Project Report

Impacts of banning protoplast fusion on the range of varieties available for organic arable cropping and vegetable production

Report as part of the COOP project on "Safeguarding organic seed and planting material – Impulses for organic plant breeding"

Module 1.3

Bettina Billmann
in co-operation with Christine Arncken, Martin Koller, Bernadette Oehen and Andreas Thommen

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Research Institute of Organic Agriculture (FiBL), Ackerstrasse, CH-5070 Frick, Switzerland,
Tel. +41 (0)62 865 72 72, Fax +41 (0)62 865 72 73, info.suisse@fibl.org, www.fibl.org

Contents

1. Summary, Conclusions and Recommendations

What is protoplast fusion (PF)?

It is the fusion of two cells without cell walls, with or without the cell nucleus, in a vegetative state by means of chemical or electrical impulses. From a technical point of view this method does not fall directly within the scope of genetic engineering, as the new combination of genetic material does not take place at the DNA level.

Where is protoplast fusion used?

In practice, PF is currently used only to breed cytoplasmic male sterility (CMS) into other plants in hybrid breeding, in species where this does not occur naturally. The technique is used for Brassica species, for forcing chicory, and in the near future probably also for rapeseed. This technique does not come into play for any of the other species with hybrid varieties (naturally occurring CMS: carrots, onions, leeks; “manual castration”: tomatoes, cucumbers; self-incompatibility system: Brassica species without CMS). Research is also being done on crossings with wild species of potatoes and sunflowers with a view to introducing resistance traits. Commercially only a few varieties of Brassica species are used that are based on protoplast fusion.

What is problematic about this and why is it being debated?

For many people, protoplast fusion is very similar to genetic engineering. The bulk of the criticism is directed towards the bypassing of the generative phase of hereditary transmission and the new combination of cytoplasm that in nature only takes place inside a plant. The organic associations *Demeter International, Naturland* (Germany) and *Gäa-Nordwest* (Germany) have banned varieties which have been bred based on protoplast fusion.

Who would be affected by a ban on protoplast fusion?

A complete ban would primarily have repercussions for organic producers in terms of crop profitability (especially in cauliflower and broccoli), seed availability, and access to breeding successes.

What recommendations have come out of this research?

According to current legal provisions protoplast fusion is not considered genetic engineering if it takes place in plant cells and if a new combination of the genetic material could also occur by natural means. Based on the information at hand, the above-mentioned cabbage species have thus not been produced by way of genetic engineering. With the exception of these cabbage types, the number of PF-free varieties of modern crop plants which lend themselves to further development is still sufficient, so that the organic sector could probably continue to be supplied with good varieties reflecting the current state of breeding. The most should be made of this opportunity and breeders should be given incentives to continue breeding PF-free varieties.

Therefore, the recommendation is that the organic agriculture sector further develop conventional varieties to the extent possible and state that protoplast fusion as a way of newly combining genetic traits is undesirable as a matter of principle. However, a ban is not considered realistic because of the relative insignificance of protoplast fusion as a breeding method and the economic repercussions associated with monitoring. Cabbage producers are advised to take orientation from the annually updated blacklist produced by the German *Demeter* Association and to voluntarily refrain from the use of CMS varieties.

2. Introduction and Problem Definition

The use of protoplast fusion in organic farming has been a topic of much debate of late. This breeding method is often seen very critically and is at times even labelled "lesser genetic engineering". The reason is that it combines genetic material under circumstances which would not occur in nature. Similar to genetic engineering there are concerns that plants may be produced which lack vitality in the long-term and which may be environmentally incompatible or even harmful.

The *Demeter International* producer association prohibited the use of protoplast fusion-based or cytoplast fusion-based CMS hybrid varieties as early as July 2005. In March 2007 the German producer association *GÄA-Nordwest* followed suit. Both prior to these bans and building on them there have been discussions amongst growers in other associations on whether to adopt the same path, such as for example the resolution of Hessian growers of November 2004.[1]

This reports aims to provide a decision-making basis for the BIO SUISSE advisory commission on vegetable production by presenting detailed information on the status quo. The debate does not concern plants derived from organic plant breeding – as set out in the IFOAM Plant Breeding Draft Standards this should be carried out without resorting to any "artificial" methods. The controversial point is rather whether conventionally bred varieties which are derived from protoplast fusion or cytoplast fusion should continue to be permitted in organic farming.

The topic of protoplast fusion is very complex and some information is unfortunately not publicly available. The information contained in this report has been carefully researched. However, no guarantees can be given as to the accuracy of the information. If despite all caution, checking and consultation there should be any errors in this report, the authors would much appreciate feedback.

3. Materials and Methods

The statements made in this expert opinion are made on the basis of a comprehensive literature review and an examination of relevant legal texts. Furthermore, interviews were held with experts and breeders on specific topics (see list of experts in the Appendix).

[1] Fischbach 2005 I

4. Introduction to Protoplast Fusion Technology

4.1 What are protoplast fusion and cytoplast fusion[2]?

In FiBL Dossier No. 2 *Techniken der Pflanzenzüchtung* (Plant breeding techniques) protoplast fusion is described as follows:

> "Protoplasts are plant cells which have lost their cell wall. They are generated by treating parts of leaves with enzymes which digest the cell wall. The protoplasts form new cell walls and divide, forming a callus and later a plant. Chemical or electrical stimuli can be used to stimulate protoplasts from different plant species to fuse (somatic hybridization). In the course of the fusion the organelles of both plants (chloroplasts and mitochondria) combine while during crossing the descendants only receive the maternal chloroplasts and mitochondria. The tetraploid plant resulting from this fusion shows the characteristics of both parental plants. During regeneration, the chromosomes and organelles of parents can become mixed, resulting in many new combinations."
> (FiBL 2001, p. 13)

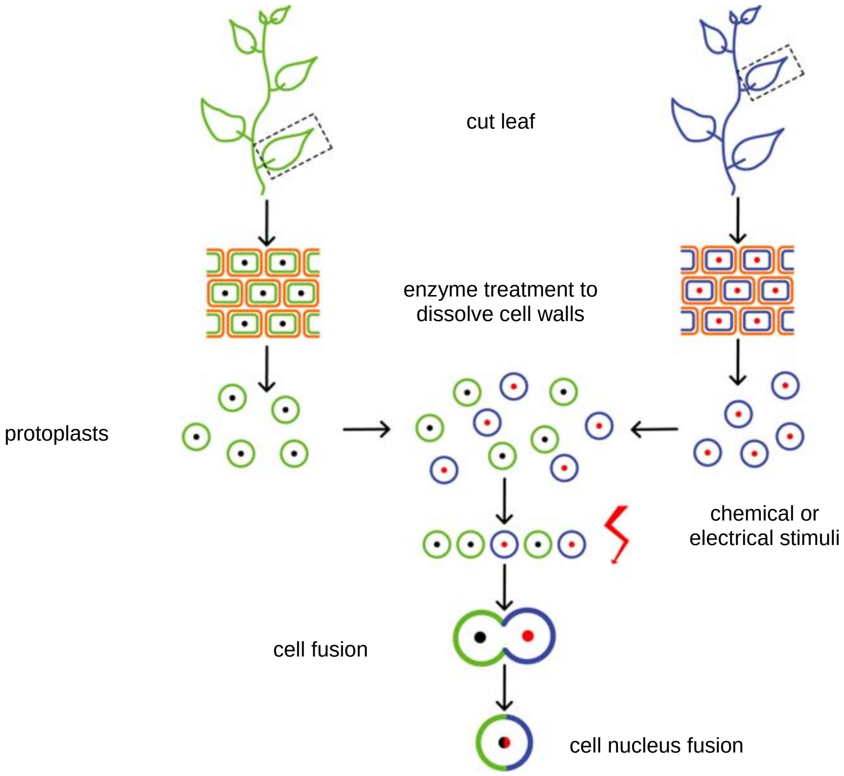

Fig. 1 **Procedure for protoplast fusion**

[2] Unless explicitly stated otherwise these two terms will be used synonymously in this report for linguistic ease and are at times abbreviated PF.

Cytoplast fusion is described as follows:

"In order to avoid the exchange of chromosomes the protoplasts can be treated in such a way that the nucleus is removed or fragmented. These so-called cytoplasts contain the organelles but no chromosomes from the donor plant (parent plant). In this way one can, for example, transfer CMS (cytoplasmic male sterility) into another plant. Companies involved in such work have documented the changes in mitochondrial DNA resulting from these procedures in detail and have patented the relevant techniques."
(FiBL 2001, p. 13)

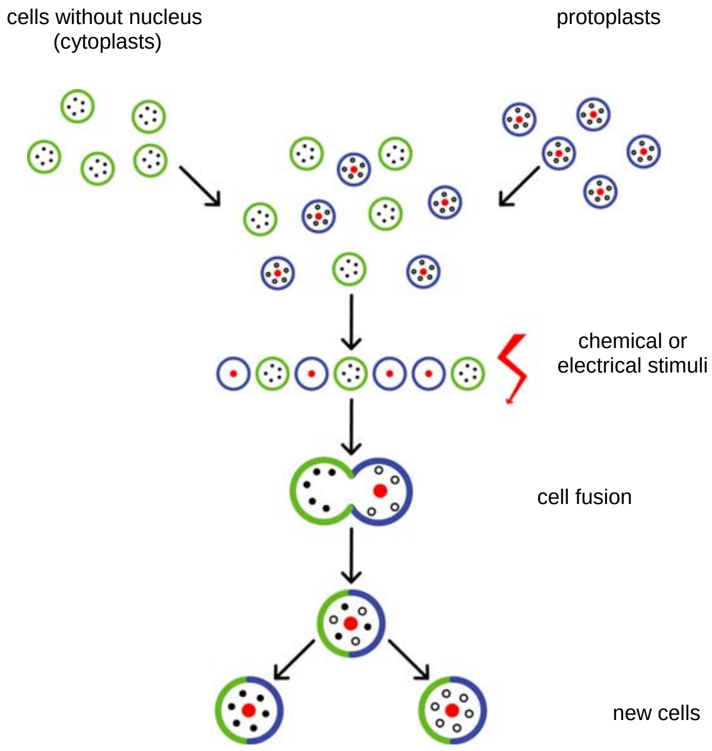

Fig. 2 Procedure for cytoplast fusion

A special type of protoplast fusion is *asymmetric fusion*:

If the aim of protoplast fusion is, as in backcrossing, to only transfer one gene from the donor, then the donor cells are irradiated; this destroys the bulk of the genetic material. Fusion then results in cells which only contain a small proportion of functioning donor genes; these are termed asymmetric hybrids.

(BECKER 1993, p.188)

It must be noted that protoplast fusion is carried out on the basis of vegetative plant cells. Therefore this technology can not directly be assigned to the field of genetic engineering where the new combination of genetic material is performed at DNA level. However, this method must be subjected to critical examination due to the fact that in the normal course of plant development a combination of genetic material of vegetative cells can only occur by way of the exchange of information between cells within a plant. Normally the recombination of genetic material only takes place following the transition of two plants into the generative phase, or following meiosis to be more precise, during which the number of chromosomes per cell is reduced to a haploid set of chromosomes.

The critical appraisal of the use of protoplast fusion contained in the FiBL Dossier notes *i.a.* that:

> The protoplasm method is used to transfer complete sections of chromosomes from another, not closely related species into a variety.
>
> Pro: A swift way of creating new combinations and traits which would not be possible in nature.
>
> Contra: This method, which is closely related to genetic engineering, transgresses natural barriers.
>
> (FiBL 2001, p. 13)

4.2 When and why are protoplast fusion or cytoplast fusion used?

4.2.1 Production of non-inbred F1 hybrids

CMS plants, *i.e.* plants with **c**ytoplasmic **m**ale **s**terility are often used to produce F1 hybrids. This trait occurs in some plants (*e.g.* radish) as a natural system within the mitochondrial DNA. CMS can also be transferred to certain plant species, *e.g.* kohlrabi, through natural crossing. However, this method results in a high percentage of unusable plants from which undesirable traits have to be bred out through backcrossing. Protoplast fusion significantly shortens this backcrossing process, making hybrid breeding considerably simpler and cheaper (Engelcke 2005).

4.2.2 Resistance transfer

In the 1990s a number of experiments were undertaken with the aim of crossing resistance traits from wild species into their crop relations. In two cases relatively good progress was made:

- Transfer of *Sclerotinia* resistance to sunflower varieties
- Transfer of *Phytophthora* resistance to potato varieties

However, the procedures in these two cases were not developed to maturity.

4.2.3 Further applications

In the literature increasing numbers of references can be found to breeds based on protoplast fusion (Oetiker 2007).

- *Tomato x potato: Topato*
- *Triazine resistance:*

  Solanum nigrum (Triazine resistant) x Solanum tuberosum (not resistant)
- *Citrus: bridging incompatibility*

The statement that nectarines were derived from protoplast fusion between peaches and plums is however a myth. Nectarines were patented as early as 1938 in the United States[3] - US Patent No. 328 -; at that time protoplast fusion had not yet been invented.

What importance does protoplast fusion have as a breeding method at present and in the future?

In the 1980s protoplast fusion was rather fashionable. Anything and everything was tried, even crossing cells of tobacco plants with animal sperm (Oetiker 2007). This wave was followed by a similar degree of disillusionment when it became evident that the regeneration of the cells, which were relatively easily created by fusion, is very difficult and entails enormous financial expense.

> Amongst the striking characteristics of active protoplasts are the swift regeneration of a cell wall as well as the development of a polarity. Both these characteristics have to be newly acquired if the regeneration into a new plant is to occur; therefore the regeneration into a complete plant following a fusion is not possible as a matter of course. The fact that the protoplasts of economically important crop plants show a lack in the ability to regenerate is a major handicap in transferring the results obtained through this method to commercial farming. (…) Interspecific fusions are relatively easy to produce. (…) Apart from a few exceptions interspecific fusions can only be regenerated if sexual crossing is also successful.
>
> (SENGEBUSCH 2003)

At present protoplast and cytoplast fusion are no longer used to any great extent in breeding. In cases where crosses have been successful however – predominantly in Brassicas – varieties continue to enter the marketplace which are based on these methods and go back to patents from the 1970s.

The future importance of protoplast fusion as a breeding method is generally considered to be rather minor: *In the times of gene transfer, the improvement of individual traits using this technique is easier to realize than using protoplast fusion since the latter is after all not the simple addition of the traits of two parents* (Seigner 2007). Breeding with the aid of marker-assisted selection has already gained much greater importance (Haring 2006).

[3] http://www.springerlink.com/content/qt3tq012t1263867/

Which plants have already been bred with the help of protoplast fusion?[4]

4.4.1 Brassicas

In Brassicas protoplast fusion only plays a role in transferring CMS. According to Prof. Michel Haring most CMS hybrids of Brassicas can be traced back to the OGURA-CMS, discovered by Hiroshi Ogura in 1968 in Daikon radish – not in European-type radish as is often stated[5] (Ogura 1968). There are however some other CMS hybrids which are based on natural crossing (Theiler 2002). Bannerot et al. (1974) and Pelletier et al. (1983) transferred and improved the cytoplasm into *Brassica oleracea* and from there at a later stage also into *Brassica napus* (Dietrich 2002).

Meanwhile, the OGURA patent has expired and is now widely used; breeders are also in the process of developing other patents (*e.g.* SAID patent) (Oehen 2007). The OGURA patent has been in use since its notification and has not posed any problems. The patent is on a pure cytoplasm containing male sterility. Nuclei and chloroplasts of these cells have been destroyed.

The emergence of the CMS line used in hybrid breeding of Brassica species at the Rijk-Zwaan company is – pursuant to the patent – described as follows:

> While CMS does occur naturally in some plants such as sunflowers or radishes, CMS was not found in headed cabbage varieties. Therefore, headed cabbage was first crossed with radish. The offspring displayed strong yellowing of the leaves, caused by the radishes' chloroplasts. In order to transfer the radish-cabbage cross' cytoplasm without chloroplasts into the headed cabbage plants, cells without cell walls, the so-called protoplasts, were fused in an electric field in the 1960s. Prior to the fusion the nuclei and chloroplasts of the radish-cabbage protoplasts were destroyed by irradiation. The result of the fusion of the radish-cabbage protoplasts thus prepared with headed cabbage protoplasts were *in-vitro* regenerated headed cabbage plants carrying the CMS trait.
>
> (FISCHER - KLÜVER 2005)

The CMS line in cabbage plants was therefore initially based on a natural cross between Asian-type radish and headed cabbage. It was only the subsequent elimination of undesirable traits that was carried out, in the manner of backcrossing, by way of cytoplast fusion. [6]

In Brassicas, hybrid varieties have largely achieved market dominance during the past twenty years. A working paper by the Louis Bolk Institute estimates a market segment of approximately 90%, so CMS is very widespread in commercial Brassicas. However, there are still varieties based on self-incompatibility as well as a number of open-pollinated varieties (Kaiser 2004).

Regarding the use of CMS varieties in cabbage production, please also refer to the section on “Share of CMS varieties in cabbage types” in the Appendix.

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[4] In this chapter reference is mostly made to plant species for which the use of protoplast fusion has been or is being discussed.

[5] The fact that European-type radish is often given as the source of CMS is due to a mix-up. Both plants are named *Raphanus sativus* in Latin, but European-type radish is the sub-species *var. sativus,* while the Asian-type or Daikon radish is *var.niger.*

[6] See also the section on rapeseed below.

4.4.2 Chicory

In the technical literature it is repeatedly stated that all chicory, *i.e.* both forcing chicory (*Cichorium intybus*) as well as endives (*Cichorium endivia*), were generated by protoplast fusion. However, this is only true for forcing chicory (*Cichorium intybus*) as there are no hybrid varieties of salad green endives on the market. This confusion is probably due to the fact that endives are termed *chicorée* in French. [7]

Regarding forcing chicory, there are "… as yet no varieties on the market to which cytobiological methods were applied. However, such varieties will enter the market in the near future. These will be CMS hybrids, with the CMS having been transferred from another species (sunflower, *Helianthus sp., Asteraceae*) by way of protoplast fusion. Moreover, this CMS type has been patented. At present sufficient numbers of varieties are still available for further development." (Kaiser 2004)

The varieties which may enter the marketplace were bred by the *Bejo* seed company. However, the company has since decided not to market these varieties. (pers. comm. Haring 2007)

4.4.3 Leeks

Some institutes such as the *Federal Institute for Crop Plant Breeding Research* (BAZ, Quedlinburg, Germany) carried out research with the aim of transferring the onions' male sterility into leeks. While this was in part successful, no practical application was placed in the market.

An enquiry made to the *Nunhems Netherlands* seed company showed that they had not used protoplast fusion technology in leek breeding: "We use the system of genic male sterility in leeks. This male sterility exists in the leeks' natural gene pool. Indeed, the male sterility we use was found in a flowering leek population. There is no connection whatsoever with protoplast fusion or genetic modification." (Communication from Inga Jessen, March 2007).

4.4.4 Potatoes

At the *Bavarian State Research Centre for Agriculture* (LfL) protoplast fusion has been used since 1990 in potatoes to produce basic seed material. More than 400 fusion hybrids are tested annually in field production. Achievements include the targeted accumulation and combination of a range of disease resistances, the use of new resistance genes transferred from wild species, the decoupling of male sterility and virus resistance (PVY) and the development of breeding lines with very high starch contents. The institute's 2004 annual report (Bayerische Landesanstalt 2005) contains the following notes on protoplast fusion; it must be noted that all the research is still at the experimental stage and that as yet no commercially suitable varieties have entered the marketplace (Schwarzfischer 2007):

[7] Endive = fr. *chicorée scarole*   Frisée endive = fr. *chicorée frisée*

Objectives

By fusing protoplasts (individual cells which had their cell wall removed) the genetic material of two selected diploid potato lines can be added together and thus characteristics of importance for breeding purposes can be directly combined. A decisive advantage of this method compared to conventional breeding is that the meiotic processes are bypassed. Especially where polygenic traits are concerned, all responsible genes are jointly passed on to the fusion product. Further advantages of the method are that sexual incompatibility and maternal inheritance are overcome.

The objectives are:

- Targeted combination of special quality characteristics (high starch content, suitability for processing, storage capability at 4°C) and resistances (potato wart, nematodes (Ro 5, Pa 3), *Phytophthora*, PVY) as well as establishment of multiplex genotypes for these traits
- Decoupling of PVY immunity and male sterility
- Enlargement of the gene pool (fusions with diploid lines from other breeders)

Method

Leaves from *in vitro* shoot cultures are chopped up and incubated in a solution containing cell-dissolving enzymes. The protoplasts generated in this process are cleaned by filtration and centrifugation, mixed at a defined cell density according to the breeding plan and fused by electro fusion. Following regeneration the hybrids are selected by flow cytometry and RFLP analysis.

Results

In the reporting year the yield in terms of successful fusion products was further increased, with 72 different combinations having been produced. Compared to the previous year an additional 25 combinations were produced and compared to 2002 the yield was more than doubled. This positive result is due to improvements in methodology which was possible to implement thanks to experienced lab personnel. Twenty-four of the fusion combinations combined good culinary types. Of the 48 combinations including starch lines, 39 aimed at suitability for processing (crisps, french fries). Forty-six combinations exhibit broad-based resistance (24 to potato wart disease, 16 to Ro1-5, 6 to *Phytophthora*). Multiplexes were developed for 10 combinations. It was possible to make increased use of interdihaploids in order to improve tuber qualities and increase yields (51 combinations). Of fusions with six Canadian breeding lines, hybrids are available for five combinations. Decoupling of PVY immunity and male sterility has been achieved. Almost all varieties exhibiting PVY immunity are sterile due to a mitochondrial gene. By abolishing maternal inheritance it was possible to establish highly-fertile plants with PVY immunity.

ANNUAL REPORT 2004 (BAYERISCHE LANDESANSTALT 2005)

4.4.5 Rapeseed

In 1994 the research institutions INRA and SERASEM registered the first rapeseed hybrid named “Synergy” in the official French seed catalogue. This registration was preceded by fifteen years of research which achieved *i.a.* that self-fertilization was prevented by transferring CMS into the maternal lines. The process was described as follows:

Through inter-species crossings between rapeseed and a radish, the researchers bred sterile male rapeseed endowed with a genome of rapeseed and a cytoplasm of radish. However, the rapeseed genome did not function satisfactorily with the radish cytoplasm, so these plants displayed abnormalities (chlorophyll deficiency, flowers without nectar which did not attract pollinators). INRA researchers, under the leadership of Georges Pelletier, then had the idea of "adding" a rapeseed cytoplasm. This was made possible in plants by applying protoplast fusion. The researchers thus obtained rapeseed with rapeseed chromosomes and a mixed rapeseed / radish cytoplasm containing the original radish gene providing male sterility. This type of rapeseed forms the basis for the majority of the hybrids cultivated in France at present, and constitutes one of the most widely exploited INRA patents (ogu-INRA patent, WO9205251, 1990).

(INRA PRESS SERVICE 2006)

Meanwhile a number of different CMS systems have been developed through crossing and protoplast fusion. However, often these can not be exploited for breeding purposes as they either can not be restored, are not environmentally stable, or the plants are generally impaired in their development. Only the CMS systems *Polima* and *Ogura* are commercially used in rapeseed production. However, the former is not stable under European climatic conditions (heat) and the latter is very expensive to grow due to high license fees (Dieterich 2002).

Despite these conditions hybrid varieties are making headway in rapeseed production. A ban on protoplast fusion as a breeding method would therefore lead to limited production in about 10 years time; at present a ban on protoplast fusion would still motivate breeders to preserve PF-free varieties (pers. comm. Becker 2007).

4.4.6 Maize

Cytoplasmic male sterility (CMS) originated from a natural mutation (Munsch 2005). This means that both the currently available hybrid maize varieties as well as “Maize Plus Hybrids” which have been attracting increasing attention for some time (see Munsch 2005) have not been created by means of protoplast fusion. The crossing in of herbicide resistance by means cytoplast fusion does also have no role. Therefore a ban on protoplast fusion is not going to affect commercial organic maize production.

4.4.7 Rye

While a CMS factor is used in the now commonplace hybrid-breeding of rye, this was not generated by protoplast fusion. Moreover, regeneration is always difficult in rye – even anther culture is hardly possible – and therefore there is no research activity in the field of protoplast fusion in rye (Miedaner 2007, pers. comm.).

4.4.8 Hops

At the Bavarian State Research Centre for Agriculture *(LfL Bayern)* the world’s first hop plants from protoplasts were regenerated. At present, *i.e.* since about 2002, no more work is being done in this field. “We were not able to regenerate true fusion plants which led us to abandon research in that field.” (pers. comm. Seigner 2007).

4.4.9 Sunflowers

In order to cross *Sclerotinia* resistances into sunflowers, crosses were attempted with wild sunflower cultivars as early as in the 1980s. However, the results were not satisfactory. Using protoplast fusion, the *Institute of Molecular Physiology and Biotechnology of Plants - University of Bonn* was successful in crossing *Sclerotinia* resistance into sunflowers (pers. comm. Friedt 2007). However, further research and patenting were not pursued for political and financial reasons (pers. comm. Schnabel 2007).

4.4.10 Barley, wheat, oats, millet, rice, forage grasses and clover

CMS has been well researched in some of these crops but information on the use of protoplast fusion in breeding is not available.

4.5 Can the use of protoplast fusion-based varieties be controlled?

Organic plant breeding uses open-pollinated varieties and hence there is no risk that protoplast fusion-based varieties may involuntarily be used.

The producers of *organic seed* however may have already made use of protoplast fusion-based varieties since organic seed has usually been derived from non-organic cultivars and only the last stage of propagation is carried out in keeping with organic standards. As breeders are as yet under no obligation to disclose information on their breeding methods, only laboratory analysis can clarify the situation.

Today's standard method for the detection of GMO products is DNA analysis with PCR (polymerase chain reaction). This technique can demonstrate whether seed, food or feed contains GMO components and what type of GMO (qualitative analysis). A prerequisite for detection is however that it is exactly known which section of the DNA was modified. A special variation of the PCR method (TaqMan PCR) can additionally establish the exact GMO content of a product (quantitative analysis with 0.1% accuracy) (ABE 2004).

These methods can also be used to detect the use of protoplast fusion and cytoplast fusion provided the relevant parent plants' DNA is well researched and can reliably be identified. Given access to equipment and procedures, the analysis is not particularly onerous. An individual analysis costs approximately €50. (Haring 2006) The genetic codes for common crop plants required for using this method should meanwhile be available. If the codes are not available, there will be corresponding development costs.

In order to establish a functioning monitoring system, it would first need to be clarified whether the genetic codes regarding the principal protoplast fusion patents – and especially the OGURA patents – are available, or if not, the relevant codes would need to be deciphered and made available to the monitoring laboratories. Moreover, it would need to be established whether the genes introduced into the crop plants through protoplast fusion may possibly have been removed again by way of backcrossing, thus rendering monitoring impossible.

5. Legal Provisions

5.1 Relevant provisions in public law

In Swiss law protoplast fusion of plant cells is not considered to be a genetic modification technique. The same holds for EU law but with the restriction that it must be possible for the plants used to also be crossed using conventional breeding techniques. Otherwise protoplast fusion is considered a genetic modification technique.

The relevant legal provision for Switzerland is the *Ordinance on the Release of Organisms in the Environment* (as of 19 July 2007), the "*Release Ordinance*". In addition, the following section also explores how the use of protoplast fusion in crop plant breeding is being dealt with in neighbouring Germany and in the EU.

5.1.1 Switzerland - Ordinance on the Release of Organisms in the Environment

According to Appendix 1 (Art. 3 c) of Ordinance SR 814.911 on the Release of Organisms in the Environment (Release Ordinance – Freisetzungsverordnung FrSV of 25 August 1999, last amended 5 December 2006) gene technology methods are defined as follows:

> Appendix 1, (Art. 3 c)
>
> **Definition of gene technology methods**
>
> 1 Methods of gene technology include, in particular:
>
> (...)
>
> c. cell fusion or hybridization techniques in which cells with novel combinations of genetic material are produced by the fusion of two or more cells through processes that do not occur under natural conditions.
>
> (...)
>
> 3 Self-cloning of non-pathogenic organisms and the following methods shall not be regarded as methods of gene technology, so long as they are not used in association with recombinant nucleic acid molecules or genetically modified organisms:
>
> (...)
>
> b. cell and protoplast fusion of prokaryotic micro-organisms that exchange genetic material by natural physiological processes;
>
> c. cell and protoplast fusion of eukaryotic cells, including the production of hybridoma cell lines and the fusion of plant cells;
>
> (...)
>
> ORDINANCE ON THE RELEASE OF ORGANISMS IN THE ENVIRONMENT (RELEASE ORDINANCE, RO) OF 25 AUGUST 1999 (LAST AMENDED 5 DECEMBER 2006)

Following this definition, protoplast fusion of plant cells is not considered a genetic modification technique. The text passages relevant to this study are found in Section 3 and describe the *cell and protoplast fusion of eukaryotic cells (…) including the fusion of plant cells (…) not used in association with recombinant nucleic acid molecules or genetically modified organisms.*

5.1.2 European Union: Deliberate Release Directive 2001/18/EC

Article 2 of the EU Deliberate Release Directive (Definitions) defines genetically modified organisms as follows:

> For the purposes of this Directive:
>
> (…)
>
> (2) "genetically modified organism (GMO)" means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.
>
> (…)
>
> Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:
>
> (…)
>
> (3)cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.(Annex 1A part 1)

However, Article 3 provides for the following exemptions:

> Exemptions
>
> 1. This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.
>
> ANNEX I B
>
> TECHNIQUES REFERRED TO IN ARTICLE 3
>
> Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:
>
> (...)
>
> (2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.
>
> (DIRECTIVE 2001/18/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL, 2001)

Therefore, the EU Deliberate Release Directive makes an additional distinction compared to the Swiss provisions in that it only exempts cytoplast fusion or protoplast fusion of plant cells where these can also “exchange genetic material through traditional breeding methods” from the provisions of the Directive.

In its practical implementation of the Directive the EU uses a very broad interpretation of this exemption in that the fusion of plant cells is effectively not regarded as a method of genetic engineering if the plants' cells are derived from plants of the same family. (Raaijmakers 2004)

5.1.3 Germany: Genetic Engineering Act (Gentechnikgesetz, GenTG)

as of 20 June 1990, last amended by Art. 1 G of the Act on 21 December 2004

The German provisions correspond to the EU Directive:

> Art. 3 Definitions
>
> For the purposes of this Act:
>
> (...)
>
> 3. Genetically modified organism (GMO)
>
> means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; a genetically modified organism is also an organism resulting from mating or natural recombination between genetically modified organisms or with one or more genetically modified organisms or other means of reproduction of a genetically modified organism, provided that the genetic material of the organism displays characteristics which are due to genetic engineering activities.
>
> 3a. Techniques of genetic modification in this sense are in particular
>
> (...)
>
> c) cell fusion or hybridization techniques where live cells with new combinations of heritable genetic material which does not occur naturally therein are formed through the fusion of two or more cells by means of methods that do not occur naturally.
>
> 3b. The following shall not be considered techniques of genetic modification
>
> (...)
>
> b) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods, unless genetically modified organisms are used as donors or recipients,
>
> 3c. Provided that they do not involve any release or placing on the market and provided that genetically modified organisms are not used as donors or recipients, the following shall not be considered techniques of genetic modification:
>
> a) cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by natural physiological processes;
>
> b) cell fusion (including protoplast fusion) of eukaryotic cells, including the production of hybridoma cell lines and the fusion of plant cells
>
> (GENETIC ENGINEERING ACT (GENTECHNIKGESETZ - GENTG) OF 20 JUNE 1990, AS AMENDED BY ART. 1 G ON 21 DECEMBER 2004)

In Germany as well as at the EU level pre-trials are commonly performed where the possibility of exchanging genetic material by means of conventional breeding techniques is to be verified.

5.2 Relevant provisions in civil law

5.2.1 IFOAM

The IFOAM Basic Standards as newly worded in 2005 state four principles in which organic/ecological farming is rooted:

- The principle of health
- The principle of ecology
- The principle of fairness
- The principle of care

Two of these principles serve particularly well to answer the question as to whether the protoplast fusion method is congruent with organic farming traditions:

The principle of health

Principle of health .

Organic Agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible.

This principle points out that the health of individuals and communities cannot be separated from the health of ecosystems - healthy soils produce healthy crops that foster the health of animals and people.

Health is the wholeness and integrity of living systems. It is not simply the absence of illness, but the maintenance of physical, mental, social and ecological well-being. Immunity, resilience and regeneration are key characteristics of health.

The role of organic agriculture, whether in farming, processing, distribution, or consumption, is to sustain and enhance the health of ecosystems and organisms from the smallest in the soil to human beings. In particular, organic agriculture is intended to produce high quality, nutritious food that contributes to preventive health care and well-being. In view of this it should avoid the use of fertilizers, pesticides, animal drugs and food additives that may have adverse health effects.

(IFOAM PRINCIPLES OF ORGANIC AGRICULTURE 2005)

According to the principle of health, the health of plants should be maintained "as one and indivisible". However, the manipulation taking place in the context of protoplast fusion violates the integrity of plants at a number of levels:

- *Plant development*: Plant cells are forced into fusion directly from the vegetative state, *i.e.* without meiosis and thus without passing through the generative state.
- *Plant cell*: The cells as the smallest living entities are removed from the plant tissue, the cell wall is chemically dissolved and the protoplasts are forced to fuse by unnatural means.
- *Genetic material*: Natural crossing barriers are overcome and - by fusing cytoplasm - genetic material is mixed which would hardly have been combined naturally (*cf.* Lammerts van Bueren 2003).

The principle of care

Principle of care

Organic Agriculture should be managed in a precautionary and responsible manner to protect the health and well-being of current and future generations and the environment.

Organic agriculture is a living and dynamic system that responds to internal and external demands and conditions. Practitioners of organic agriculture can enhance efficiency and increase productivity, but this should not be at the risk of jeopardizing health and well-being. Consequently, new technologies need to be assessed and existing methods reviewed. Given the incomplete understanding of ecosystems and agriculture, care must be taken.

This principle states that precaution and responsibility are the key concerns in management, development and technology choices in organic agriculture. Science is necessary to ensure that organic agriculture is healthy, safe and ecologically sound. However, scientific knowledge alone is not sufficient. Practical experience, accumulated wisdom and traditional and indigenous knowledge offer valid solutions, tested by time. Organic agriculture should prevent significant risks by adopting appropriate technologies and rejecting unpredictable ones, such as genetic engineering. Decisions should reflect the values and needs of all who might be affected, through transparent and participatory processes.

(IFOAM PRINCIPLES OF ORGANIC AGRICULTURE 2005)

According to this principle, precaution and responsibility are key concerns in making technological choices in organic farming and it is expressly pointed out that “unpredictable [technologies], such as genetic engineering” should not be employed.

5.2.2 Demeter International

At the application of the *Forschungsring für Biologisch Dynamische Wirtschaftsweise* (Research Group for Bio-dynamic Farming) a ban on CMS hybrids generated by protoplast fusion was decided in June 2004 and came into force on 1 July 2005. In addition to ethical considerations, the decision was also taken on qualitative, cultural, and economic grounds (FORSCHUNGSRING 2006 I).

Following inquiries with a number of seed companies a blacklist of non-permitted protoplast fusion varieties was compiled which was published both in the *ÖKOmenischer Sortenratgeber* (a list of cultivars recommended for organic growing; Arbeitsgemeinschaft Ökologische Gartenbauberatung 2006) and on the website of the Research Group for Bio-dynamic Farming (http://forschungsring.de/fileadmin/wissenstransfer/pdf/CMS-Orientierungsliste_Nov_2007.pdf). Other measures planned on the basis of the ban are set out in Section 8.3 of this report.

Demeter press release of 16 December 2004

Demeter settles seed issue: No varieties from cytoplast fusion technology, the "lesser genetic engineering" – Focus on breeding and growing biodynamic varieties

Seed breeding has become a very important issue for biodynamic farming. "As the pioneers of organic farming who have just celebrated our 80th anniversary we once again live up to our aspiration of being at the vanguard of consistent organic quality", comments Demeter Managing Director Dr. Peter Schaumberger on this development. More than 20 years ago Demeter growers and farmers begun to intensively scrutinize the origins of the seed they use and look for alternatives to industrial varieties. The initiative of individuals resulted in biodynamic breeding work, the fruit of which can now also be found in the marketplace. With a well-rounded marketing strategy the biodynamic carrot varieties Robila, Rodelika and Milan are now available on the fresh vegetable shelves of organic food shops and are getting much attention from consumers and the press. Similarly there are biodynamic cereal varieties, as approved by the German Federal Variety Certification Office, and the first goods produced from these are available, such as for example breads from the Dottenfelder Hof in Bad Vilbel or other baked goods from ErdmannHAUSER. With resolute specifications for producers the biodynamic community now prescribes in their standards that varieties generated by means of cytoplast fusion techniques are excluded from Demeter production. This amendment will come into force in July 2005. Initially this change will primarily affect vegetable varieties. (…)

HTTP://WWW.DEMETER.DE/PRESSE/PRE_ARCHIV_ID.PHP?PRESSE_ID=314&PAGEGROUP=DEMETER.DE&PAGEACTION=HOME

5.2.3 Bioland (German Association)

According to Eckhard Reiners, Head of the Crop Production Division, *Bioland*[8] currently does not plan to change their standards. Reiners is of the opinion that "a scientific/legal decision as to what constitutes GMO and what does not was taken with Directive 2001/18/EC. The organic sector should not deviate from this in its definition of genetic engineering" (Fischbach 2005 I).

Since CMS hybrids generated by protoplast fusion are, as already outlined above, legally defined as not being genetically modified organisms, these varieties are permitted for use on Bioland holdings.

At a meeting of the *Bioland* division for vegetable production at the end of July 2006 the following statement was decided upon after lengthy discussions:

[8] *Bioland* is one of the biggest German organic growers associations

- Protoplast fusion is not genetic engineering. However, it is a further step in a worrying direction in terms of breeding techniques which are far removed from the principles of organic farming. Most of the varieties available in the marketplace and used in organic growing were bred using these conventional methods. Only during the last stage of propagation the seed is grown in keeping with organic standards. Despite the concerns it is not deemed necessary to specifically prohibit protoplast fusion as a new type of modern breeding techniques in the Standards. One of the considerations in this decision has been that a ban could not be policed by examination of the seed.
- Transparency and freedom of choice are principles demanded by Bioland at all levels of food production. Therefore it should be possible to include notes on breeding techniques for every variety in the organicXseeds database. Moreover, seed companies must be urged to disclose such information on their varieties.
- A close eye must be kept on the further development of breeding techniques and especially on protoplast fusion. For established varieties, studies should be instigated in order to assess risks such as potential chromosome jumps.

(REINERS 2006)

5.2.4 Naturland (Germany)

The *Naturland* organic farming association views the breeding method as “not without difficulties” but it seeks a European or possibly even global ruling. Prior to requesting clarification at EU level and asking for a statement from IFOAM the following statement was published in the *Naturland-Nachrichten,* the association’s newsletter, in June 2007:

Varieties generated by protoplast fusion (CMS varieties) not permitted at Naturland

Protoplast fusion is a breeding method in plant breeding where characteristics of other, related or unrelated species can be crossed into a plant via protoplasts. An example is the transfer of male (pollen) sterility (CMS) from radishes into cabbages. In hybrid breeding this trait is used to create female lines without pollen which ensures that all pollen is derived exclusively from male lines grown beside it.

To this end two protoplasts (plant cells without cell walls) are fused and the cell containing CMS has its nucleus destroyed prior to the fusion so that only the CMS information is transferred. Due to its similarities with genetic engineering methods, this method has occasionally been termed “lesser genetic engineering”. Many experts, including the Federal Association of German Plant Breeders, are of the opinion that protoplast fusion methods equal genetic modification at least in cases where genetic material from another species is being transferred. For this reason, varieties which have been bred with the aid of protoplast fusion are not permitted by Naturland.

We must point out that varieties of a number of vegetable types, and cabbages in particular, which were generated using protoplast fusion have already entered the marketplace (cauliflower, romanesco, broccoli, white cabbage, spring (pointed) cabbage, red cabbage, Savoy cabbage, Brussels sprouts, kohlrabi). If we don’t position us against this situation there may well be a risk that for some types only varieties from protoplast fusion will be developed in the future. These varieties do not necessarily have to be labeled as such. An up to date blacklist of all know varieties derived from protoplast fusion (and which thus are not permitted by Naturland) can be downloaded from the Internet at http://forschungsring.de/fileadmin/wissenstransfer/pdf/Negativliste.pdf or can be obtained from your advisor or at the Naturland offices.

(VOGT-KAUTE, FRITZSCHE-MARTIN 2007)

5.2.5 ECO-PB

ECO-PB (European Consortium for Organic Plant Breeding, www.eco-pb.org) has been considering the possibility of banning protoplast fusion for some time. The main reasons for such a ban would be ethical considerations:

Ethical objections to the use of protoplast fusion

- The integrity of plants is violated

  Protoplast fusion goes beyond the level of the organized cell, which is biologically seen the smallest living entity, and affects the cell coherence and organization. The cell wall is dissolved and cells are separated from the living context of the plant or tissue.
- The genetic integrity of plants is violated

  With protoplast fusion natural crossing barriers can be forced. This technique is mostly applied when two species differ so much that a successful crossing cannot be achieved under natural circumstances.

(RAAIJMAAKERS 2004)

Other reasons for banning protoplast fusion are its proximity to genetic engineering and thus the possible loss of credibility of organic products.

The Louis-Bolk Institute in the Netherlands, one of the members of the consortium, suggests that an assessment of breeding techniques for organic farming should take orientation from the following three basic principles of organic farming: closed internal farm nutrient cycles, natural self-regulation, and biodiversity. As applied to breeding this means:

- Natural ability of plants to reproduce
- Adaptability to environmental conditions
- Genetic diversity which respects the natural authenticity and characteristics of species

(WWW.OEKOLANDBAU.DE/ ZÜCHTUNGSMETHODEN UND -TECHNIKEN, STAND 6.2.2006)

ECO-PB has not yet taken a decision as to how to proceed.

6. What would be the impact of the continued use of protoplast fusion in organic farming?

6.1 Impacts on humans

Are plants bred using protoplast fusion or cytoplast fusion unhealthy?

"If one manipulates the genetic material (DNA) of a natural food plant, new proteins will be produced." (Liedtke 2006). It is obvious therefore that genetically modified plants always contain a proportion of "new" types of proteins – so-called foreign proteins[9].

Whether or not plants bred by means of protoplast fusion or cytoplast fusion contain foreign proteins has not been clarified and given the possible multitude of fusion results as described above, this would indeed be very difficult to ascertain. However, one can not assume that when genetic material is combined from nuclei and cell organelles only "well-known" gene sequences result which synthesize "known" proteins. There is a high probability that foreign proteins are produced.

"The insertion of individual new traits is carried out without sufficient knowledge of the overall regulation of a plant's development above the level of the genome (epigenetics). Therefore one does not know beforehand how the plant as a whole will react to this intrusion with the individual new genetic trait." (Liedtke 2006)

As a rule it must be assumed that genetic manipulation of a plant using DNA from another species will disrupt and destabilize its biological organization, previously coordinated to correspond to the needs of its existence. In the millennia of plant evolution there have been many of such individual "trial and error" events, so-called mutations. Many of the "new versions" resulting from these mutations did not survive. Therefore, in order to rule out possible health hazards, assessments are required of the performance of protoplast fusion-based varieties over a number of generations.

6.2 Impacts on the environment

The likelihood that protoplast fusion generates plants with disrupted systems (*e.g.* disruption of plant physiology, resistances, decoupling from the biological environment, new diseases) is much higher than the likelihood of general 'improvements'." (Liedtke 2006) Ultimately, long-term trials would likely confirm the hypothesis that protoplast fusion-based varieties can not stand their ground over a number of generations and that they may even be environmentally damaging.

[9] Foreign proteins are associated with, in some instances significant, toxicity compared to native proteins. They are components of *i.a.* numerous highly effective animal and plant toxins where one of their effects is to inhibit essential enzymes.

6.3 Impacts on breeders

Breeders who serve the conventional market can continue their work unimpeded and remove open-pollinated varieties from the market according to their needs. There is no labelling obligation for protoplast fusion-based varieties. The existing gene pool becomes increasingly dominated by “artificially” generated varieties. Moreover, there is no liability regime in place.

As a result of the unimpeded use of conventional varieties, *plant breeding specifically for organic farming* is not supported in its efforts to breed varieties compatible with organic farming methods. The rising number of protoplast fusion-based varieties in the conventional gene pool increasingly limits the possibility to access this pool for breeding new varieties, in other words organic plant breeding must be able to access a larger “internal” gene pool in the future.

6.4 Impacts on farms

Farm holdings are most likely to suffer limitations in terms of the diversity of varieties they can access as has been the case with the breeding of hybrid varieties. The latter has resulted in a significant drop in the number of open-pollinated varieties on offer for some crop types. The situation for carrots can serve as an example: “While in 1985 the share of hybrid varieties in the EU catalogue of varieties (…) was 37%, it had increased to 73% by 1999.” (www.oekolandbau.de/ Züchtungsmethoden und -techniken, accessed 6 February 2006)

Many open-pollinated varieties which may have been well suited to organic farming have vanished. Major potential for farming has already been lost and the diversity of (open-pollinated) varieties continues to decline.

The same situation may come to pass in some crops in that protoplast fusion-based varieties are substituted for conventionally bred hybrids. In areas where PF-varieties have successfully been introduced it is likely to become increasingly difficult to source PF-free varieties.

6.5 Impacts on trade

Customers who purchase organic products expect that proper action is taken on their behalf in terms of health and respect for nature. The slightest hint of the possibility that genetic engineering may have been used in organic produce will diminish the customers’ trust in the integrity of organic farming. While protoplast fusion within species barriers legally does not constitute genetic engineering, the layman will possibly be left with unease as to the “naturalness” of this breeding method, especially if the term “lesser genetic engineering” remains in public use.

It is questionable whether the advantages of PF-based varieties can make up for this likely loss of confidence.

7. What would be the consequences of a ban on protoplast fusion in organic farming?

7.1 Economic consequences

7.1.1 Plant breeding level

The 2001 FiBL Dossier No. 2 *Techniken der Pflanzenzüchtung* (Plant breeding techniques) contains the following comments on the consequences of a rejection of protoplast/cytoplast fusion:

- *At the level of varieties: Only a small number of modern varieties of cabbage, endive, leek and chicory*[10] *would no longer be available to organic farming.*
- *At the level of breeding: Protoplast fusion has not yet achieved great importance. Organic plant breeding objectives can be achieved without it. However, cytoplast fusion is very important for introducing CMS.*

In any case, in some crop types, a ban on protoplast fusion as a breeding method will likely lead to a decisive reduction in the genetic potential available for breeding with corresponding repercussions in terms of breeding methodology:

> It is expected that the certified "organic breeding programmes" will be isolated programmes existing side by side with the customary breeding programmes. While the customary programmes allow for gene flow between programmes (including from organic to non-organic programmes), the organic programmes will then become isolated islands where improvements can only originate from the recombination of alleles within their own populations. (…) Since gene flow to and from other programmes will be very limited, these programmes will need to have large effective population sizes in order to allow for sustained selection successes. Moreover, the programmes must be designed in such a way that the loss of advantageous alleles is small. (…) The situation would be further exaggerated if the various organic farming associations devised differing evaluations of breeding methods. In that case even gene flow between certified "organic breeding programmes" may become impossible.
> (LÉON 2002)

Furthermore, a ban on protoplast fusion would entail liabilities if the ban was legally binding. This would not apply to bans at the association level.

7.1.2 Practical level

Some advisors as well as many practitioners – particularly in organic vegetable production – fear grave economic consequences of a ban since a major determinant of the success of a crop is the choice of cultivar (Koller 2006). One of the prime concerns is whether the recommended CMS-free varieties will meet the requirements of cost-efficient management (*e.g.* machine trimming). As part of a discussion amongst practitioners on the use of PF-based CMS hybrids the following points were also made:

[10] As the enquiries outlined in Section 4.4.1 of this report have shown, this has no relevance for endive and leek varieties.

- “CMS hybrids will let us make advances in breeding; one should not isolate oneself from new developments.
- In cabbages we can hardly expect good organic varieties without CMS.
- The Demeter Association rejects CMS breeding involving protoplast fusion; this could entail uncertainty amongst customers if there are differences in the associations’ standards.
- Even if one association rejects the procedure, other associations do not necessarily have to follow suit.
- CMS breeding should be subjected to critical examination but should not be dismissed off-hand.
- If we can not use CMS some crops will no longer be grown. These will then be imported; that can’t be the intention.
- Rules must apply to the whole of the EU; there must not be a rule for Germany alone.”

WWW.OEKO-KOMP.DE/INDEX.PHP?ID=2128&LANGUAGEID=1

7.2 Administrative consequences

7.2.1 Organization of inspections

Due to potential liabilities a legally binding ban on protoplast fusion would likely entail a disclosure of breeding methods on the part of breeders. In that case both the blacklist contained in the *ÖKOmenischer Sortenratgeber* (a list of cultivars recommended for organic growing; Arbeitsgemeinschaft Ökologische Gartenbauberatung 2006) and the breeding information given at www.organicXseeds.com would be more accurate than before. In this way the situation would become more transparent for both producers and inspectors.

The possibility of introducing PCR monitoring (see Section 5.5) would allow for random sampling with a view to verifying information provided by breeders. However, the financing of these measures would need to clarified.

7.2.2 Co-existence with PF-based varieties

As it is not certain in how far pollen and thus genetic material from protoplast fusion-based varieties spreads in the environment, a ban on protoplast fusion of plant cells could lead to similar co-existence problems as have occurred due to the use of genetic engineering.

Obviously this only affects plants which enter the generative stage such as cereals, oilseeds and legumes. This difficulty doesn’t arise for most vegetable types as they are harvested in a vegetative state and do not flower and set seed in the field. However, the question of incorporation of plant matter into the soil would need to be addressed in this context.

8. Which solutions are viable?

8.1 Approach based on legal provisions

This option is the approach currently taken in the organic farming sector. If this approach was chosen, CMS hybrids generated by means of protoplast fusion and potentially some future varieties into which resistances have been crossed by means of protoplast fusion would continue to be permitted in organic farming, provided that crossing could also take place by natural means. All other varieties generated by means of protoplast fusion would need to be considered GMO and declared as such and they would therefore be prohibited for use in the organic sector.

8.2 Ban on protoplast fusion in organically propagated seed

By definition, organically bred varieties have not been generated by means of protoplast fusion! The organic varieties listed in the organicXseeds database can therefore be used without any constraints.

However, only during the last stage of propagation is organic seed commonly used in organic farming grown in keeping with organic standards. As the original breeding method is irrelevant at that stage it is conceivable that protoplast fusion may have been used in some instances. Therefore the points made in Section 8.3. below with regard to non-organic seed also apply here.

8.3 General ban on protoplast fusion in organic farming

A general ban on protoplast fusion as a breeding method would entail significant restrictions in organic farming for some of the crop types given above; a ban would therefore need to be well prepared and may need to include transition periods in some cases. At present this is particularly relevant to organic vegetable production:

"Seed from hybrid vegetable varieties in the generation of which the cell fusion techniques mentioned were applied, are (…) often not recognizable as such. The possibility that such seed may unwittingly be used in one's holding is causing much uncertainty at present amongst growers. The dependence on major seed companies which use such breeding techniques is seen as a major problem." (Wilbois 2006)

Confirmation from breeders could give growers certainty in this regard. In mid-2005, at the initiative of the Demeter Association, the official organic seed database organicXseeds was amended to allow for such declarations. Organic farmers would have been given the opportunity to recognize PF-free varieties and consider this information in their choice of cultivars. Unfortunately however only a few seed breeding companies have followed the request to label their PF-free varieties.

As a consequence of their ban on protoplast fusion-based varieties, the Demeter advisory service has been planning a number of measures for organic vegetable production in the German-speaking area. The measures are outlined in the context of a report submitted in 2005; in the long-term the other organic associations would need to participate in these measures:

- Obtain information on all CMS varieties / protoplast fusion-based varieties ("compile blacklist") and make this list available to all organic vegetable growers for their annual seed orders.
- Establish legal certainty for all Demeter holdings regarding information sources on known CMS varieties which, in accordance with Demeter Standards, must not be used; to be achieved by means of subsequent decisions.
- Initiate/organize variety trials with "non-CMS hybrids" and open-pollinated varieties at a variety of research institutes.
- Publish summary of variety trials and recommendations for growers as to which varieties should increasingly be cultivated in order to change/bundle demand.
- Create a coordination centre / centre of excellence for issues surrounding varieties and breeding in organic vegetable production to advise growers and also maintain dialogue with breeders.

(REGNAT 2005)

Unfortunately the issue of controls was not yet considered in these plans. Inspectors would need to be equipped with the annually updated blacklist provided by the Demeter Association or by similar institutions. The organicXseeds database would need to contain the relevant information for producers. And last but not least random testing to confirm information provided by breeders by means of PCR (see Section 5.5) would need to be established and financed so that producers would not be burdened with the cost of controls in addition to other restrictions.

Overall, the situation that would arise from a general ban on protoplast fusion is not seen as hopeless, even in vegetable production: "Firstly, the situation as regards modern vegetable varieties is by no means so dramatic that the organic sector could no longer be supplied with very good, disease-resistant varieties which have not been generated by means of these methods. (…) Secondly, with a share of about ten percent of Germany's vegetable production the organic vegetable sector is such an important market for seed that it can freely voice its requirements and assert its future needs. However, to this end a clear message on the part of the organic sector is needed, stating that varieties generated by means of cell fusion techniques do not have a place in organic farming." (Wilbois 2006)

9. Conclusions and Recommendations

On the question of whether varieties generated by means of protoplast fusion should be permitted in organic farming the following summary can be given:

Legal situation

- According to the legal provisions in force, protoplast fusion of plant cells is not considered genetic manipulation in Switzerland. In the EU, in addition, evidence has to be furnished showing that the genetic material could also be newly combined by natural means. According to the enquiries made for this report, the abovementioned cabbage types have therefore not been created by means of genetic engineering.

Necessity

- In agricultural practice protoplast fusion is currently exclusively used to create CMS hybrids. It is only used to any considerable extent in vegetable production, and in cabbages in particular (up to 100% for some types; see Appendix!). While some varieties of forcing chicory (*Cichorium intybus*) were bred, these were not launched in the marketplace.
- Efforts to establish CMS systems in rapeseed are still at the level of basic research. There has been research on crossings with wild species of potatoes and sunflowers with a view to introducing resistance traits but these efforts were shelved at a basic level for financial and/or political reasons.
- A ban would have the greatest impact on organic cabbage producers with, in some instances, dramatic repercussions in terms of crop profitability, seed availability, and access to breeding successes. CMS varieties often have better external qualities[11] and are easier to process; especially wholesalers and large-scale supermarket retailers do not tolerate any losses in this regard!
- Up to 70% of the economic success of a crop is dependent on the choice of cultivar – this also holds true for organic growing[12]. Therefore the choice of varieties should not unnecessarily be made more difficult for producers.
- For all types CMS-free varieties are still available at present which could be further developed.

Environment

- To date no unacceptable environmental impacts are known.
- As it is not certain in how far genetic material from protoplast fusion-based varieties spreads in the environment, a ban on protoplast fusion for oilseeds and cereals could lead to similar co-existence problems as have occurred due to the use of genetic engineering.

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[11] If there are more rejects, a larger area has to be cropped for the same quality class.
[12] Laurense Tuinbouw-Adviesgroep, Breda (NL) at a meeting of the *Verein Ehemaliger Wädenswiler* (an alumni network) in 1996

Human health

- To date no unacceptable impacts on human health are known.
- However, there is a possibility that PF-based varieties have lower vitality which could impact on the holistic quality of the foods produced thereof.
- In order to be able to provide more specific information in this regard, relevant trials and the application of newer methods to assess vitality and quality are required.

Holism

- In protoplast / cytoplast fusion the intermixing of genes from nuclei and/or cytoplasm bypasses the generative phase. Such a combination of cytoplasm of vegetative cells from different plant species, as well as the plants thus generated, do not occur in nature.
- Since cells produced by means of protoplast fusion can only be regenerated if the originating plants are close relatives, it is not beyond the realms of possibility that the new species could naturally occur.

Organic farming traditions

- According to the 2002 IFOAM Basic Standards organic plant breeding should be “a holistic approach that respects natural crossing barriers and is based on fertile plants”.
- The majority of seed used in organic farming however is based on non-organic cultivars – only at the last stage of propagation does it become organic seed.
- The Demeter producer association prohibited the use of protoplast fusion-based or cytoplast fusion-based CMS hybrid varieties from July 2005, albeit without being in a position to present a solution to the monitoring issue. The same is true for the statement issued by Naturland in Germany in June 2007.

Feasibility of controls

- In principle, PCR (Polymerase Chain Reaction), the standard method for the detection of GMO products, could also be used to detect the use of protoplast or cytoplast fusion. However, this requires that the DNA of the respective parent plant has been well researched and can reliably be identified. This option has yet to be assessed and established, should the need arise, but it could be very costly.
- Given access to equipment and procedures and following the establishment of the method, the PCR analysis itself is not particularly onerous. An individual analysis costs approximately €50. In all probability these tests are not yet available on a routine basis.
- Varieties identified as “PF-generated” in this manner would need to be compiled in a blacklist on an annual basis and made available to both producers and inspectors.

Consumer acceptance

- Without doubt the new varieties will increase at least external product qualities while costs remain steady or may even fall due to cheaper production and processing methods.
- However, the use of protoplast fusion does not need to be declared and consumers who wish to purchase naturally bred products would be faced with uncertainties. These consumers would likely be concerned if organic produce was to increasingly involve methods which are often termed as being "in the proximity of genetic engineering".

Summary and recommendations

Generally it can be said that protoplast fusion is no longer a breeding method considered to be of promising usefulness. Apart from patents on the transfer of CMS from radish into other Brassicas, there are no significant patents in the market. The reason for this situation is the great difficulty of regenerating plants from protoplast fusion products – it is rarely successful. The current legal position in Switzerland and the EU is that the existing patents are not considered to be genetic engineering patents.

Especially due to the bypassing of the generative development phase, the use of protoplast fusion with the aim of crossing plants is not congruent with organic farming traditions and can give rise to uncertainty amongst both producers and consumers. With the exception of certain cabbage types and varieties, the current number of PF-free varieties amongst modern crop plants which lend themselves to further development is still sufficient and therefore the organic sector could continue to be supplied with very good varieties reflecting the current state of breeding. The most should be made of this opportunity and breeders should be given incentives to continue breeding PF-free varieties.

For these two reasons, the recommendation is that the organic agriculture sector state that protoplast fusion as a way of newly combining genetic traits is undesirable as a matter of principle and thus limit its use as much as is possible. However a ban should not be considered because of the relative insignificance of protoplast fusion as a breeding method and the economic repercussions associated with the development and implementation of a monitoring system. It would doubtlessly be more purposeful to invest in progress and to use available funds for breeding programmes rather than for policing a ban.

Producers are recommended to voluntarily refrain from the use of CMS varieties. For the time being, vegetable producers should continue to take orientation from the annually updated blacklist published by the German Demeter Association. In the long term a pan-European list, in the compilation of which other organic farming associations and institutions should also become involved, would be most favourable to provide certainty.

It is recommended that in the long term a technical committee be established which would analyse the rapid biotechnological developments in breeding research on an ongoing basis and make recommendations for the assessment of breeding methods.

10. Outlook

The discussion on protoplast fusion as a breeding method in organic farming can not be conducted without taking a look at the current situation in molecular biology.

The science of the heritability of traits – and thus ultimately also agricultural genetic engineering – is currently experiencing a phase of radical change. In her book *Zellgeflüster* (Cell Whispers – journeys through the new realms of science) the author Floriane Koechlin describes it as "a floating state": "On the one hand, agricultural genetic engineering is still rigidly attached to the old *central dogma of the gene*[13]. (...) On the other hand, research in molecular biology has long cast off the chains of that narrow dogma; it has allowed for a 'holistic' view at the cellular level, opening up incredible insights into life processes." (Koechlin 2007, p. 182 f)

In this context, the book entitled *Evolution in Four Dimensions* by Eva Jablonka and Marion J. Lamb (Jablonka 2005) is currently getting much attention. It provides a detailed description of the mechanisms of epigenetics. According to Darwin, evolution is based on traits occurring by chance which persist if they give an organism a competitive advantage. This theory remains undisputed as far as traits are concerned which are inherited by way of genes. However, there are more and more indications of the existence of "epigenetic" factors which can influence the way genes function in ourselves and other living beings.

For example, at the University of Basel a group of researchers working with Professor Emeritus Barbara Hohn has provided the first ever evidence that plants can inherit environmental responses. Using the plant *Arabidopsis thaliana* they showed that plants passed on the memory of stress, *e.g.* from UV light, for at least four generations without any changes in the genome being detected. If these plants are crossed it is sufficient for one parent to carry the memory of stress in order for the offspring to be supplied with the "knowledge" of past negative experiences. (Molinier et al. 2006)

The mechanisms governing these processes are not yet known in detail. Initial research indicates that environmental factors such as radiation, nutrition and temperature play an important role. The question as to which other factors may be of significance is not yet resolved. To understand this phenomenon it may be helpful to refer to Alexander Lauterwasser and his *Water Sound Images*: "In science, the ordering principles of such fields of vibration with their quiescent structures are increasingly being regarded as codes or matrices for the most diverse *Gestalt* formations: Living development is not an additive stringing together of building blocks; it rather arises from a holistic process." (Lauterwasser 2006)

Regine Kollek, a molecular biologist at the University of Hamburg, Germany (cited in Koechlin 2007, p. 158 ff) summarizes: "It is quite undisputed nowadays that it is not the genes which guide life processes. Basically, genes are merely the suppliers of biochemical substances required by the cells at their particular developmental phase and functional state. This is a complete reversal of the hierarchy. The genes are important for manufacturing proteins but ultimately they are molecules like any other. They are activated by the cell when their products are required. (…) Neither genes nor epigenetic mechanisms determine the events; rather, they

[13] Put very simply, the *central dogma of the gene* says that genes are the "Book of Life", determining all life. Nowadays the understanding that genes are dynamic and flexibly adapt to the environment is increasingly gaining ground.

are in the service of the whole system's dynamics. Cells and living beings are complex, interactive, developing and self-organizing systems."

Based on these considerations an entirely new type of breeding research is being implemented by the organic grower Ute Kirchgaesser at the biodynamic market garden of the *Bingenheim Community* in Hesse (Germany). Plants, including lettuces and dandelion, are treated with a variety of sound intervals (*e.g.* thirds and fifths) from the time of germination. The parental plants and several generations of offspring are assessed in terms of total yield and share of marketable produce as well as occurrences of pests and diseases. One of the results was that the treatment of leafy vegetables with thirds led to increased vegetative growth of the untreated later generations. The treatment with fifths resulted in slower but very harmonious growth, provided the intervals were not applied too intensively (Zukunftsstiftung Landwirtschaft 2005).

The results from this and other research looking at positive influences on seed are likely to open up entirely new dimensions in breeding which go far beyond technical aspects such as the use of protoplast fusion.

11. Glossary

CMS hybrids

CMS stands for cytoplasmatic male sterility and means that in the flowers' stamens no viable pollen is produced. This trait allows for the production of inbred lines required for F1 hybrids as there is no self-fertilization in the maternal parent. CMS is based on the modification of mitochondrial DNA which, in conjunction with certain nucleic genes, leads to pollen sterility but which in conjunction with other nucleic genes results in fully fertile plants.

Heterosis

When two parents with a high degree of homozygosity are crossed there can be a significant increase in the performance of the F1 generation compared to the performance of the two parents, an effect termed heterosis.

Hybrid breeding

Hybrid breeding is based on the creation of inbred lines using maternal, paternal, and maintenance lines. The maternal line should be self-sterile for it to be exclusively pollinated by the paternal line. In most cases, the paternal line is self-fertile and is only used to pollinate the maternal line. The maintenance line (or restorer line) is used for the continued propagation of the maternal line; it contains the "restorer genes" which serve to overcome the maternal line's self-sterility.

Compared to OP seed, hybrid seed results in more uniform populations and, due to heterosis, in higher yields. To ensure purity of the F1 hybrid seed, it is a prerequisite of hybrid breeding that when seed is produced there is no self-fertilization in the maternal line.

Hybrid rye varieties are currently not permitted under the standards of DEMETER INTERNATIONAL and BIO SUISSE (Arncken 2005).

OP varieties

OP stands for "open pollinated". This allows for cross-fertilization of the female flowers and leads to greater diversity in the genetic material of the next generation.

“Originally the best plants in each population were selected and grown on to obtain seed. This method of selective breeding has a longstanding cultural-historic tradition and has led to major yield increases as well as to the diversity of crop plants. The resultant varieties are called OP varieties. This method continues to be used and is in some instances combined with individual selection, combination breeding, or backcrossing. It is of importance in organic plant breeding. Seed from OP varieties can continue to be saved and propagated for seed production.” (Theiler et al. 2002)

Restorer gene

Restorer genes are nucleic genes which overcome pollen sterility. In contrast, maintainer genes are nucleic genes which in conjunction with CMS maintain pollen sterility. As a general rule, hybrid varieties must not be pollen sterile as for all crop species where the seed is the actual crop (cereals, oilseeds, legumes) full pollen fertility is required in the population for normal seed development. Therefore the hybrid variety’s paternal line must be a restorer. Where the generative parts of the plants are not harvested, as is the case in most vegetable types, the restorer characteristics of the paternal line are of no significance.

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13. List of Experts

Name	Institution	Topic
Christine Arncken	FiBL Switzerland (CH)	Agricultural crop plant breeding
Prof. Dr. Heiko Becker	University of Göttingen, Faculty for Agricultural Science (D)	Rapeseed
Dr. Beat Boller	Research Institute Agroscope Reckenholz-Tänikon ART (CH)	Forage grasses, clover
Dr. Ulrich Darsow	Federal Centre for Breeding Research on Cultivated Plants (D)	Potatoes
Friedemann Ebner, Amadeus Zschunke	Sativa AG Rheinau (CH)	General topics of organic breeding and seed propagation
Prof. Dr. Wolfgang Friedt	Justus-Liebig University Giessen, Institute for Plant production & Plant breeding (D)	Sunflowers
Prof. Dr. Hartwig Geiger	University Hohenheim, Institute for Plant Breeding, Seed Research and Population Genetics (D)	Rye
Prof. Dr. Michel Haring	University of Amsterdam, Faculty of Science, Swammerdam Institute for Life Sciences (NL)	General questions Options for monitoring/control
Inga Jessen	Nunhems Seeds (NL)	Leeks
Prof. Dr. Edith Lammerts van Bueren	Louis Bolk Institute (NL)	General questions
Martin Koller	FiBL Switzerland (CH)	General topics Vegetables
Bernadette Oehen	FiBL Switzerland	Questions of patent law, especially OGURA Patent
Prof. Dr. Jürg Oetiker	Faculty of Science of the University of Basel (CH)	General topics re protoplast fusion and cytoplast fusion procedures
Maaike Raaijmakers	Biologica (NL)	General topics
Rudolf Regnat	Demeter Bavaria (Bayern) (D)	Demeter ban and approach
Prof. Dr. Heide Schnabel	Formerly at Institute for Molecular Physiology and Biotechnology of Plants, University of Bonn (D)	Sunflowers
Dr. Andrea Schwarzfischer	Bavarian State Research Centre for Agriculture, Institute for Plant Production and Plant Breeding (D)	Potatoes

Dr. Elisabeth Seigner	Bavarian State Research Centre for Agriculture, Institute for Plant Production and Plant Breeding (D)	Hops
Andreas Thommen	FiBL Switzerland (CH)	General topics www.organicXseeds
Jan Velema, Taco van der Maaren	Vitalis Biologische Zaden bv (NL)	Cabbage etc.
Prof. Dr. Gerd Weber	University Hohenheim, Institute for Plant Breeding, Seed Research and Population Genetics (D)	Maize
Dr. Klaus-Peter Wilbois	FiBL Germany, ECO-PB (D)	General topics

14. Brief Description of the Project

This report was compiled in the context of the COOP Naturaplan project “Safeguarding organic seed and planting material – Impulses for organic plant breeding”. The project was carried out at the Research Institute of Organic Agriculture (*Forschungsinstitut für biologischen Landbau*, FiBL) from 2003 to 2006. Project Module 1.3 presented here on “Impacts of banning protoplast fusion on the range of varieties” was compiled between April 2005 and April 2006; due to more recent developments further additions were made in April 2007.

Problem definition

Genetic engineering methods such as protoplast fusion which cross species barriers are prohibited in organic farming. According to the organic plant breeding draft standards protoplast fusion within species barriers should also be banned. It will not be possible to enforce this ban without industry self-declaration as there is no legal obligation to declare. Therefore, organic farming associations and the seed industry have to come to an arrangement at the European level. Following this, the potential impact of such a ban on the spectrum of varieties can be assessed.

Short description and objective

The aim of the project is to seek clarification on the implementation of a ban on protoplast fusion and its impacts on the spectrum of varieties, with a view to achieving initial self-declaration on the part of the breeders.

Contacts

Project management:	Bettina Billmann
FiBL staff involved:	Christine Arncken, Bernadette Oehen,
	Martin Koller, Andreas Thommen
Manuscript correction:	Dr. Bernhard Speiser

15. Acknowledgements

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16. Appendix

Share of CMS varieties in cabbage types

Author: Martin Koller

In order to assess the share of CMS hybrids in cabbage types, the variety recommendations contained in four lists (two organic, two non-organic, see Table 1) were analysed for four cabbage types (cauliflower, broccoli, white kohlrabi and white cabbage).

Not all varieties could clearly be allocated to either the CMS or non-CMS group; these were marked with a question mark and allocated based on the relevant companies' breeding programmes. Information contained in the companies catalogues and personal communications from company representatives were taken as baseline information in this regard.

Table 1: Share of CMS varieties in all recommended varieties by segment (2006)

Segment	Swiss vegetable manual [14]	Swiss list of recommende dorganic varieties [15]	Recommended varieties for growers in Rhineland-Pala-tinate, (D) [16]	List of cultivars recommended for organic growing [17]
Cauliflower white / early	100%	60%	86%	0%
Cauliflower white / summer	80%	67%	75%	60%
Cauliflower white / autumn	71%	40%	33%	50%
Cauliflower white / winter	100%	0%	-	-
Cauliflower green	100%	100%	60%	50%
Cauliflower purple	0%	0%	0%	0%
Cauliflower orange	100%	-	100%	-
Cauliflower / Romanesco	67%	100%	33%	0%
Broccoli /early	50%	50%	100%	33%
Broccoli / summer-autumn	75%	75%	100%	50%
Kohlrabi white / tunnel early	33%	0%	75%	20%
Kohlrabi white / summer-autumn	40%	0%	40%	29%
White cabbage / early	0%	0%	40%	0%
White cabbage /summer-autumn	50%	0%	33%	25%
White cabbage /storage 1-2 kg	60%	67%	50%	50%
White cabbage /storage >2kg	-	-	0%	20%
White cabbage /industry	0%	0%	36%	33%

[14] Theiler R. et al. (2007): Sorten. In Verband Schweizer Gemüseproduzenten (2007): Handbuch Gemüse, Bern
[15] Koller, M., Weidmann G. (2007): Biogemüse: Empfohlene Sorten für die Anbausaison 2007 /Ausgabe 2. FiBL, Frick
[16] J. Schlaghecken, I. Koch, J. Kreiselmaier (2006/7): Anbau und Sortenhinweise für Rheinland-Pfalz. www.hortigate.de (Only main varieties were considered)
[17] Arbeitsgemeinschaft Ökologische Gartenbauberatung (2005): Ökomenischer Sortenratgeber 2006-07

The share of CMS varieties is particularly high for early cauliflowers and broccoli. Coloured cauliflower varieties (green, orange, Romanesco) were also often bred using CMS technology (with the exception of purple cauliflower).

In kohlrabi varieties the share of CMS varieties is still low but many new varieties were bred using CMS technology. The white cabbage segment has the highest share of uncertain allocations with 9-21% varieties being doubtful (up to 19 varieties per segment).

A closer analysis of the varieties listed shows that most of the non-CMS varieties listed are older varieties. With the exceptions of the seed companies Bejo and probably Rijk Zwaan for white cabbage, all new varieties are CMS hybrids.

Table 2: Seed companies which bred the recommended varieties, sorted by CMS and non-CMS breeding programmes (2006)

Segment	Companies with CMS breeding programme	Companies without CMS breeding programme	No information
Cauliflower, white	Seminis, Clause, Enza, Syngenta, Rijk Zwaan, Nickerson Zwaan	Bejo	
Cauliflower, coloured / Romanesco	Clause, Rijk Zwaan, Seminis	Bejo	Tozer, Takii
Broccoli	Seminis, Syngenta, Sakata	Bejo	
Kohlrabi, white	Rijk Zwaan, Enza	Bejo	Takii
White cabbage, fresh	Clause, Syngenta, Nickerson Zwaan	Bejo, Bingenheim, Rijk Zwaan (?)	
White cabbage / industry		Bejo, Rijk Zwaan (?), DSP	

“With CMS breeding programme” usually means that all new varieties bred by the company are CMS varieties. “Without CMS breeding programme” usually means that all varieties – and as a minimum the organic varieties – are **not** CMS varieties.

What impact would the discontinuation of the use of the listed CMS varieties have?

- *Marketable yield*: For cauliflower, broccoli and, in parts, kohlrabi the new varieties produce greater marketable yield (for example, better self-wrapping in cauliflower). A smaller marketable yield has a drastic impact on the crop’s profitability.
- *Seed availability*: For vegetable types that are difficult to propagate, the supply situation in terms of non-dressed seed (NCT) and organic seed is difficult. If only one or two companies can supply marketable varieties supply security is not guaranteed.
- *Access to breeding successes*: For example, club-root resistant cauliflower and white cabbage varieties are currently only available from CMS breeding programmes.