomict. The apomixis is of the pseudogamous aposporic type. Thus, pollination is necessary for parthenogenetic embryo development, for endosperm is developing only after fertilisation. Therefore, pollination is involved in all reproduction and so, also strict apomictic genotypes may produce pollen in sufficient amounts to be able to fertilise sexual types in their immediate surroundings. Completely sexual types lacking dominant apomixis alleles are rare and they are often growing poorer than apomictic types (Hintzen & Van Wijk 1985). In a recent model, genetics of apomixis could be explained by a five-locus model (Matzk et al. 2005). Parental lines with a higher rate of sexual reproduction are used in breeding cultivars. In sexual reproduction, not only normal diploid but also triploid embryos can be formed, the latter by a combination of an unreduced 2n egg cell and a haploid sperm cell. Triploid progeny is subsequently capable of reproduction through the apomictic mode, in turn enabling all sorts of hybrid swarms to persist.

It is possible to induce sexual reproduction by heat treatment, e.g. by applying high night temperatures and air humidity (cf. Han & Funk 1968). Apomictic reproduction is often associated with chromosome number, which is highly variable, with both polyploids and aneuploids occurring. However, a relationship with chromosome number is not found in all studies (e.g. Gröber et al. 1978 found no significant relationship).

Cultivars are variable in their level of apomixis. Percentages for specific cultivars could be found in USA registrations of cultivars published in the journal Crop Science, e.g. Rugby II averages 85%, but generally levels of 93-95% apomictic are reported, with a range of 90-99% (Douglas-Bede et al. 2001). However, levels of apomixis were not reported systematically in the published registrations, e.g. no data were mentioned by Bonos et al. (2004).

Apomictic frequencies used to be laborious to assess by studying ovule development. However, more recently, a reasonable estimation could be made using an auxin test but this could underestimate frequencies found in the progeny (Mazzucato et al. 1996). Also molecular markers were shown to be effective to identify progeny from sexual reproduction, such as SCAR markers for two genes involved in apomictic development, *Parth1* and *Sex1* (Albertini et al. 2001), and seed protein electrophoresis or RAPDs. However, tests were performed on little material from manual crosses. In this way, Barcaccia et al. 1997 could show by performing crosses without emasculation that completely sexual types were completely outbreeding, whereas with the facultative apomicts only part of the aberrant progeny could be attributed to outcrossing. Wu et al. 1984 and Wu & Jampates 1986 used isozymes for cultivar identification and so could indirectly give indications of sexual reproduction: seed lots contained 2-15% of variants, in one case even up to 63%. However, in this case, seed admixtures could not be excluded as a cause of the occurrence of variants.

Only very recently, a first test of outcrossing under field conditions using transgenic herbicide resistance as an efficient marker for hybridisation was published by Johnson et al. (2006). With a highly apomictic line as pollen donor, they found overall outcrossing rates in facultatively apomictic accessions of 0-15.8% depending on cultivar and year. Outcrossing diminished with distance to 0-0.13% at 53 m, but there were too few scores to fit a mathematical function on the data.

Poa pratensis is very common in The Netherlands in grasslands and road sides. The distribution of one other recently recognised species of the complex, *P. angustifolia*, is not yet completely known. Johnson et al. (2006) detected hybridisation with *P. angustifolia*, but not with other species of the Dutch flora, *P. annua*, *P. trivialis*, *P. compressa*, *P. nemoralis*, although the latter was reported previously to hybridise with *P.pratensis* in the USA.

In conclusion, as a facultative apomict, *P. pratensis* may show sexual reproduction also in cultivars selected for apomixis, normally at a rate of 1 to at most, 10%. Scanty evidence indicated that sexual reproduction mostly follows an outbreeding mode. Recently published evidence shows that outcrossing at a short distance, using a highly apomictic pollen donor can amount to 15%, but this has only been tested on the progeny of relatively few plants per accession (18 each from 6 accessions, including the closely related *P. angustifolia*) (Johnson et al. 2006). This confirms that with the pseudogamous apogamy characteristic for *P. pratensis,* even a highly apomictic genotype can be an effective source of pollen for outcrossing on compatible sexual individuals.

Triticum aestivum L.: wheat (NL Tarwe)

Flowering starts in the spikelets located in the middle of the spike and part of the florets in the spikelets at both ends of the spike are often not setting seed. Part of the florets are cleistogamous, but chasmogamous (open) florets mostly occur in the spikelets on the upper half of the spike. Within these spikelets, only the basal florets are chasmogamous and the remaining florets cleistogamous, except sometimes again the uppermost florets. Despite the partial cleistogamy, 30-80% of the total pollen produced by a spike is usually shed outside of florets. Flowering lasts for 2-6 days and is often taking place in two rounds. In the second round unfertilised florets open again for 2-3 days and in the latter case the stigma may protrude outside of the floret (Waines & Hegde 2003).

Outcrossing was reported as usually less than 1%, but may reach up till 10.6%. The high figures were found in experimental set-ups where the receptor plants are outnumbered by the donors having the hybridisation marker used (e.g. Hucl 1996: up till 6%) or have been sown at high density in large numbers, such as in the four-cultivar mixes used in farmers' fields in France (Belhaj Fraj et al. 2003: 1.3-6.3%). The highest value of 10.6% was attained by bagging spikes of donor and receptor plant together in a greenhouse cultivation. In the context of GMO biosafety research, the experimentators hoped to achieve a sort of efficient "worst case" scenario for assessing the highest gene flow possible. This method did, however, not have a high predictive value for findings in the field (Lawrie et al. 2006).

There is also variation between cultivars in the laxness of their spikes and the consequent degree of opening of florets, and also of the degree of anther extrusion. Related to such variation, for instance, Canadian spring cultivars were shown to vary in outcrossing rates from 0-6.7% (Hucl 1996). Waines & Hegde (2003) quote a report from 1917 on a cv. Fulcaster that at one time showed an outcrossing rate of 34%. Such a high figure may be attributed to male sterility, which has been found more often. Problems with off-types and production loss in the cultivar Moulin in the 1980s were apparently caused by environmentally induced male sterility, probably by a combination of low temperatures and light intensity at the time of meiosis. Breeders have also introduced male-sterility from other species, such as *T. thimofeevi*. On such male-sterile lines, seed settings of 40-70% can be achieved, but this drops off quickly with distance (cf. De Vries 1974, Khan et al. 1973).

Since wheat pollen is quite heavy, outcrossing also quickly diminishes with distance on normally male-fertile varieties. By pollen trapping, Khan (1973) showed that 90% of pollen did not reach farther than 6 m, and that there was a fast decrease after 3 m with a maximum distance recorded as far as 60 m; pollen numbers assessed by trapping correlated significantly with seed set on male-steriles. Thus, Hucl & Matus-Cádiz 2001 and Hanson et al. 2005 found zero outcrossing beyond 30 m on normal varieties. On the other hand, Matus-Cádiz et al. 2004 found 0.01% at 60-100 m, and an occasional observation at 300 m downwind.

The stigma remains receptive for 2-13 days, but is best on day 3-4. This period can be longer under moist and cool conditions, but then there are also more cleistogamous flowers. Matus-Cádiz et al. 2004 associated low outcrossing rates downwind in one year with the hotter and dryer weather, which may have desiccated the pollen faster. Under stress there is generally more pollen scattering, as was also observed in the Fabaceae (common bean, see under that section above) (Waines & Hegde 2003).

There is practically no feral occurrence of wheat in The Netherlands and also no cross-fertile wild relatives, such as representatives of the genus *Aegilops* elsewhere in Europe (cf. Zaharieva & Monneveux 2006).

In conclusion, wheat usually shows a low outcrossing rate of $\leq$1%. However, varieties with significantly higher rates occur, up till around 10%, depending on inflorescence characteristics and local wind conditions. Breeders' experiences are that there are usually no isolation distances necessary for seed production, or 3-10 m at most for pedigreed seed, which are for instance, also the directives in Canadian seed production (Hucl & Matus-Cádiz 2001).

Hordeum vulgare (barley)

The flowers basically develop cleistogamously, that is, pollination occurs before flower opening and even while the spike is still enclosed by the sheath. However, like in wheat, there is variation in the degree of flower opening and anther extrusion. In barley, this variation is wider: in an extreme case, Robertson & Deming (1931) reported for a winter line with the rather intriguing name "*Hordeum deficiens nudideficiens*"in a given year an outcrossing rate of 20.7%. The high outcrossing rate was ascribed to the florets staying open for several days after fully heading. Moreover, winter barleys are known to systematically have higher outcrossing rates than spring types (Doll 1987).

Outcrossing rates for spring barley vary mainly between 0.35-2%. The higher levels in winter barley are mostly in the range of 2.5% to up to 12.5%. Higher anther extrusion is seen as the most probable cause for the higher outcrossing rates of winter type barley. Abdel-Ghani et al. (2005) hypothesise that a functional explanation for this is that spring barley is most likely to face drought during anthesis and therefore it is advantageous to have cleistogamous flowering. Stamen protrusion also generally becomes less with drought.

Pollination on male-sterile types reached values in barley of e.g. 64-92% as reported by Gorastev & Popova (1977). Ritala et al. (2002) studied outcrossing with distance in Finland on male-steriles and normal types using a transgenic herbicide tolerance marker. On the male-sterile plants, 100% outcrossing was achieved at 1 m, which dropped to 3% at 50 m, at which distance the rest of the fastly diminishing absolute number of seeds was derived from background pollen. Male-fertile plants were only tested at 1 m from the transgenic pollinator and showed rates between 0-7% depending on wind direction. 7% Outcrossing is rather high for a spring type and is perhaps related to the northerly climatic conditions. Abdel-Ghani et al. (2004) and Brown et al. 1978 showed a significant positive relationship of outcrossing rates with moisture (precipitation rates) and a negative relationship with high temperatures.

Molecular marker studies on wild populations in the Middle East are in line with a selfing nature for barley, e.g. a low observed heterozygosity of 0.037 in Brown et al. 1978).

There is no wild occurrence of barley in The Netherlands, and also no cross-fertile wild relative, that is, no fertile progeny could be produced by crossing with *H. murinum, H. marinum, H. secalinum* or *H. jubatum* (Eastham & Sweet 2002)

In conclusion, barley shows outcrossing values contrasting between spring and winter type. Whereas the spring type has low rates and will pose low likelihoods as pollen donor with its mostly cleistogamous flowers, the winter type shows higher rates up till 12.5%. Thus, according to breeders' experience, spring barley does not need any specific isolation measure in seed production, but winter barley does so with a recommended distance of 250 m.

Asteraceae

Lactuca sativa L.: lettuce (NL Sla)

Flowers are protandrous and opening for a short period early in the morning. The style pushes out the pollen from inside the tubularly fused ring of anthers that have dehisced inwards, and when about 2 mm above the anther tube, the stigma branches curl back upon themselves taking up pollen from the sides of the style. Pollen also becomes available for insect visitors as soon as the style extrudes from the anther tube and can then in principle be transferred to other flowers.

Outcrossing rates were reported to vary between 1.3-7.4% at a close range, that is just enough to keep the inflorescences separate (Thompson 1933, Thompson et al. 1958). In The Netherlands, an average of 1.3% was found in a field trial in the EU project "ANGEL" representing a "worst case" scenario of gene flow between crop and wild forms, that is, a wild receptor plant surrounded by several potential pollen donors of the crop (Hooftman et al. in prep.).

A highest average outcrossing rate was found in Switzerland by D'Andrea et al. (in prep.) also in the framework of the EU project "ANGEL", specifically on drought-stressed plants during a warm summer period in 2003 and therefore, was attributed to poor pollen production by the receptor plants. However, another factor could be insect activity: average outcrossing was significantly lower during a more rainy period, when, not only flowers were observed to open poorly, but insect activity was also low. Nectar production has not been shown for lettuce, but in most studies, insects, such as syrphids and bees, have been observed on the flowers and will most likely be the main pollen vectors.

Lettuce's close wild relative *L. serriola* L. (prickly lettuce, NL Kompassla) is very common in The Netherlands and most of Europe, and to all practical purposes could be considered conspecific with *L. sativa*, as it is freely crossable with the cultivated form (De Vries 1990).

In conclusion, relatively few studies have appeared on outbreeding in lettuce. They indicate that levels of 1-5% are normal both within *L. sativa* and *L. serriola* with *L. sativa* as pollen source. Breeders' experiences are that outcrossing in isolated seed production areas in California and Australia only take place when insects are present and generally remains below 1%, but there are no publications about this. In a small experimental set-up using blackflies as pollinators, outcrossing was not more than 0.1%. However, in view of the insect visitors found in the field (mainly syrphids), it is questionable whether the optimal pollinator was used.

Helianthus annuus (sunflower)

A quick literature scan indicated that sunflower is basically an outbreeder. The wild progenitor is self-incompatible, which has only been partially overcome during the development of crop variants. Modern inbred material shows a selfing rate of between 80 and 100% (cf. Gandhi et al. 2005). In The Netherlands, sunflower only occasionally emerges from seed spillage, e.g. at river beaches or on sparsely vegetated dunes.

Concluding remarks

Not unexpectedly, all purportedly selfing species discussed in this report were shown to be perfectly capable of outcrossing. This has a certain evolutionary logic, since a low rate of outcrossing would sufficiently contribute to creating the variability that would be advantageous for survival in a changing environment. Outcrossing rates vary with genotype, experimental design, pollinating agent, and other environmental conditions. Generally, outcrossing rates are higher in the wild conspecific taxa than in crop races and in the regions of origin of a crop species than elsewhere, the latter for instance, because of lack of optimal pollinating insect species outside of regions of origin.

The species considered in this study also widely vary in the rates of outcrossing found on average. At one extreme, *Vicia faba* effectively shows a mixed mating breeding system, that is, it is completely self-compatible, and has a flowering system favourable to outcrossing. Therefore, a considerable and varying amount of outcrossing is found, comparable to e.g. *Brassica napus*, which was not treated in this report because of the already extensive body of knowledge on that species. At the other extreme, pea (*Pisum sativum)* shows a selfing system with basically cleistogamous flowers and so only a limited amount of outcrossing, usually considerably lower than 5%. Between these extremes, each species shows its own characteristics and variability in outcrossing rates. For instance, barley shows a distinction between its winter and summer types, with the winter types showing the highest outbreeding. The purportedly selfing species that most obviously deviates from this selfing nature is the common bean *Phaseolus vulgaris*. Quite some publications reported outcrossing rates significantly higher than 5%, depending on genotype, but also on environmental conditions. Apparently, the species may outcross more frequently under conditions of high insect pressure and a warmer climate like in its area of origin in South and Middle America. However, under Dutch conditions, outcrossing was considered to be low by breeders, that is, in the order of maximally 0.05% using a separation distance of 50 m, even though that still poses a need for attention in seed production to off-types. Moreover, outcrossings from genotypes lacking obvious morphological differences will be easily missed in seed production practice. It must be concluded that each crop has to be considered by itself for decisions on containment measures.

For species of the families Fabaceae, Linaceae and Asteraceae, insects were an important factor in effecting cross-pollination. Insect species vary, a.o. depending on crop species, e.g. Syrphids appeared most frequently on lettuce and bumble bees more frequently on the Fabaceae discussed here. There is also some discussion about effectiveness of specific insects for pollination, such as honeybee on bean or flax. Most discussion was about a role of thrips in pollination. Juveniles can already be present in closed flowers of bean or flax thereby being the first to export pollen when leaving the flowers upon opening (cf. Antunes et al. 1973 or Mackie & Smith 1935, Henry & Tu 1928, resp.). However, Dillman (1938) doubted the role of thrips in pollination. Park et al. (1996) trapped thrips with pollen on their bodies, but could not establish pollen transfer. Recent reviews (e.g. Mound 2005) confirm a role for thrips in effecting pollination in such diverse plant families as Cycads, Moraceae and Annonaceae. On the other hand, also for other Fabaceae than the ones discussed in this report, conflicting views are found in recent literature (cf. Anathakrishan (1993) in favour of a positive role vs. Raju & Purnachandra Rao (2002) regarding thrips as mere foragers.

There are a few possible limitations with extrapolating the results on outcrossing to implementation. First, adamount to the present assessment is the capacity to act as pollen donor of the cultivar or genotype to be used in transformation. However, the focus of most studies is the outcrossing on the cultivars acting as pollen receptors. Since for most of the species, a reasonable number of publications have appeared, one could assume that a representative picture will have emerged. Nevertheless, it has to be taken into account that often a limited number of donor types have been used that have attractive markers to assess hybridisation in the progeny, such as a blue aleurone layer in the seeds of wheat or dominant flower colours in Fabaceae and *L. usitatissimum*. For *L. usitatissimum,* the reciprocal study by Robinson (1937) showed higher outcrossing rates with the white-flowered variety acting as donor for the blue-flowered variety than the other way around. On the other hand, Metz et al. (1993) in their extensive study on faba bean did not find a clear influence from the pollen donor, whereas there were significant differences between receptors. With molecular markers or the introduction of transgenes, basically all varieties become available as donors in gene flow research. In this regard, observations in transgenic *Arabidopsis thaliana* become most relevant, namely the considerably varying rates of outcrossing (up till 12%) found between different *Arabidopsis* lines produced by separate transformation events with the same transgenic construct (Bergelson et al. 1998), the only difference between the lines expected thus being varying insertion sites for the transgene. No clear explanation for these differences in outcrossing rate has been put forward and as far as could be ascertained. After some debate about the generality of the conclusions from these small-scale experiments, no follow-up to this research has been published either, as far as could be ascertained.

The second point concerns the actual practice in research situations with newly developed transgenic plants, when one needs to consider that this will take place in enclosures like greenhouses. In some cases, like for multiplication of lettuce for germplasm collections, already additional measures may apply to keep free from aphids in order to avoid transfer of viruses (I. Boukema and L. de Groot of CGN, pers. comm.). Under such conditions outcrossing will be less likely for lack of insects and wind to effect pollen transfer, as explicitly stated even for *Phaseolus vulgaris* in the review by Bliss (1980). Consequently, it is treated as such in standing breeding practice. However, little further evidence for this position could be retrieved from the literature and this may need attention in future research. An indication for the possibility of outcrossing in the greenhouse for lettuce may come from the rare occurrence (in the order of 0.1%) of highly heterozygotic individuals in expectedly homogeneous cultivars in a large molecular marker data set on the CGN collection. This may be attributed to outcrossing during seed multiplication in the greenhouse before uptake of the cultivar into the collection (Van Treuren, pers. comm.). On the other hand, Haskell & Paterson (1966) did not find any outcrossing in a greenhouse experiment in Scotland with tomato, which also is known as a basically selfing species. Recently, Watanabe et al. (2006) studied the issue with the highly outcrossing, wind-pollinated maize: they showed that covering a pipe-frame greenhouse frame with a 1-mm nylon fine mesh could reduce siring of plants outside by 77% at close range, but the reduction at larger distances was less effective.

A few species remain for which only little data on outcrossing could be extracted from both the literature and experts, and these mostly concern the apomicts: *Poa pratensis*, *Boechera* (*Arabis*) *holboellii* and *B. stricta* (*A. drummondii*), but also *Thlaspi caerulescens*, and*Thellungiella halophila*. *T. halophila* does not occur in The Netherlands and is restricted to extremely salty habitats. Likewise, though locally occurring in The Netherlands, *T. caerulescens* is restricted to habitats containing high levels of heavy metals, such as Zinc.

B. holboellii and *B. stricta* are a special case as they make up an evolutionary complex with polyploid apomictic forms in both *B. holboellii* and the hybrid between the two species, *B. xdivaricarpa*. Most likely, sexual forms in *B. stricta*, where they are apparently the norm, and *B. holboellii* are basically selfing, but it is difficult to discern any amount of outcrossing. However, both basic species are native to North America. However, the other apomictic species, *Poa pratensis,* is a common component of grasslands in The Netherlands. Like in the above North-American species, sexual forms occur, and although not easy to ascertain, these are most probably for the greater part outcrossing. Only very recently, a field experiment was published indicating low rates of outcrossing within the species and to a few relatives using a transgene as marker, but this was performed on a limited number of plants per species and accession (Johnson et al. 2006).

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Glossary

Allogamy	Outcrossing breeding system
Autogamy	Selfing breeding system
CGN	Centre for Genetic resources, The Netherlands, the Dutch genebank
Chasmogamy	Pollination taking place with flowers opened
Cleistogamy	Pollination taking place in closed flower buds
Endosperm	Where present, tissue providing food reserves for the embryo in seeds, arising from fertilisation of maternal nuclei in the ovule (predecessor of the seed) by one of the two sperm nuclei from the pollen tube, the other fertilising the egg cell leading to embryo formation
Herkogamy	Pollination system with a spatial separation of female and male flower function
Isozymes	Enzymes occurring in several isoforms, encoded by the same locus, that can be detected by electrophoresis followed by an enzyme-specific staining reaction; as the isoforms are supposedly not subject to selection, their variation is regarded as representative of “neutral” genetic variation in the populations studied
ITS	Internal Transcribed Spacer, non-coding part of the repetitive sequences encoding ribosomal RNA, the sequence variation of which is often used for reconstructing related species phylogeny, this variation can rather efficiently be accessed by PCR using universal primers based on highly conserved sequence parts of the ribosomal RNAs themselves
PCR	Polymerase Chain Reaction, a molecular-biological method in vitro amplifying specific DNA sequences targeted by so-called primers recognising the ends of the desired sequence
Protandry	Flowers having male parts maturing before female parts
Protogyny	Flowers having female parts maturing before male parts
RAPD	Random Amplified Polymorphic DNA, multilocus molecular marker method scoring presence or absence of specific PCR products, relatively easy in application by using random primers and separation of PCR products by agarose gel electrophoresis
SCAR	Sequence Characterised Amplified Region, molecular marker showing either presence or absence of a PCR product by using specific primers usually developed by sequencing RAPD-based polymorphisms
SSR	Short or Simple Sequence Repeats (also called microsatellites), repeats of 1-8 basepairs (eg dinucleotide (GA)n or trinucleotide (TCT)n occurring regularly throughout eukaryotic genomes that are often highly variable in number of repeat units between alleles; they can be scored by sizing PCR-amplified loci; since basically every allele can be positively identified, heterozygotes can be detected