Federal Environment Agency – Austria

ANALYSIS OF OBSERVED ADVERSE EFFECTS FROM THE RELEASE OF GENETICALLY MODIFIED ORGANISMS

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PREFACE

Observed negative effects from the release of genetically modified organisms have not been systematically dealt with in the international debate, researchers are divided on the risks involved. For the Federal Environment Agency the scientific surveying and assessment of the negative effects that have actually occurred is an important prerequisite for risk assessment, especially in view of precautionary environmental protection and environmental control.

In the controversial discussion about the use of genetic engineering in agriculture and for the production of foodstuffs the expected and documented positive impacts of GMOs (e.g. the reduction in plant pesticides, saving of renewable resources) play an important role. However, the documentation and evaluation of positive impacts necessary for evaluating instruments which go beyond risk assessment as such and environmental control, such as life cycle analysis or the socio-economic evaluation of products, were not within the scope of this study.

In the present study the literature cited and other scientific findings from various research activities were condensed into a systematic documentation of the observed negative effects ensuing from the release of genetically modified organisms. Up to now this topic has been mainly discussed in the media, but neither at national nor at international level has there been any co-ordination of release-related biosafety research. Also, effort to consolidate and process the data and results gained has been limited so far. The present study tries to remedy this lack and shows the need for action to improve the systematic gathering of data necessary for risk assessment and monitoring.

Due to its technical competence and expertise, underlined by its well-known and highly acclaimed critical work in the area of risk assessment and the impact of genetically modified organisms on the environment and human health, the Öko-Institut Freiburg was commissioned to carry out the present study.

CONTENTS

SUMMARY

When it comes to identifying signs of (undesirable) environmental effects of transgenic organisms release experiments occupy a central role. This is why the gathering, communication, and discussion of release data relevant for the environment should be of central importance. Gathering of such data is faced with certain difficulties: it is estimated that world-wide ecological data are only collected in less than 1 % of releases of genetically modified organisms. Neither at the national nor at the international level has there been any co-ordination of release-related biosafety research. Also, efforts to consolidate and process the data and results gained have been limited so far. The present study tries to partly fill this gap.

Microorganisms

As of yet it has not been possible to investigate the impact of genetically modified microorganisms (GMMOs) on the ecosystem as a whole. However, the specific characteristics of microorganisms, such as short generation times, the possibility to adapt to unfavourable environmental conditions, and the capacity to exchange genetic material, have led to the development of several indicators of potential damage resulting from the release of genetically modified microorganisms. These indicators include

- persistence or capacity for survival,
- dispersal,
- population dynamics,
- competition between different microorganisms,
- impacts on biotic communities in the surrounding environment.

Today safety assessments during the release of GMMOs focus on the persistence and dispersal of released microorganisms and of recombinant gene products.

The capacity of GMMOs to establish themselves is influenced by biotic factors such as food supply, predators, density of bacteriophages, competing microorganisms or population density or by abiotic factors such as temperature, pH-value, oxygen demand, water and salt content and soil conditions. Some microorganisms are able to survive over long periods of time under bad environmental conditions by forming spores or cysts. Other microorganisms do not form such permanent states, but change into a state in which they are viable but not culturable (VNC). Many scientific papers have disproved the widely expressed view that GMMOs are at a disadvantage due to additional genes, referred to as extra burden. In actual fact, GMMOs have at least a transient capacity to survive. In many cases, however, the capacity for survival is likely to be more durable.

Another important aspect in the risk assessment of GMMOs is their introduction into other habitats, once they are released into the environment. Thus microorganisms from unfavourable environmental compartments can be transported into an environment offering better conditions for survival. This transport can take place via wind, running waters, rain water as well as when GMMOs become attached to tools or harvested goods. In addition, organisms such as protozoa, insects (e.g. spring-tails) and other soil animals, such as earthworms, may serve as vectors, too. Field experiments revealed evidence of both vertical transport into deeper soil layers as well as lateral transport.

Another risk associated with the release of GMMOs is the elimination of species from the autochthonous microflora. An indicator of the fitness of GMMOs is their capacity to eliminate their wild relatives. Small-scale and farm-scale experiments have shown that depending on the case GMMOs may have a lower, equivalent, or higher competitiveness.

When considering the risks from the release of GMMOs it has to be taken into account that the released microorganisms may themselves acquire genes from other microorganisms and thus change their ecological properties. On the other hand, GMMOs may pass on their transgenes to endogenous microorganism populations. In general, microorganisms can exchange genetic material via conjugation, transduction or transformation. All three ways of transfer were found with GMMOs.

So far, only few investigations of the environmental effects of GMMOs in various environmental media have been carried out.

An assessment made by different authors has produced the following conclusion: GMMOs may

- successfully compete with indigenous microorganisms of disturbed ecosystems,
- transfer their new genes onto indigenous organisms; and these genes can also be expressed in their new hosts,
- influence general and specific metabolic activities and the turnover of biomass,
- influence the structure of biotic communities and their function within various habitats,
- change interactions between symbionts and organisms of different trophic levels and
- produce metabolites which may have unexpected impacts on the environment.

Due to the still patchy knowledge in microbiological ecology it is very difficult to assess these impacts.

Plants

The uncontrolled dispersal of transgenic plants is normally considered a risk. This is mainly based on agronomic considerations. The dispersal potential of genetically modified plants is considered an ecological risk, too, although there are no uniform assessment criteria. With preliminary assessments the parent plant, and the genes and gene constructs transferred by genetic engineering play a key role. However, the impact of the intended modification of genetic traits on the dispersal behaviour of GMOs is still largely unclear. When it comes to the use of genetically modified plants the vertical transfer of transgenes via intraspecific and interspecific hybridization in related cultivated and wild plant species is a much discussed safety aspect. In Europe, comprehensive investigations on the hybridization potential and probabilities were mainly focused on oilseed rape and sugar beet. In the framework of “normal” release experiments, no systematic investigations of hybridization were carried out. However, there were special research activities into the hybridization potential of transgenic plants.

Different views exist on the occurrence of **oilseed rape** outside the cultivated areas in Central Europe. In Canada rape is often found as a second growth in cultivated areas (DOWNEY, 1999).

With rape the prevailing opinion is that there is a possibility of establishment. Since various investigations of herbicide-tolerant rape did not reveal any difference in the competitive traits of transgenic and conventional rape, the growing wild of transgenic rape will have to be reckoned with. In addition, rape is a native plant of Europe together with a number of cross-fertile relatives.

The results from crossing experiments reveal that in wild herb populations gene flows may well start from rape. Potential hybridization partners of *Brassica napus* can be found not only in the genus *Brassica* but also in other genera of the family of the cruciferous plants. Under field conditions it was possible to cross transgenic oilseed rape with turnip rape (*Brassica rapa*), Indian mustard (*Brassica juncea*), black mustard (*Brassica nigra*), *Hirschfeldia incana*, synonym *Brassica adpressa*, wild radish (*Raphanus raphanistrum*), and charlock or wild mustard (*Sinapis arvensis*).

Sugar beet has a potential to grow wild. In several European countries and in the USA they can be found as a weed. With beta beet there are no hybridization barriers within the species. Cross-fertilisation is possible both between differing wild forms and between wild and cultivated forms. This was confirmed by related research carried out by the German university of Aachen. No hybridization barriers were found neither between the transgenic variants of the beta beet nor between other cultivated or wild forms. According to the scientific community, a dispersal of transgenes following commercialisation is inevitable.

Transgenic herbicide-resistant **soya beans**, which have been commercially cultivated since 1996, show a series of unintended modifications, which are probably due to position effects or pleiotropisms. These modifications include changes in the phytohormonal balance of plants and significantly higher lignification of the stems. As temperature rises this leads to the splitting of the stems and consequently to harvest losses.

The commercial cultivation of **cotton** has also brought about a number of partly unexpected problems. In 1997, in some areas herbicide-resistant cotton showed a malformation of the capsules followed by premature shedding of the capsules. According to research of the US Department of Agriculture, this insect-resistant species in certain areas brought about an infestation of secondary pests, which before had hardly ever caused any damage. This, in turn, led to the application of large amounts of pesticides.

The fact that pests may become **resistant** through insect-resistant plants is very important for risk assessment. Under field conditions this building of resistance has not been widespread. Laboratory tests, however, give rise for concern. More recent investigations into how *Bt*-resistance in *Bt*-sensitive pests is passed on to new generations allow the conclusion that this is not only a recessive inheritance. This means that existing resistance management plans that are based on the refugium strategy (where traditional crops are cultivated on adjoining areas which serve as refugia for sensitive pests) need to be revised. In addition, there is some indication that resistant pests take longer to reach sexual maturity, which would make the refugium strategy redundant.

When it comes to the use of herbicide-resistant plants, discussions focus on the potential **resistance of field weeds** caused by outcrossing. First data from the large-scale cultivation of rape in Canada confirm the results of field experiments. Multi-herbicide-resistant rape plants were found as second growth in secondary crops as well as outcrosses onto related wild relatives. Due to a potential gene transfer and the expected increase in the use of a few selected complementary herbicides (increased selection pressure) the problems caused by resistant weed ensuing from the cultivation of transgenic herbicide-resistant plants may prove to be far more pressing than the ones commonly associated with resistance to herbicides.

Research into the risks associated with **virus-resistant transgenic plants** focuses on the agronomically motivated question of whether genetic modification techniques to confer virus resistance may or may not change the dispersal and the evolution of viruses and whether plant viruses and more damaging viral plant diseases and epidemics could be brought about in the process. Here, three types of risk can be distinguished, which were partly investigated in experiments: recombination between cloned viral sequences and infected viruses, heterologous encapsidation and synergies between the cloned gene or gene product and the infected virus. Under greenhouse conditions proof could be established of the formation of virus recombinants in transgenic plants. Two of the investigated recombinants showed an increased host range; in three cases changed symptoms were ascertained. Heterologous encapsidations occur with transgenic plants that express a coat protein. Heterologous encapsidations were found both between viruses of the same virus group and between viruses of different groups. Synergies remain to be investigated.

Up to now field experiments to study the effects of transgenic plants on **non-target organisms** have predominantly been carried out by companies wanting to place transgenic plants on the market. No effects have been ascertained. Laboratory or greenhouse investigations,

however, indicated possible direct and indirect damage to beneficial animals through transgenic insect-resistant plants.

Investigations of the effects of insect-resistant plants on decomposers living in the soil were mainly made for the cloned *Bt*-toxin. Springtails are damaged in high concentrations. Proteinase inhibitors, cloned to fend off insects, had toxic effects on this group of organisms, too. Effects on soil microorganisms were only investigated in individual cases.

The last years have seen a strong increase in activities regarding transgenic **trees**. Until 1998, 116 release experiments had been carried out officially with 24 tree species in 17 countries worldwide. The stability of expressions, a common problem in all transgenic organisms, is a special problem in trees because of their longevity. Instabilities were found in field experiments with trees even after a relatively short time. Pleiotropic and position effects were found to be relatively common in trees under field conditions. First investigations of released poplars in Schleswig-Holstein with regard to their effects on mycorrhiza showed significant differences between transgenic and non-transgenic plants. The hybridization potential and hence the risk of an unintended dispersal of recombinant gene sequences has to be evaluated separately for each tree species. It is well known, though, that especially the genera Populus, Eucalyptus and Pinus that are intensively used in genetic engineering, have high hybridization potentials. In China insect-resistant poplars that express the *Bt*-toxin were found to have been damaged after 2 years by insects that until then had been considered insignificant. In field experiments with transgenic plum trees (*Prunus domestica*) expressing a viral coat protein, it was found that resistance had been overcome only after a short time.

Animals

It is commonly held that fish will be the first transgenic animals to be commercially used. It is not within the foreseeable future that other transgenic animals will be placed on the market for the purpose of agricultural use. Hence there have been no investigations of potential ecological effects. With transgenic fish the greatest advances have been made with modifications aimed at increasing growth by integrating growth hormone genes and with modifications of the cold tolerance, which can be achieved by transferring gene sequences coding for anti-frost-proteins (AFP).

Genetic modifications of fish are accompanied by sometimes considerable pleiotropisms and/or position effects. These include:

- tumours
- changes of fin and vertebra shape
- skull deformities
- abnormal gill growth
- missing body segments
- atrophies of nape and tail.

The stability of the expressions is another problem that remains to be solved with transgenic fish. There is not a single publication documenting stable and sustained gene expression with regard to the increase of growth in the F_1 generation. Another phenomenon occurring in all transgenic fish is mosaicism, which means that a modified individual consists of both cells with and cells without the transferred gene sequences.

Laboratory experiments and the experimental keeping in closed breeding ponds allow some conclusions as to the ecological risks. With traditional breeding in commercial fish farms it is quite common and very difficult to avoid the escape of individuals or their offspring. With cat-

fish it was found that transgenic individuals crossed without any problem with non-transgenic individuals. Similar results were obtained for other species. Therefore, an unwanted transfer of modified gene sequences into natural populations is very likely.

Moreover, with transgenic fish a change was observed both in the quality and the quantity of the food spectrum. Both competitive behaviour and sexual selection change due to the above-average size of transgenic fish. There are no investigations into the potential effects on predators or other members of aquatic coenoses.

RECOMMENDATIONS

Processing the data available on the effects observed with release experiments has shown that they are very patchy and have not been gathered in a systematic way. Access to these data is for the most part very difficult, and sometimes impossible. Company data are normally not made publicly available, and if so, then only in an aggregated way, which does not allow an assessment of the design of the experiment nor of its validity. Comparisons with other investigations are not possible either. Overall, research is very costly and it has to be assumed that only parts of the available data are actually gathered.

In order to obtain a better and quicker overview of the release-related ecological investigations carried out so far, efforts should be made to reliably publish the collected data including information on their aggregation, systematisation and central registration. Ecological investigations are used in notification procedures and provide an important basis for monitoring as foreseen in the amendment of Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms (2001/18/EC). In addition, the Biosafety Protocol adopted in Montreal in 2000 requires the making available of data for national risk assessments in the importing states via the Biosafety Clearing House Mechanism. Here too it is necessary to systematise and gather together the results of release experiments and the experience from commercial cultivation.

Different countries adopt different approaches when it comes to informing the public about applications for the release or placing on the market of GMOs and the relevant scientific findings. To make these decisions understandable a high degree of transparency is needed.

There is a strong need for harmonisation at a high level.

From the above, the following recommendations for genetically modified plants can be deduced:

- Joint development of validated procedures for release-related biosafety research initiated at OECD and/or EU level in order to ensure relative comparability of data collected in different cultivation areas
- EU-wide definition of basic data to be required from the notifier for each release experiment. These should include at least random soil investigations in order to establish proof of the recombinant gene constructs (e.g. zero control, time line of the degradation, persistence, potential accumulation especially with multi-annual releases on the same or adjoining plots) as well as monitoring of pollen dispersal and the setting of pollen traps.
- EU-wide definition of data which must be gathered depending on the specific modifications of traits and the plant species (e.g. documentation of the residence of seeds / vegetative propagation units and possible problems with second growth with frost-resistant or frost-tolerant crop plants, investigations of the effects on selected soil organisms and herbivorous insects as well as their predators).

The (scientific) communication of observed effects should be improved through

- Foundation of a scientific journal, which gives an overview of new publications, available both on-line and in printed form. Here it would be particularly desirable to make sure that when implementing the amendment to Directive 90/220/EEC (2001/18/EC), attention be paid to the inclusion of mainly publications verified by "peer reviews" in the ecological data that are important for the approval of marketing, and thus making the data available to the interested public and allowing for independent risk assessment.
- Establishment of a publicly accessible database sorting entries by organism and region for the EU. This database could be maintained by DG Environment or the EU Commission and could play an important role in implementing the Biosafety Protocol. The OECD Biotrack Database could be used as a foundation. This database should contain the plants used in field experiments in Europe as well as the changes in their characteristic traits and the results of release-related biosafety research. The regions where they are cultivated and/or released should be given. Furthermore there should be information on cross-pollination partners and their regional distribution. With insect-resistant plants the regional distribution and the importance of target organisms as agricultural pests should be taken into consideration too, as well as the occurrence of non-target organisms that might be impaired by the genetically transferred traits. In the long run such a database could play an important role in the planned monitoring, given that the database would provide up-to-date knowledge and allow for quick integration of phenomena observed during monitoring, as well as information obtained from release-related research.
- When implementing the amendment to Directive 90/220/EEC (2001/18/EC) efforts shall be made to ensure that all national and European-wide expert opinions of scientific advisory bodies will be made publicly available. This is already being done e.g. by the Commission itself and in the United Kingdom.

Beyond that, a Europe-wide co-ordination of release-related biosafety research should be sought. Open questions should be identified at EU level and be dealt with in joint multi-country projects by ecologists, microbiologists, population biologists, agricultural scientist, nature conservation specialists and molecular biologists. Socio-economic aspects should be included in the respective individual projects.

As a consequence, when elaborating concepts and methodologies for resistance management plans, their implementation and related costs should be determined, as well as the changes in agricultural practice the implementation of such management plans would bring about and ensuing structural changes. Comparative analyses of different forms of agricultural cultivation using methods derived from life cycle assessments can provide valuable input into socio-economic assessment (see also KLÖPFFER et al., 1999) and should be promoted more strongly.

In future the importance of resistance management plans will increase, not only with regard to insect-resistant plants – and here we are mainly referring to plants expressing the *Bt*-endotoxin – but also with regard to herbicide-resistant plants (also see KORELL et al., 1997). Based on the concern that cultivating different crop species with the same herbicide-resistance gene would create a high selection pressure leading to the development of resistant weeds, several proposals have been developed which consider regional cultivation management plans covering several growing seasons in order to prevent the relevant weeds from developing resistance. The same applies to crop species with cross-fertile relatives which are endowed with different resistance genes and are cultivated on adjoining plots. Here wild relatives might develop differing resistances, which may cause new agronomic problems (KORELL et al., 1997). Should these considerations lead to the implementation of resistance management measures as part of risk management, it has to be borne in mind that the necessary planning and control measures will probably entail considerable cost. Therefore, the socio-economic consequences of large-scale cultivation should be modelled as early as possible and results integrated into the decision-making process.

The work and results of the release-related research should be continuously evaluated by an accompanying group of experts from the scientific community, industry, environmental organisations and public authorities. This group should comprise no more than 10-15 experts coming from different EU Member States. This group should have a fixed-term mandate.

In this group it would be possible to address and air opinions on more general aspects as well as to pinpoint open questions. These could include, for example, cumulative phenomena or possible synergistic effects from the effects produced by genetically modified organisms, which up to that point had not been considered, neither by the notifier nor in the framework of related research. The evaluations of this group would contribute to decision-making and the standard procedure of giving consent to the placing on the market and to the further development of research and industrial policies at both Community and Member State level.

In order to guarantee independent financing of this group, the authors of this study propose to establish a fund to which industry and public authorities could contribute.

In connection with the time-limited approval according to EU Directive 2001/18/EC, provision should be made for the imposition of further conditions, should new scientific finding render this necessary. In principle it should also be possible to revoke an approval within the given period of consent.

Detailed suggestions

Information required in notifications concerning releases of genetically modified organisms (concerning Annex III A and Annex III B of the amendment of EU Directive 90/220/EEC, 2001/18/EC).

In addition to the information which will be required according to Annex III A and Annex III B of the Directive 2001/18/EC, the following points should be included in the lists:

- A complete list of all laboratory, microcosm, mesocosm, and greenhouse experiments carried out by the notifier including information on the chosen methodology, the design of the experiment, the original data and the evaluation made in order to find out how the step-by-step principle was implemented.
- Information on sequence homologies between natural vectors/plasmids and the vectors constructed for the modifications in order to allow an assessment of a potential integration following a possible horizontal gene transfer via homologous recombination
- Information on possible sequence homologies between vector/gene construct and the DNA of microorganisms in the various habitats with which GMOs (may) interact.

This additional information could be laid down in the form of future guidelines. They might considerably facilitate risk assessments by providing a quick overview of the biosafety investigations carried out as well as of the ones missing, at the same time ensuring comparability with data and results of other notifiers as well as of related research.

Additional Information (regarding Annex IV)

Further information which should be provided in the event of notification for placing on the market:

- Data regarding agronomic and ecological parameters gathered during release experiments, the methodology applied and analyses carried out.
- Information on whether prior releases revealed phenotypic differences or differences in the genetic stability of GMOs, which were not observed in the greenhouse.

Scientific Committees

The scientific assessments of many observed phenomena often vary. In order to better reflect this dynamic process in the scientific debate, it should be possible for minority votes to be put forward (and subsequently published) in these scientific committees. To ensure better transparency, attention should be paid to correctly citing data and work which underpin the arguments when publishing the statements of the scientific committees.

In order to ensure maximum transparency and independence of members of the scientific committees, lists containing the names and professional activities of the members should be made publicly accessible.

1 INTRODUCTION AND SCOPE OF THE PROBLEM

Release experiments play an important role in connection with the expected ecological effects of genetically modified organisms (GMOs) and the risks associated therewith. They become all the more important when considering that with regard to many ecological effects of organisms it does not seem possible to extrapolate from laboratory and greenhouse data to field conditions. In fact, more often than not projections of the environmental behaviour of both transgenic and non-transgenic organisms miss the mark (COUNCIL OF EXPERTS FOR ENVIRONMENTAL ISSUES (*Rat von Sachverständigen für Umweltfragen*), 1998).

The reasons for this can partly be found in unsolved methodological problems. And some phenomena simply cannot be investigated in the laboratory or in the greenhouse. It is important to remember that data obtained in the laboratory or greenhouse are interpreted against the background of the knowledge on traits that can be identified only there and their relation to environmental effects of organisms. Failed attempts to correlate laboratory and greenhouse data with effects in the field also point to gaps in knowledge about ecological and ecosystem processes. It is generally held that these processes defy accurate projections since they are governed by many unpredictable parameters, such as the weather, mutations and, last but not least, anthropogenic modifications. This is why even in retrospect it is sometimes difficult to explain ecological phenomena.

When it comes to the deliberate release and placing on the market of GMOs this complex problem should be addressed with a keen sense of responsibility with a view to risk prevention, as foreseen in national and international legislation. However, although it is a fact that it is impossible to make accurate projections, it is important to develop methods that might lower the degree of uncertainty in order to come closer to the aim of damage prevention. Releases of GMOs and the accompanying ecological research are only **one** instrument to achieve this aim. Investigations in laboratories, mesocosms and greenhouses and finally investigation into the environmental behaviour of non-transgenic organisms and their biotic communities constitute further instruments. Eventually the post-approval monitoring, which still needs to be further developed both in terms of concept and method, will allow conclusions and revisions of preceding steps.

Here it is of central importance to continually integrate new knowledge gained in these different areas and to adhere to the sequence defined in the so-called step-by-step procedure. This means that in order to prevent or at least minimise the risks, decisions on the first step for loosening the containment (greenhouse experiments) should be based on the maximum available information obtained in contained systems, and so forth. With each decision about continuing the step-by-step procedure the latest knowledge (of the ecology of the relevant non-transgenic parent organisms, practices of agriculture and forestry) has to be taken into account.

In practice, there still is a relatively long way to go to reach this ideal. This can be demonstrated with the subject of this study: If release experiments are accorded a central role in identifying signs of (undesirable) environmental effects of transgenic organisms, the gathering, communication and discussion of environmentally-relevant release data ought to be of key importance.

However, the gathering of such data fails to meet expectations: it is estimated that world-wide ecological data are gathered in less than one per cent of all GMO releases. In the Federal Republic of Germany it is assumed that 15 % of all field experiments are accompanied by biosafety research (SUKOPP & SUKOPP; 1997). Furthermore it has to be admitted that release experiments mainly serve to gather data which are interesting from an agronomic point of view, i.e. regarding the intended marketing of the GMOs. In the Federal Republic of Germany there is no co-ordination of ecological release-related biosafety research (BRANDT, 1998). In the USA and at EU level there are research programmes on the risks from trans-

genic organisms, which focus on some key topics and hence provide some co-ordination. They, however, cover only part of the activities in this field and also projects on non-transgenic organisms (SCHÜTTE et al., 1998a).

The scientific communication of the environmental effects of transgenic organisms observed during releases is not gathered together in specific magazines, but dispersed over the most different publication media. It is often the case that data are not accessible for a long time, or never, because research projects frequently span several years and the results are mainly intended for the notification authorities. There are no databases designed specifically for effects observed with release experiments.

The first and so far only step towards gathering together information at an international level has been made by arranging symposia entitled ”The biosafety results of field releases of genetically modified plants and microorganisms”, which have been held every other year since 1990. The focus of these symposia goes beyond the environmental effects observed with GMO releases as such and covers ongoing projects and release-related laboratory and greenhouse research. However these symposia are far from covering all relevant research activities. One of the symposia which took place in Braunschweig, Germany, in 1998 focused on the presentation of agronomic data for the marketing of GMOs, whereas aspects of biosafety were marginalised. In Germany occasional conferences are held, which are dedicated to release-related research projects on plants and microorganisms (technical discussion on the “state of safety research regarding the release of genetically modified plants”, Hannover, 16 December 1997; BMBF Workshop “Release-related biosafety research with genetically modified plants and microorganisms”, Braunschweig, 25-26 May 1998). At the end of 1998 the German Federal Ministry of Health published a study summarising the state of release-related research (BUNDESGESUNDHEITSBLATT 1998).

There are similar and equally scattered activities of communication on environmental effects observed with releases of transgenic organisms in other countries within the framework of international conferences (e.g. PAN-EUROPEAN CONFERENCE 1993). However, the focus here is not exactly nor exclusively on the topic of environmental effects of release experiments.

The present study tries to close this gap. Within the scope of this study, as many relevant projects as possible were identified and data collected world-wide. In the light of the expected effects, individual cases are described in detail and discussed by means of selected examples of experiments with microorganisms, plants and animals. Also, by taking into consideration information on the environmental behaviour of GMOs obtained through means other than release experiments, conclusions are drawn and suggestions made that should be considered in risk assessment. Finally ways of research are documented and evaluated in order to facilitate future surveys and assessments of environmental effects observed with the release of transgenic organisms.

2 EFFECTS FROM THE RELEASE OF GENETICALLY MODIFIED ORGANISMS

2.1 Microorganisms

2.1.1 General investigation in the light of expected effects

There are more and more discussions about the use of genetically modified microorganisms (GMMO) and their potential for resolving many different problems. For example, enzymes are producued today with the help of GMMOs on a large scale. Although the production takes place in fermenters, it cannot be avoided that time and again genetically modified production organisms are released into the environment (DE VOS, 1998). But there are also deliberate releases of GMMOs constructed especially, e.g. to break down pollutants in soil and groundwater or as biological pest control in agriculture or as live vaccine in medicine.

Although the last years have seen an overwhelming increase in knowledge about microbial ecology, it is still impossible to predict the behaviour of GMMOs in the environment. For an assessment of the ecological consequences of GMMO releases into the environment, a definition of characteristic ecosystem structures and functions is needed. Also, the ecological function of microorganisms in their natural habitat is a key prerequisite for the projection of ecological effects (FÖRSTER, 1998). To date there are no generally acknowledged ecosystem endpoints for assessing the environmental risks from the release of GMMOs. Up to now many methods and parameters have been suggested and also applied in order to assess the ecological effects of the introduction of GMMOs. But the sensitivity of many of these methods is so low that only “catastrophic” changes in the ecosystem can be detected (FÖRSTER, 1998).

To date it has been impossible to investigate the effects of GMMOs on the ecosystem as a whole. However, the specific characteristics of microorganisms, such as quick generation times, adaptability to unfavourable environmental conditions, and the capacity for exchanging genetic material, have led to the development of several indicators pointing to potential damage or at least to the degree of impact resulting from the release of GMMOs. According to KLUEPFEL (1992) there are five ecological parameters relevant for the biological safety of GMMOs and recombinant DNA:

- persistence or capacity for survival,
- dispersal,
- population dynamics,
- competition between different microorganisms,
- impact on biotic communities in the surrounding environment.

Today safety assessments during the release of genetically modified microorganisms (GMMOs) focus on the persistence and dispersal of released microorganisms and of recombinant gene products. The considerations regarding the associated risks not only apply to microorganisms which are explicitly released for agricultural applications or for soil remediation experiments, but also to microorganisms which are used in fermenters, since it must be assumed that living GMMOs are released together with the discharge from these installations (DE VOS, 1998). In the following an overview is given of the capacity for survival of GMMOs and the persistence of isolated DNA in different environmental media. Based on this overview, current knowledge on the possibilities and limits of natural gene transfer and the dispersal of transgenes will be summarised.

2.1.1.1 Capacity for survival and dispersal of GMMOs

Many scientific papers have disproved the widely expressed view that GMMOs are at a disadvantage due to additional genes, referred to as extra burden (see AWONG et al., 1990; BARCINA et al., 1992; BOUMA & LENSKI, 1988; CHAO & FENG, 1990; GOLDSCHMIDT et al., 1994; KOZDROJ & PIOTROWSKA-SEGET, 1995; KOZDROJ, 1996a; LENKSI et al., 1994; REGAL, 1988 and 1994; SOBECKY et al., 1992; VAN ELSAS, 1992). In how far GMMOs do have effects on the environment mainly depends on how long they survive, their potential establishment, reproduction and dispersal. A series of investigations on the survival of transgenic microorganisms has shown that that they are able to survive over longer periods of time in different environmental media, e.g. sludge treatment plants, aquatic systems, soil or the digestive tract (summarised in ECKELKAMP et al., 1998a; TAPPESER et al., 1999).

Survival

The survival in the environment of recombinant microorganisms and the persistence of their genes depends on several associated factors. The capacity of GMMOs to establish themselves is influenced by biotic factors such as food supply, predators, density of bacteriophages, competing microorganisms or population density or by abiotic factors such as temperature, pH-value, oxygen demand, water and salt content and soil conditions (SMALLA et al., 1989; VAN ELSAS, 1992; DOYLE et al., 1995). Other important factors for their survival are the specific inherent genetic fixes of GMMOs (e.g. the formation of permanent states, spores) and the new traits directly or indirectly conferred via the transgenes. Some microorganisms are able to survive over long periods of time under bad environmental conditions by forming spores or cysts. Other microorganisms do not adopt such permanent states, but can change into a different temporary state in which they are viable but not culturable (VNC) (ECKELKAMP et al., 1998a; FÖRSTER, 1998; TAPPESER et al., 1999).

The above gives an account of how difficult it is to monitor and thus make statements on the capacity of GMMOs to survive in the environment. The validity of statements on the capacities of GMMOs for survival or dispersal depends mainly on the detection procedure chosen and the possibilities to survey different environments and stages of life. The examples of releases of genetically modified *Pseudomonas fluorescens* perfectly illustrate these difficulties. In 1993 and 1994 Thompson et al., inoculated sugar beet seeds with genetically modified *P. fluorescens* and released them (THOMPSON et al., 1995). The GMMOs successfully populated the roots and leaves of the sugar beets and could be detected throughout the whole growing season (270 days). The population dynamics of the GMMOs on the leaves of the sugar beets significantly varied in the different years of investigation. In 1993 with a share of less than six per cent the GMMOs only constituted a small part of the total Pseudomonadales population. Although after the first growing season the genetically modified *P. fluorescens* could be detected neither in the soil nor on the overwintering plants, their share rose to 81 % in the following year. Similar effects were found by DE LEIJ et al. after the release of wheat grains inoculated with transgenic *P. fluorescens* (DE LEIJ et al., 1995a). In this experiment after 319 days the number of GMMO cells dropped to the detection limit. Wheat grains germinating after the harvest or newly sown grains were nevertheless successfully populated by the GMMOs. LILLEY & BAILEY (1997a) also found both in the greenhouse and under field conditions population fluctuations of transgenic *P. fluorescens* strains, which depended on plant development. Following inoculation, the GMMOs first showed a reduced fitness on the leaves and the surfaces of the roots of young sugar beet plants. With increasing age of the inoculated plants this effect reversed: in the field GMMOs on leaves and roots of older plants were found to be more competitive than the wild type, in the greenhouse only on the roots.

Even after having been not detectable for a while GMMOs may reappear later and successfully colonise habitats. Changes in physiological characteristics, the capacity for adaptation, variations in the initial physiological condition and differing environmental parameters make

assessments of the capacity for survival under natural environmental conditions very difficult[1]. Adaptations often occur after longer periods of time. This is why safety assessments have to be long-term projects in order to achieve significant results. It is common practice to conclude investigations after a few weeks. At that point it is frequently determined that the number of cells of the released GMMOs rapidly decreases and that only a small share of the released GMMOs is able to survive. The frequent conclusion that GMMOs are not able to establish themselves in the investigated environment must be viewed with a critical eye: there are a number of examples showing that under changed environmental conditions, even after an apparently severe initial reduction of the number of cells, sometimes even below the detection limit, GMMOs may propagate again and entail a re-increase in the number of cells (CLEGG et al., 1995; GILLESPIE et al., 1995; KLUEPFEL et al., 1994; SJORGEN, 1995; THOMPSON et al., 1995). In general, literature published in the course of the last years indicates that the chances of survival of GMMOs have frequently been clearly underrated. This statement holds true both for GMMOs explicitly constructed for use in the field and the ones intended for use in contained systems (both points are discussed in detail in JÄGER & TAPPESER, 1996; ECKELKAMP et al., 1998a; TAPPESER et al., 1999).

In order to monitor the survival rate of GMMOs in the environment additional biological safety concepts were developed with the aim that bacteria released into the environment would eventually be eliminated by means of self-elimination. To date these "suicidal" systems have not been 100 % effective and it has to be expected that some mutants survive (RAMOS et al., 1995)[2]. In field experiments in Germany, for example, *Sinorhizoium meliloti* were genetically modified in such a way that they showed a reduced life span. The integration of a luciferase gene into the chromosomal recA-gene, which inter alia confers recombination, should have brought about a biological containment (SELBITSCHKA et al., 1994). These bacteria also showed reduced capacity for survival, albeit only in mode ecosystems. Under real release conditions this characteristic was not found when comparisons were made with the unmodified parent strains (DRESING et al., 1995) (see also chapter 2.2.4).

Dispersal of GMMOs

Another important aspect of the risk assessment of GMMOs is their dispersal from the release site into other habitats. Through this movement microorganisms may be transported from unfavourable environmental compartments into an environment where more favourable environmental conditions prevail. This transport can take place via wind, running waters and rain water as well as through attachment to tools or harvested goods. In addition, organisms such as protozoa, insects (e.g. spring-tails) and other soil animals such as earthworms, may serve as vectors (SCHMIDT 1991; CLEGG et al., 1995; HEIJNEN & MARINISSEN, 1995; WEISKEL et al., 1996). These organisms are able to actively move and they have their own intestinal flora, which may interact with the ingested microorganisms (BYZOV et al., 1993). This also applies to larger animals, mammals and birds, which contribute to the dispersal of microorganisms by transporting them over longer distances either in their digestive tract or stuck to their body surfaces (e.g. in fur or on feathers). In earthworms a non-genetically modified *P. fluorescens* strain could be detected in faeces for 50 days (HENSCHKE & SCHMIDT, 1989). In earthworms which were kept in microcosms with genetically modified *P. fluores-*

[1] It is often assumed that GMMOs that are released into the environment for a particular purpose, such as the degradation of pollutants in the environment, disappear from the environment if the environmental conditions change. It is forgotten though that GMMOs have the capacity to adapt to certain environmental conditions. Velicer showed that the chance of survival of GMMOs equipped with a transgene for the degradation of 2,4-D was independent of the presence of 2,4-D (VELICER 1999). The author concludes from these experiments that GMMOs can adapt to changing environmental conditions and can persist in the environment.

[2] Appropriate biological containment systems were developed in order to reduce the probability by four orders of magnitude (under 10^{-7}) for transfers of chromosomally integrated genes (MUNTHALI et al., 1996).

cens the GMMOs were detected for 15 days (CLEGG et al., 1995). LILLEY et al., have ascertained that leaf-colonising *P. fluorescens* can be transferred from infected to non-infected sugar beet plants by caterpillars of the genus owlet moth (*Memestra brassicae*) (LILLEY et al., 1997). Insects may not only disperse GMMOs that colonise leaves but also those which live in the root area. The latter may enter the plant via cracks in the roots and then be transported to the plant parts above ground (KLUEPFEL et al., 1994). From there they may also be ingested by herbivorous insects. In insects (Southern corn worm) feeding on transgenic corn plants colonised by *P. aureofaciens* GMMOs could be detected for 12.5 days. Furthermore they were able to transfer the GMMOs to non-infected plants (KLUEPFEL et al., 1994). It was established in an experiment that grasshoppers (red legged grasshoppers, *Melanoplus femurrubrum*) both in the field and in microcosms had the capacity to take up GMMOs colonising the rhizosphere from the corn plant parts situated above ground and to retain them in the gastro-intestinal tract for about a week and then to pass them on to non-infected corn plants. In the open environment the probability of a transfer increased with time from the GMMO uptake, although the absolute number of GMMO cells in the insects decreased (SNYDER et al., 1999). Kluepfel and Snyder (KLUEPFEL et al., 1994 and SNYDER et al., 1999) concluded from their results that phyllophagous insects are capable of taking up and transferring bacteria which originally belonged to the rhizosphere. Since this implies that also classic plant pathogens can be dispersed by insects over long distances, it can be assumed that released GMMOs from the rhizosphere can be transported to new host plants and into new habitats via the same means.

Vertical transfer in soil also entails risks. Groundwater may be contaminated with GMMOs, which may entail a risk to human health, or GMMOs may be transported into habitats where they have a higher chance of survival (NATSCH et al., 1996). From laboratory and microcosm studies it was concluded that after their release GMMOs are kept in the soil matrix and hence remain at the release site. The question remains as to whether this conclusion is justified or not. SMITH et al., (1985) have shown that under certain conditions microorganisms can be transported in soil quickly vertically with the macropore stream. Investigations with GMMOs also yielded differing results. In microcosms DE LEIJ et al. (1994) found that following inoculation of wheat grains with transgenic *P. aureofaciens* most of the GMMOs remained in the upper 15 cm of the rhizosphere of wheat plants, whereas a small part could be isolated even at a depth of 60 cm. The situation in the open environment is similar: in most cases the released GMMOs remain in the topmost soil layers. In the investigations carried out so far only a small number of GMMO cells were transported to a depth of 15 to 75 cm (HEIDENREICH, 1998). However, GMMOs may also reach lower soil layers. TROXLER et al. (1997a) investigated 2.5m field lysimeter columns after inoculation of *Pseudomonas fluorescens* CHA0 and found that the vertical transport of bacteria into deeper soil layers depended on the heaviness of rainfall. With normal rainfall transport was very slow and the inoculated *Pseudomonas* strain CHA0 was only transported through the lysimeter following a rainstorm after 200 days. However, if heavy rainfalls are simulated after the inoculation, the bacteria are transported much quicker into deeper soil layers (NATSCH et al., 1996). From this the authors draw the conclusion that under natural conditions, especially after heavy rainfalls, a vertical transfer of *Pseudomonas* into groundwater cannot be excluded (NATSCH et al., 1998a).

But GMMOs can also be transported **laterally**. Some investigations into lateral dispersal have shown that GMMOs remain at the release site or are only dispersed up to 40 cm (e.g. AMARGER & DELGUTTE, 1990; COOK et al., 1990; KLUEPFEL et al., 1991; THOMPSON et al., 1995). DE LEIJ et al. (1995a) ascertained the presence of transgenic *P. fluorescens* released with wheat grains one to two meters away from the release site. DANE & SHAW (1996) found released transgenic *Xanthomonas campestris* even at a distance of up to eight meters from the inoculated cabbage plants.

2.1.1.2 Competitiveness, damage of non-target organisms and ecosystem functions

A potential risk associated with the release of GMMOs is the elimination of species from the autochthonous microflora. An indication of the fitness of GMMOs is their capacity to displace the corresponding wild-type strain. This could happen either via direct competition for the same ecological niche or indirectly through the production of secondary metabolites.

Experiments in microcosms and in the field have revealed that, depending on the case, GMMOs can show a lower, the same or a higher competitiveness (HEIDENREICH, 1998). During an investigation period of 14 days KOZDROJ (1996b) found recombinant Tn5-mutants of *P. fluorescens* in soil and the rhizosphere to be less competitive than the corresponding non-transgenic parent strain and a naldixin-acid-resistant *P. fluorescens* strain. RAAIJMAKERS et al. (1995) found that in the radish rhizosphere a transgenic *P. fluorescens* strain may well compete with non-transgenic *Pseudomonas* strains. Lux-marked *P. fluorescens* investigated by CIRVILLERI & CALDERA (1998) were found to establish themselves on bean leaves as easily as the corresponding wild-type strains. DE LEIJ et al. (1995 a,b) observed that non-transgenic and transgenic LacZY/ Kan^r-marked *P. fluorescens* inoculated on summer wheat lead to a transient disturbance of the cultivable autochthonous microflora. CAROLL et al. (1995) and NATSCH et al. (1998b) observed similar transient population fluctuations following the release of GMMOs. However, in both cases the release of corresponding non-transgenic bacterial strains also caused significant population fluctuations in the investigated soil samples. Quickly growing microorganisms such as fluorescent *Pseudomonads* or yeasts were particularly affected. A transgenic *P. putida* strain developed for crop protection purposes, which produces the antifungal substance PCA (phenazine-1-carboxylic-acid), as well as a released non-transgenic bacterial strain transiently changed the fungus population in soil (GLANDORF, 1998). The effect of the GMMOs proved to be more long-lasting: the reduction of the fungus population ceased after 27 days following the release of non-transgenic strains, while remaining effective for up to 90 days with GMMOs.

Competitiveness strongly depends on the environmental conditions. KLUEPFEL et al. (1994) discovered that competition between transgenic LacZY-marked *P. aureofaciens* depended on the inoculated host plant species and the water content of the soil. Where corn roots were inoculated there was no difference in competitiveness between the two strains. Similar competition experiments with wheat revealed the competitive capacity to be dependent on soil moisture: if soil moisture remained below 12 % the transgenic strain was able to displace the non-transgenic parent strain (KLUEPFEL et al., 1994). Selection pressure can have a decisive influence on the competitive capacity of GMMOs. A transgenic *P. fluorescens* gene with a lactose marker gene produced by FEDI et al. (1996) in the absence of lactose in the rhizosphere of sugar beet has the same competitive strength as the non-transgenic parent strain inoculated at the same time. When lactose was added the population of the GMMO significantly decreased when compared with the parent strain, but stabilised at the same level after 27 days (FEDI et al., 1996). Also DOYLE et al. (1991) observed that the microflora in nutrient-poor soils may change as a result of GMMO inoculation. *P. putida* transgenes constructed to degrade the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were able to transiently reduce the populations of sporogenous and chitin-using bacteria. The application of the corresponding herbicide did not affect these population fluctuations. The release of GMMOs permanently reduced the total number of soil fungi. The rate of this reduction strongly increased with the presence of the herbicide. Without 2,4-D the effect was visible after 53 days, in soil enriched with 2,4-D already after 18 days. The degradation of the herbicide leads to an accumulation of a toxic intermediate product (2,4-dichlorophenol) in soil (DOYLE et al., 1991).

Apart from the microflora the release of GMMOs can also influence higher organisms. The release of rhizobia with improved nitrogen fixation may change the plant population through an increase of the nitrogen content in soil and thus, for example, lead to the establishment of new weeds (GIDDINGS, 1998).

2.1.1.3 Persistence and dispersal of gene constructs

In general, microorganisms are able to transfer genetic material within one species and between different species. These gene transfers are part of natural evolution and have led to the fact that today different bacteria species contain the same or similar antibiotic-resistance-genes (ECKELKAMP et al., 1998b). With risk considerations regarding the release of GMMOs it has to be taken into account that released microorganisms may themselves acquire genes from other microorganisms and thus change their ecological characteristics. On the other hand, GMMOs may confer their transgenes onto endogenous microorganism populations.

Persistence

The probability of gene transfer processes taking place largely depends on the amount and above all on the **stability of isolated DNA**. ECKELKAMP et al. (1998a) and TAPPESER et al. (1999) have summarised the current state of the scientific debate on DNA stability in various environments. As a rule, in sewage treatment plants isolated nucleic acids are degraded quickly and completely. Under certain conditions, however, even there intact DNA can be discovered over longer periods of time. Especially the fixation on sewage sludge floccules allows a longer persistence of isolated DNA (AARDEMA et al., 1993; GROSS et al., 1994). In aquatic media “naked” DNA is also to a large degree protected from degradation if it is associated with particles. In particle-poor waters, however, free DNA is generally degraded very quickly (AARDEMA et al., 1983; ALVAREZ et al., 1996; MIESCHENDAHL & DANNEBERG, 1994; ROMANOWSKI et al., 1993a). Depending on the soil type, and especially the mineral content, nucleic acids can be very persistent in soil. Several transformation experiments (KHANNA & STOTZKY, 1992; LORENZ & WACKERNAGEL, 1987; LORENZ et al., 1988; LORENZ & WACKERNAGEL, 1992; 1994; PAGET et al., 1992) show that in soils the DNA structure and the DNA sequences with complete genes, respectively, very often remain intact for a long time. In contrast to previous assumptions, nucleic acids are not completely fragmented in the digestive tract, but may enter the blood via the gastric epithelium and thus be incorporated into leucocytes or cells or body organs (SCHUBBERT et al., 1997).

Dispersal

Microorganisms can exchange genetic material by means of conjugation, transduction or transformation. All three kinds of transfer also occur with GMMOs. The transfer of genes from GMMOs onto other organisms is favoured under certain environmental conditions. Dead organic material under cultivated plants, nutrient-rich compost, biofilm on leaves, stones in waters or near roots are often conducive to gene transfer processes (ECKELKAMP et al., 1998a). Through the transfer of GMMOs into autochthonous microorganisms the transgene probably persists longer in the environment than the GMMO itself. Once the transgene has been transferred into another, possibly unknown microorganism, the consequences of such a transfer cannot be predicted.

- In nature, **conjugation** is probably the most effective gene transfer mechanism. Physiologically active, living cells are a prerequisite. In most cases plasmids are transferred, sometimes also chromosomal genes. For safety reasons, plasmids which are used in the laboratory usually do not have the essential structural characteristics for an autonomous conjugation (non self-transmissible tra^-/mob^- or mob^+ plasmids). However, if GMMOs are released, the self-transmissible plasmids occurring in autochthonous organisms may cause a conjugative transfer of non self-transmissible plasmids from GMMOs. LILLEY & BAILEY (1997b) found the transfer of a conjugative plasmid from the autochthonous microflora to a genetically modified *P. fluorescens* strain inoculated on sugar beet. Thus the probability of a transfer of plasmids from GMMOs can be reduced but not totally excluded. Especially under selection pressure, for example through the presence of antibiotics or herbicides, the

probability of a transfer of non self-transmissible plasmids increases (NATSCH et al., 1998a). The incorporated plasmids stemmed from 5 genetically different groups of large conjugative mercury-resistant plasmids.

- If conjugative plasmids from the autochthonous microflora are integrated in GMMOs, transgenes located on the bacterial chromosome can be mobilised. TROXLER et al. (1997a) have shown that in the presence of conjugative plasmids chromosomally located marker genes can be transferred from *Pseudomonas aeruginosa*.
- This allows the conclusion that the localisation of recombinant genes on the chromosome cannot completely prevent a horizontal gene transfer. For the mobilisation of chromosomal genes it is a prerequisite that the GMMO itself has a conjugative plasmid or incorporates one from the surrounding environment. The frequency at which conjugative plasmids are incorporated is unknown. Selection pressure is another important factor influencing transfer frequency (NATSCH, 1998).
- **Transduction** is a bacteria-induced DNA transfer between bacteria. It has been found under natural conditions in fresh and salt water, on the surfaces of leaves of plants and in yoghurt starter cultures (RIPP & MILLER, 1995; CHIURA, 1997; KIDAMBI et al., 1994; HELLER et al., 1996). Bacteriophages frequently infect more than one kind of bacteria. Transduction can contribute to gene transfer processes, especially in an environment with high phage titre and a high concentration of hosts, which can be found, for example, on particles of micro-environments. Transduction allows the complete transfer of genes and even plasmids between different bacterial hosts (ECKELKAMP et al., 1998a).
- If recombinant DNA persists in the environment for a longer period in time, it may be incorporated into the cells of the autochthonous microflora via **transformation processes**. In order for this to happen individual species of the autochthonous microflora must develop competence naturally. It is not sufficiently known which organisms have this capacity. The environmental conditions which lead to the development of competence are also only known for a few cases. Another prerequisite for a successful transformation is to overcome the restriction system of the recipient bacteria (ECKELKAMP et al., 1998a). However, for a recombinant DNA to establish itself in the natural microflora, it not only has to be incorporated but also has to be integrated into a replica. The integration into the bacterial genome seems to depend on the degree of sequence homology. This very often prevents the successful transformation with foreign DNA. However, this mechanism does not present an absolute barrier, since even in heterologous nucleic acid molecules there may be regions with matching sequences. Furthermore, homologous areas can be produced by means of transposable elements.
- The successful transformation of plasmids does not depend on matching sequences. Here the availability of suitable origins of replication and requisite promoters is of key importance. With the integration of the DNA, both the place of potential integration and the expression of the coding sequences can have an influence on the transformed bacteria and their survival (ECKELKAMP et al., 1998a).
- In investigations of sewage sludge samples from a treatment plant in Bavaria vector sequences were detected in sewage sludge organisms, which were identical to the sequences from the cloning vectors. This suggests that these sequences were incorporated via transformation processes from autochthonous microorganisms (LFU BAYERN, 1994).

2.1.1.4 Secondary effects from transformation

With genetic modifications it is frequently not possible to place transferred genes in the recipient genome. This is why transgenes at the site of their integration can destroy genes, change the interaction between genes or establish new interactions (JÄGER, 1994). The function of the newly introduced genes can be influenced by the new environment, given that the information content of the genes is not only determined by the sequence of the nucleotides but also by the environment in the genome. It is still impossible to predict pleiotropisms or position effects[3]. Whereas it has become relatively easy to decode the sequence of the nucleotides, the analysis of the complex higher contextual dependencies associated with genetic information. Such effects can modify the ecologically important characteristics of GMMOs. Many authors assume that secondary effects of transformation reduce the fitness of the GMMOs concerned and thus rather reduce than increase the risk (REGAL, 1988; WILLIAMSON & FITTER, 1996). However, in some instances this assumption has already been disproved. The transformation of a rhizobia strain with a *Bt*-toxin gene by Giddings (GIDDINGS et al., 1997) has made it easier for the transgenic GMMO to colonise pea nodules than for the non-transgenic parent strain. This characteristic of the GMMO was a side effect of the transformation and could not be explained by the cloned *Bt*-sequence which was to confer a higher pest resistance only. Moreover, this characteristic of the GMMO remained stable over a period of three years.

2.2 Microorganisms, case-specific

2.2.1 Survey

In 1995 Doyle et al. wrote a first review that summarises the knowledge gained of the effects of GMMOs in various environmental media (DOYLE et al., 1995). Although in the meantime genetically modified microorganisms are routinely dealt with on different scales, from laboratories to commercially used devices, in 1995 only 15 to 20 GMMOs had been tested for their environmental effects (see table 1).

In their evaluation the authors arrive at the following assessment: GMMOs are able to

- successfully compete with indigenous microorganisms of disturbed ecosystems,
- transfer their new genes onto indigenous organisms, which in turn can be expressed in the new hosts as well,
- influence general and specific metabolic activities as well as the metabolic rate of biomass,
- influence the structure of biocoenoses and their functions in various habitats,
- change the interaction between symbionts and organisms of various trophic levels,
- create metabolites that have unanticipated effects on the environment.

With respect to the conceptual design of such tests the authors emphasize that a larger number of various tests would be necessary to actually identify and assess potential environmental effects.

[3] POSITION EFFECT: A modification in the normal expression of a gene due to a change its position in the genome. PLEIOTROPISM: The control of several apparently unrelated characteristics by a single gene.

Tab. 1: Overview of release experiments with microorganisms with ecological issues (status 1995)

Organism	Medium tested	Source
Erwinia carotovora subsp. Carotovora	aquatic	SCANFERLATO et al., 1989; SCANFERLATO et al., 1990
Pseudomonas sp. B13FR1	aquatic	WAGNER-DÖBLER et al., 1992
Alcaligenes sp.	aquatic	FULTHORPE and WYNDHAM, 1989, 1991, 1992
Pseudomonas putida	activated sludge	McCLURE et al., 1991a,b
Erwinia carotovora subsp. Carotovora	soil	ORVOS et al., 1990
Streptomyces lividans	soil	WANG et al., 1989; WANG et al., 1990
Pseudomonas solanacearum	soil	AUSTIN et al., 1990 WILLIAMSON and HARTEL, 1991
Azospirillum lipoferum	soil	BENTGEN et al., 1989; BOLTON et al., 1991a,b; FREDRICKSON et al., 1989, 1990
Pseudomonas cepacia	soil	BEG et al., 1991
Pseudomonas fluorescens	soil	TREVORS 1991
E.coli (two strains) *Enterobacter cloacae* *Pseudomonas putida (two strains)*	soil	DOYLE and STOTZKY, 1993; STOTZKY et al., 1992
Pseudomonas syringae	plants	LINDOW and PANOPOULOS, 1988
Lactobacillus plantarum	plants	SHARP et al., 1992
Clavibacter xyli subsp. Cyrodontis	plants	FESTER, 1992
Pseudomonas aureofaciens	plants	ENGLAND et al., 1993
Bradyrhizobium sp.	plants	NAMBIAR et al., 1990
Rhizobium leguminosarum bv viciae *R. leguminosarum bv trifolii*	plants	SKOT et al., 1990

source: DOYLE et al. (1995)

Some of the above studies were carried out in microcosms and greenhouses, a few were designed as release experiments, such as tests with what is called the ice-minus bacterium *P.syringae.*

2.2.2 Ice Minus: *Pseudomonas syringae*

One of the first and most well-known releases of GMMOs was carried out with the „Ice-Minus" strain of a genetically modified *pseudomonas syringae* (frostban), which was to curb frost damage in plants (LINDOW, 1987). The GMMO contained a deletion in the so-called iceC gene. The protein this gene produces acts as cristallization germ in the freezing of water. In agriculture, this property leads to frost damage, since the bacterium enhances ice formation even at temperatures of slightly below 0° C. By deleting the iceC gene, the formation of ice crystals was to be prevented. In the laboratory, the Ice-Minus GMMOs were able to successfully compete with their parent organisms and could thus curb frost damage. The research team of Lindow et al. also sprayed the GMMO on open strawberry and potato fields and studied the population dynamics of the bacteria on the plants (LINDOW & PANOPOULOS, 1988). In the soil, the GMMOs showed no high capacity for survival. In the open envi-

ronment, the Ice-Minus bacteria could no longer be detected at a distance of 30 metres from the release site. Also, the bacteria could not be traced in the surrounding vegetation nor in the surface water. On wheat growing in the vicinity there had been colonizations of highly concentrated autochthonous Ice-Plus *P. syringae* strains prior to the spray experiments, which may have prevented the colonization of GMMOs. Still, the GMMOs were only identified by means of selective media and special DNA probes. The authors gave no indication as to the detection limit for these techniques. The data on capacity for survival have thus to be considered with a certain degree of uncertainty (VON SCHELL, 1992) .

2.2.3 *Klebsiella planticola*

A research team around soil ecologist INGHAM has tested capacity for survival and effects of genetically modified klebsiellae in various types of soil by means of an experimental design that imitates as exactly as possible the habitat and the nutritive environment. *Klebsiella planticola* is a harmless soil bacterium of safety level 1 that has been endowed with an additional gene to produce alcohol (the pyruvate-decarboxylase gene from *Zymomonas mobilis*). To examine their capacity for survival and competitiveness, the genetically modified Klebsiellae were applied in soil columns of different arable soil types. Two of the results of the complex experiments are outlined as follows.

1. The genetically modified Klebsiellae survived in all of the tested soil types, even if alcohol production did not take place in all soil types. The presence of growing plants had a positive effect on the survival of the genetically modified Klebsiellae and changed the interaction between the various organisms of the soil flora and fauna.
2. In certain soils, the tested genetically modified *Klebsiella planticola* caused plants growing on them to die. This effect appears to be due to a chain of effects. After the inoculation of the transgenic strain the nematodes in the soil that feed on bacteria and soil fungi multiply significantly. Particularly fungivorous nematodes appear to cause the death of plants by an action mechanism that is not yet clarified.

It can thus be assumed that the effects of genetically modified organisms are not only expected to occur directly, that is from the immediate effect on a specific target organism, but may be triggered by action chains and shifts in the population density and population fabric of other groups of organisms (HOLMES, 1995; HOLMES et al., 1998).

2.2.4 Rhizobia

In a joint project, in which five research teams were involved, transgenic *Sinorhizobium melliloti* were released in Germany. In 1994, the releases were carried out in soil columns, in 1995 they took place in an open soil environment (KELLER et al., 1997). The transgenic bacteria were two model strains. Both were transformed with the constitutively expressed *luc*-gene from the North American glow-worm (*Photinus pyralis*). In strain L 1 the foreign gene was inserted into the endogenous *RecA*-gene, which causes these bacteria to be *recA* deficient, whereas in the *luc*-transgenic *S.mellioti* strain L 33 the recA function remained unimpaired (SELBITSCHKA et al., 1994).

Under laboratory conditions in liquid cultures and microcosms the $RecA^-$ strain naturally showed increased sensitiveness to DNA-damaging agents and a lower capacity for survival as well as nodulation competitiveness compared to the $RecA^+$ strain, which was considered a wild type. The release experiments served to test the use of the $RecA^-$ strain as safety strain (KELLER et al., 1997).

In the field experiment with soil columns expectations seemed to materialize to the extent that only the $RecA^+$ strain showed a slight growth (10^6 CFU/g soil in twelve months) after a de-

crease in the cell number at the beginning of the experiment (from 10^6 to 10^4 CFU/g soil in six months). The RecA$^-$ strain, by contrast, remained at the constant level of 10^4 CFU/g soil for six months after the initial decrease in cell number.

In open soil release the cell number of the RecA$^-$ strain decreased more significantly in the first three months, to 3 x 10^3 CFU/g soil, whereas the RecA$^+$ strain retained only 3 x 10^4 CFU/g soil. After seven months, however, both strains reached 10^4 CFU/g soil. This colonization density remained over several years. In the aggregate, no differences could be identified between the various strains in open environments (MIETHLING et al., 1999). In one case, however, heat stress had a more adverse effect on the RecA$^-$ than on the RecA$^+$ strain (DRESING et al., 1998). Moreover, transgenic bacteria were also found outside the area on which they had been applied. It is assumed that this is mainly due to aerosol formation at the time of application (KELLER et al., 1997; LOTZ et al., 1999). However, probably due to washing out bacteria can also be dispersed over a distance of more than 100 metres in two to four years (SIEBER et al., 1994). On areas that had not been inoculated with transgenic bacteria they were found at 10^2 CFU/g soil, in the rhizosphere of clover and a non-host plant they were found at 10^5 CFU/g soil root wet weight.

In the faeces of insects on the inoculated areas some GMMOs were identified (KELLER et al., 1997).

In 1995 and 1996 rhizobia release experiments were also carried out in Italy. These rhizobia also bore only one marker gene (lacZ). The nodulation capacity of the geneticlally modified rhizobia was partly lower, the capacity for survival of the tested strains was, however, strong. After one year, in all inoculated plots of land as much as 10^2 to 10^4 CFU/g soil could be identified (NUTI et al., 1997).

In the course of this project methods were developed that are able to detect an influence of genetically modified rhizobia on endogenous populations of cultivable rhizospheric bacteria. Still, conclusive findings have not yet been made (TICHY & SIMON, 1999).

On the whole the project described is highly appropriate for elaborating investigative and reviewing techniques. Still, it may contain only few indications of the behaviour of the respective bacteria, since the commercially interesting interferences and modifications aimed at influencing nodulation capacities and nitrogen fixation performance will surely produce other environmental effects under establishment, dispersal and competition conditions.

A report by associates of the Environmental Protection Agency of the USA (who are not mentioned by name) points out some inconsistencies and problems with respect to the approval of a rhizobia strain. The report is not so much about observed effects, but rather focuses on safety tests that have not been carried out or further evaluated. The authors mention among others:

- gene transfer investigations of closely related bacteria such as Agrobacteria and Phylobacteria, but also of animal pathogenic relatives such as *Bartonella* and others;
- representative colonization experiments with other leguminous plants, in particular those that cause weed problems (PEER, White Paper, 1995).

In the aggregate it was found that the population capacity of other leguminous plants was caused by the RMBPC strain, which is due for commercialization (COMMERCIALISATION REQUEST, Dec. 1994).

2.2.5 *Pseudomonas sp.*

RAMOS et al. (1997) tested pollutant-decomposing transgenic *Pseudomonas putida* that were cultivated in pots in two field experiments. The experiments were supposed to demonstrate the efficiency of a biological safety system inherent in these bacteria. This suicide system is linked to the genetically conferred capacity of bacteria to decompose 3-methyl-benzoic acid (3 MB). As soon as the substrate 3 MB is missing, the *gef* gene conferred onto the pseudomonads from *Escherichia coli* is to be switched on. The *gef* protein that is fitted into the cell membrane leads to cell death by causing the membrane potential to disintegrate.

More specifically, the above procedure rests on a complicated genetic construction: The *gef* gene was linked to the *plac* promotor. The pm promotor, which controls the genes of the 3 MB-decomposing operon, was merged with the *lacI*-gene, which codes for the *lac*I-repressor. If the substrate-dependent P*m*-promotor is active, the gef-gene is to become inactive by means of the *lac*I-repressor. If the P*m*-promotor, however, is inactive in the absence of substrate, no lacI-repressor is formed and no gef-protein is synthesized.

So much for the theory, which was confirmed in laboratory experiments with two transgenic *P. putida* strains.[4] GMMOs with pollutant-decomposing operon without the suicide system grew equally well with or without 3MB. By contrast, the transgenic strains with the suicide gene grew only when 3MB was added. These findings were also made in liquid minimum media as well as in terrestrial and aquatic mesocosms.

For the release experiments with these bacteria carried out in Spain from 1995 to 1996 variations with and without 3MB as well as with and without plant growth (*Zea mays*) were chosen. The experiments ran for 112 days, and no dispersal of GMMOs was found outside the pots. Surprisingly, both “safety strains” with the suicide system were able to colonize the roots of the plants, independently of whether or not the soil contained 3MB.

This may possibly be interpreted as evolutionary adjustment as VELICER (1999) could observe in field experiments with natural isolates of the genus of *Burkholderia*. Two strains that had acquired the capacity of decomposing the herbicide 2,4-D were tested for their capacity to make better use of alternative C-sources in the course of time and thus achieve a fitness benefit. Out of twenty tested lines two showed a distinct fitness gain. Velicer concludes from his findings that adaptation to anthropogenic substrates can increase competitiveness insofar as at the same time, due to pleiotropic effects of adaptation, better use is made of natural substrates. He assumes that this increases the likelihood of genetically modified organisms to persist in the environment, even in the absence of anthropogenic substrate (or if it has been decomposed).

GERMIDA et al. (1998) also found phenotypical adaptation over time in release experiments with genetically modified *Pseudomonas aureofaciens* with lacZY as marker gene. The degree of adaptation was, however, rather low. In the soil lysimeter, however, the strain proved highly persisent under field conditions.

In experiments with *Psueudomonas fluorescens*, which fluoresce in the process of decomposing naphtalene, the capacity for survival of these bacteria in aerosols was examined in Oake Ridge. These bacteria showed the highest capacity for survival, and thus dispersal potential, in conditions of low relative humidity and light wind. Once established in the soil, they proved highly persistent (FORD et al., 1999)[5].

Interesting findings were also made by TCHELET et al. (1999). The *Pseudomonas sp* strain P 51, which is able to synthesize 1,2,4-trichlorobenzene (TCB), was tested in microcosms that

[4] One strain (*P. putida* EEZ 30) carries the suicide gene in the chromosome and the control element on a mob$^+$-tra-plasmid. In the other strain (*P. putida* CMC4) both elements are integrated in the chromosome.

[5] There are no indications as to the testing period. It just refers to “over long periods”.

were filled with TCB and non-TCB sewage sludge of a sludge treatment plant. The other test system consisted of a water-saturated non-sterile soil column. In the soil column the bacteria dispersed entirely, grew to a concentration of $2x10^6$ cells in the soil and continued decomposing TCB. In the TCB sludge columns the bacteria titre fell directly after the inoculation by ten raised to the power of two. After more than two days the cell number fell below the detection limit. There was hardly any decomposition of TCB. In the non-TCB column the cell titre remained at 10^5cells/ml (inoculation concentration 10^7) over eight days, but afterwards also fell below the detection limit. These experiments demonstrate how strong the influence of the environment is on the capacity for survival of individual strains and that, depending on the environment, different effects or enzymatic actions occur.

2.3 Plants

2.3.1 General Research in the Light of Anticipated Effects

2.3.1.1 Dispersal

The (undesired) dispersal of transgenic plants is basically considered a risk, an assumption which is governed by agronomic considerations: There is a certain concern that increased efforts are necessary to fight volunteers and the growth of transgenic weeds and -grasses. The dispersal potential of genetically modified plants is, however, also considered an ecological risk, for which there is no uniform assessment standard. The displacement of other animal and plant species is generally considered a damage. There is, however, disagreement as to whether the increase of certain fitness parameters (capacity for survival, seed production, etc.) or the potential of displacement of individual colonizations of organisms beyond the level of non-transgenic reference plants is to be considered an indication of future displacement procedures to be expected in the long run.

SUKOPP & SUKOPP (1993) found medium-term time lags for the establishment of exotic plants, i.e. 32, 68 and 147 years for annual or biennial species, persistent shrubs and wood species. Considering these time lags, during which continued measurings can hardly be carried out, it appears inevitable to include indications of delayed displacement procedures in risk assessment.

Whether or not the integration of genes and gene sequences into entirely new, genetic contexts, which by way of evolution were separated from each other a long time ago, carries an ecological risk remains unknown and is mostly denied. It has not yet been clarified what impact the confrontation of rhizospheric organisms with plant roots expressing the entero-bacteria-typical kanamycin resistance gene *npt II* has on their population dynamics and -genetics, irrespective of the possibility of a horizontal transfer of this gene. If this view is taken seriously, the reference point of the non-transgenic control plants, with which the transgenic plants are to be compared in the process of risk assessment, will no longer apply; the comparison with control plants is, however, often left out and there is no uniform method of carrying out the comparison (PURRINGTON & BERGELSON, 1995; BERGELSON et al., 1996). In such a case the issue alone of whether the transgenic plant surpasses the non-transgenic reference plant in terms of dispersal potential would no longer apply without the fact that the integration into ecological contexts of DNA and protein sequences separated so far by way of evolution is considered a novelty *per se* that requires assessment of the consequences.

Preliminary assessment of the dispersal potential of transgenic plants generally includes the known properties of non-transgenic parent plants and the intended new properties to be created by the transgene(s). “Risk candidates” among plants are those that already have a certain potential for running wild. The problem is that such assessments strongly vary from

country to country and from region to region and that they require in-depth knowledge and update surveys on the flora in the region where the intended release or commercialization is to take place. Moreover, at least in some cases, differences in the properties of the parent varieties play a role. For instance, it is assumed that the high degree of variation in terms of persistence capacity of rape seeds is due to such differences (SCHLINK, 1994). Eventually, cultivation techniques have an impact on persistence and dispersal of cultivated plants. Rape seeds, for instance, may develop secondary dormancy in deeper layers of the soil (DIETZ-PFEILSTETTER et al., 1999) and it is assumed that weed growth in an unknown, yet considerable part of cultivated plant populations is due to the unintended anthropogenous dispersal of diaspores (SNOW et al., 1998).

Apart from the parent plant, genes and gene constructs transferred by means of genetic engineering play a vital role in preliminary risk assessment. However, the impact of the intended genetic property modification on the dispersal behaviour of GMOs remains largely unclarified. There are few investigations on the subject, and expectations as to the dispersal potential of GMOs as well as the interpretation of data vary among different experts. There are extensive discussions on the question of whether the expression of a transgene in itself has an impact on the energy balance of transgenic plants strong enough to put them at a disadvantage in relation to non-transgenic reference plants in terms of dispersal potential (genetic load- or excess baggage theory). Even if this issue is not conclusively clarified scientifically, there is little doubt about its significance in risk assessment: The integration of transgenes **may** at least have no negative impact on the energy balance of the respective plant, regardless of the possible fitness-affecting potential of the specific genetically conferred quality (e.g. BERGELSON et al., 1996; HAILS et al., 1997). Thus, for the purpose of risk assessment, it must be assumed that genetic modifications do not in themselves interfere with the dispersal potential of the plants and that usually lines that are selected in the cultivation process show no fitness impairment in relation to parent lines.

Eventually, most genetic modifications pursue the declared aim of making plants more resistant to pest organisms, diseases and abiotic stress, thus to factors that contribute to curb dispersal potential. However, there is surprisingly little basic ecological knowledge on the issue. The impact of pest-, disease or stress resistance on the dispersal potential of plants is only marginally investigated into, irrespective of whether or not genetically conferred qualities are at work (e.g. RAYBOULD et al., 1998; SNOW et al., 1998). Against this background it is hardly surprising that assessments of the dispersal potential of plants vary.

The following are examples of transgenes that are likely to increase the dispersal potential of plants:

- resistance to insects, fungi, viruses and diseases
- resistance to heat, cold, salt and stress
- modified seed lipids (LINDER & SCHMITT, 1995)
- herbicide resistance (ECKELKAMP et al., 1997a; SNOW et al., 1998).

„Side effects" of genetic modification do not readily lend themselves to preliminary assessment of the impact on the dispersal potential of transgenic plants. They manifest themselves in that transgenic plants often do not appear to simply exhibit the sum total of properties of the non-transgenic parent plants and the deliberately genetically conferred properties. The reasons for this may lie in the uncontrollable integration site of the transgenic constructs, in the fact that their number can be selected only afterwards, giving rise to mutagenesis, as well as position effects. Pleiotropic effects as well as somaclonal variation in plants that regenerate from cell cultures must also be taken into consideration.

Preliminary assessement of the dispersal potential based on the known properties of the parent plants and transgenic constructs must thus be complemented by testing the GMOs them-

selves. Release experiments, among others, would be an appropriate device. Still, preliminary laboratory and greenhouse experiments appear inevitable, for reasons of safety as well as considering the small experimental effort. Laboratory and greenhouse experiments have the further advantage that effects observed there can be correlated to certain factors more clearly.

2.3.1.2 Gene Transfer

The vertical gene transfer of transgenes by intraspecific and interspecific hybridization onto related species of cultivated and wild plants is another, much discussed safety aspect of the utilization of genetically modified plants (ELLSTRAND & HOFFMANN, 1990; RAYBOULD & GRAY 1993, 1994; SNOW & MORAN-PALMA, 1997). In Europe, large-scale tests on hybridization potential and probabilities were carried out for rape and sugar beet, since they are native plants. The possibilities of gene flow from cultivated plants into non-cultivated wild forms of the same species or into related species present themselves differently in the various regions of the world, since apart from the question of whether the release takes place in a "centre of origin" or "centre of diversity", also differing surrounding weed flora and thus differing hybridization partners must be considered in relation to the same species when assessing the risk. Special concern arises when considering the possibility that vital agricultural weeds that are related to the corresponding useful plant may acquire fitness-enhancing qualities by means of outcrossing (DANIELS & SHEAIL, 1999). In the course of "normal" release experiments there have usually been no investigations on hybridization. Still, research projects have been carried out that address this very issue.

2.4 Examples of Plants with High Dispersal and Hybridization Potential

2.4.1 *Brassica napus* (Rape)

2.4.1.1 Possibilities of Establishment and Dispersal

The occurrence of rape outside the cultivated land of Central Europe is assessed differently. It is often assumed that the colonization is of an ephemeral character, and more permanent colonizations – as grow along streets or railway tracks – persist due to the steady supply of seeds from transport losses and/or disorders of the habitat (CRAWLEY et al., 1993). ADOLPHI (1995), however, rules this out as far as colonizations in the Rhine Region are concerned, which have established themselves there. In Canada, rape widely occurs outside cultivated land (WARWICK, 1997).

Rape seeds are hardy up to –20 °C. They are germinable over a long period of time, which is shown by the fact that the second growth (volunteer oilseed rape) of the secondary crop must be eliminated (TORGERSEN, 1996).[6] Given its high propagation potential, its growth behaviour and its germination ecology, rape is very similar to field weeds (SCHLINK, 1994).

For some years the increased occurrence of rape outside arable land has been observed. Rape colonizations often occur in ruderal sites, field fringes and traffic routes (SEBALD et al., 1990; TORGERSEN, 1996). TOMIUK et al. (1996b) conclude from more recent developments that rape "is basically likely to establish itself". Since in various investigations with herbicide-tolerant rape no differences have been found between transgenic rape and conventional rape in terms of competitive behaviour, it must be assumed that transgenic also carries a potential of running wild (AGREVO, 1996, S. 23; FREDSHAVN et al., 1995).

[6] SCHLINK (1994) found that after 1.5 years as much as 70 % and after 5 years up to 58 % of the rape seeds in the soil were germinable. Such high persistence rates are otherwise only achieved by weed seeds (MAYER et al., 1995).

2.4.1.2 Release Experiments on the Dispersal Potential

In large-scale release experiments CRAWLEY et al. (1993) tested two transgenic rape lines, one resistant to the antibiotic kanamycin and the herbicide glufosinate, the other resistant to kanamycin only , in comparison to a non-transgenic control line as well as to the wild rape relative *Sinapis arvensis L*..The experiments took place at four different sites and in three different climatic zones of England.

CRAWLEY et al. (1993) conclude from these experiments that the transgenic plants are not superior to the non-transgenic ones in terms of their dispersal behaviour. HAILS et al. (1997) describe in detail the findings of these release experiments in relation to the capacity for survival of the seeds that have been planted into the soil in two different depth layers. They arrive at the conclusion that the capacity for survival of the seeds of the transgenic plants was lower than that of the non-transgenic control line. However, after two years of experiment, this result was significant in only five of twelve habitats.

LINDER & SCHMITT (1995) investigated the impact of genetically modified seed lipids on the dispersal potential of the plants taking the example of rape. In release experiments at a site in Georgia the capacity for survival of buried transgenic rape seeds with an increased stearin acid content surpassed the capacity for survival of the seeds of non-transgenic controls (non-segregated control lines and parent lines). At another site in California, by contrast, no difference was observed between the transgenic and non-transgenic seeds. In greenhouse experiments the possibility of increased dispersal potential of the transgenic plants did not manifest itself. The germination rate of the buried transgenic seeds did surpass that of the non-transgenic parent plants, yet the transgenic germ buds fell behind the controls in terms of growth rate and biomass.

In the greenhouse the seeds of another transgenic rape line with increased lauric acid content differed from the parent plants only with respect to the time lag of the growth rate: The retarded growth of the seed buds in the first two weeks was made up for in the following two weeks.

By comparison, hybrids from the transgenic plant with increased lauric acid content and turnip rape proved equal to their wild parents in greenhouse experiments and did not surpass non-transgenic hybrid control plants in terms of germination rate and growth rate (LINDER & SCHMITT, 1995).

STEWART et al. (1997) claim to be the first to publish a report on release experiments with transgenic plants in which a dispersal-enhancing effect of the transgene was investigated and found (see chapter 2.4.2.2, BARTSCH et al., 1995). In these experiments, transgenic insect-resistant rape plants with a *Bt*-gene (one line with a strong and one line with a weak transgenic expression) were compared on cultivated plots with non-transgenic commercial parent lines (OSCAR and WESTAR) with seeds sown in the winter. Infestation with pest organisms was simulated in plots separated by tents. The transgenic plants showed better survival of strong pest infestation than the control plants. The differences in terms of survival were not significant where the infestation was only minor. However, the transgenic plants on an average produced more seeds with moderate or strong pest infestation. At non-cultivated sites only two transgenic plants survived the winter.

2.4.1.3 Possibilites of Hybridization

Rape is a native plant of Europe with a variety of cross-fertile relatives. The outcrossing of rape into adjacent rape fields was underestimated for a long time. The possible reasons for this may be that hybridizations vary in terms of specific genetic structure and environmental parameters. Different experimental designs may also lead to differing assessments of the hybridization rate. Thus, completely different data on the cross fertilisation rate of rape are found in literature (SCHEFFLER et al., 1993; FELDMANN, 1997; TIMMONS et al., 1995a).

The experiments by the above research teams demonstrate that the outcrossing rates depend to a great extent on the experimental design: In general one can say that as the field size increases the probability rises that transgenes are dispersed over vaster distances.

Furthermore, the results of crossing experiments prove that gene flow may occur from rape into weed populations. Potential hybridization partners of *Brassica napus* are not only found within the genus of *Brassica*, but also in the larger family of crucifers (SCHEFFLER & DALE, 1994). The potential hybridization partners of rape are weeds that probably are all largely cross-fertilized. According to DARMENCY (1994), this high cross-fertilisation rate facilitates the dispersal of transgenes from rape into the related surrounding weeds.

Gene Flow from Rape to Rape

The National Institute of Agricultural Botany (NIAB) in Cambridge, England carried out accompanying investigations on the gene flow between transgenic and conventional rape species in variety tests for the national list. Two glufosinate-resistant winter rape varieties and one glyphosate-resistant winter rape variety were tested. Simultaneously, five non-transgenic, commercial varieties were integrated in the experiment. Accompanying testing was carried out at four different sites. Gene flow from transgenic to non-transgenic varieties was documented in three experimental designs. The frequency of incrossing generally decreased with distance, but showed a high degree of variability in relation to the non-transgenic varieties. Particularly the variety of Synergy was highly “susceptible” to pollen cross fertilisation even at a greater distance. Hybrids double-tolerant to glufosinate and glyphosate were found at all sites (SIMPSON et al., 1999). Double-resistant hybrids were also found in Canada under commercial cultivation conditions (DOWNEY, 1999). In early 2000 even the occurrence of triple resistant rape plants was reported from Alta in the Federal State of Alberta, Canada (Western Producer, Saskatchewan, Canada, February 10, 2000).

Experiments with male sterile pitcher plants at a distance of 400 metres from the experiment field demonstrate that rape pollen are carried by the wind over wide distances and also remain fertile under these conditions (SIMPSON et al., 1999). In further experiments by the Scottish Crop Research Institute the regional dispersal of rape pollen to adjacent fields was tested under the most realistic agricultural conditions. In an area of 70 km² male sterile pitcher plant colonies during the flowering period were set up at 52 sites. They were positioned at distances of between zero and 4000 metres from the transgenic crops. On the whole a region was selected in which a large part of the agricultural area is cultivated with conventional rape.

In all pitcher plants the incrossing of transgenic pollen was found, with frequency decreasing with distance.

However, partly even at longer distances a high degree of variability in terms of incrossing frequency was found (e.g. between 13 % and 57.9 % at a distance of 500 metres). The authors conclude from these findings that in every cultivation region there is a continuing network of cross-hybridization between rape plants.

In their opinion, the strong gene flow will lead to volunteer oilseed rape and weed populations, which will serve as a gene pool for newly introduced genes, regardless of whether cultivation takes place at closer or further distances (THOMPSON et al., 1999).

Hybridization with Cross-Fertile Relatives

Under field conditions it was possible to achieve hybridization of transgenic rape with **turnip rape** (*Brassica rapa*), Indian mustard (*Brassica juncea*), black mustard (*Brassica nigra*), *Hirschfeldia incana*, (synonymous with *Brassica adpressa*), wild radish (*Raphanus raphansitrum*), and wild mustard (*Sinapis arvensis*) (described in detail in ECKELKAMP et al., 1997a as well as CHÈVRE et al., 1999).

MIKKELSEN et al. (1996) found that under field conditions herbicide-tolerant rape may spontaneously hybridize with turnip rape (*Brassica campestris*). It took only two generations for this outcrossing to produce fertile, transgenic, herbicide-tolerant offspring with wild plant properties. Thus, when it comes to agricultural cultivation, it may be assumed that transgenic rape distinctly hybridizes with turnip rape. These findings were confirmed in further experiments (SNOW & JØRGENSEN, 1999).

Hybridizations of rape with wild radish (*Raphanus raphanistrum*), which occurs in Austria, are also possible (EBER et al., 1994; DARMENCY et al., 1995; LEFOL et al., 1996). In an experiment approach the outcrossing produced fertile, transgenic, herbicide-tolerant offspring with wild plant properties in four generations (CHÈVRE et al., 1997). Even under Australian conditions interspecific hybridization between *Brassica napus* and *Raphanus raphanistrum* was found. Further investigations on the hybridization potential with the agricultural weeds *Brassica tournefortii* and *Diptotaxis murialis*, which are important in Australia, are planned.

Hybridizations between transgenic secondary crop rape and ruderal rape populations with **Indian mustard** (*Brassica juncea*), which grows wild, but is often grown as intermediate crop, are also possible (SCHEFFLER & DALE, 1994).

In Switzerland, it is estimated that there is a high probability of gene flow from rape into **hedge mustard** (*Eruca sativa*) (JACOT, 1994).

Viable hybrids may also be created from hybridizations of rape with wild mustard (*Sinapis alba)*, (JACOT, 1994; SUKOPP & SUKOPP, 1994; FISCHBECK, 1995). In further experiments in England and France it was again confirmed that it was basically possible to hybridize *Brassica napus* and *Sinapis arvensis*. Still, hybrids could only be produced, if *Brassica napus* played the female part (MOYES et al., 1999).

Hybridizations between sterile rape plants and *Hirschfeldia incana* under field conditions also produced viable hybrids (DARMENCY, 1994). A gene flow of transgenic rape to *Hirschfeldia incana* is forecast particularly for the mediterranean area, where *Hirschfeldia incana* often occurs (LEFOL, 1996).

In Canada, the possible incrossing of transgenes into *Erucastrum gallicum* is investigated with great attention. Hybridization with rape as the female part produced strong, very fertile plants in the greenhouse, which are likely to have a high chance of survival in field conditions. Investigations on further back-crossing generations have as yet not been completed (DOWNEY, 1999).

2.4.2 Beta vulgaris ssp. vulgaris (sugar beet)

2.4.2.1 Dispersal Potential

Sugar beets have a potential of running wild. In many European countries and in the USA they are found as weeds. Hornsey and Arnold report of their occurrence on road edges, in the middle of paths as well as on fallow land (HORNSEY & ARNOLD, 1979, page 279). The growth of weed beet populations on cultivated land takes place via bolting in the cultivated biennial forms. BARTSCH et al. (1999) report of the growth of a weed beet population after only twelve years of sugar beet cultivation in crop rotation with wheat and maize on an area of more than 80,000 plants/hectares close to the Belgian border near Aachen.

2.4.2.2 Hybridization Potential

Beta-beets do not have any crossing barriers within their species. There are crosses among the various cultivated forms as well as between wild forms and cultivated forms (inter alia, DE VRIES, 1992). This is also what German accompanying research projects have found,

which were carried out at the University of Aachen. No crossing barriers were found between transgenic variations and other cultivated or wild forms. The authors estimate that a dispersal of the transgenes after commercialization is sure to take place (POHL-ORF et al., 1999.

BARTSCH et al. (1995) appear to be the first to carry out accompanying investigations with genetically modified plants that were to be protected by the transgene against natural enemies. They compared transgenic sugar beets resistant to the beet necrotic yellow vein virus (BNYVV)[7] with non-transgenic plants of the same cultivation line as well as with a conventionally cultivated beet necrotic yellow vein virus-resistant sugar beet variety. In the first release experiments the transgenic plants did worse with virus infestation than the non-transgenic ones, which is apparently due to inbreeding in the genetically modified sugar beets. In further release experiments transgenic sugar beet hybrids with virus infestation were, however, superior to the virus-sensitive control plants. The conventionally cultivated virus-tolerant sugar beets were, however, most successful in competition experiments in two consecutive years of investigation (PARKER & BARTSCH, 1996).

2.5 Target Effects: Resistance Development/Increase in Virulence in Pests or Pathogens

Genetically modified plants are developed, inter alia, in an attempt to combat as specifically as possible target organisms that cause problems in agricultural cultivation. In this expertise target effects shall refer to those effects of transgenic plants on the viruses, bacteria, fungi, insects and weeds that they are supposed to fight. As of yet, risk analyses of environmental effects of genetically modified plants on target organisms have focused on two aspects:

2.5.1 Resistance Development of Surrounding Weeds and Plant Pest Organisms

There is a certain concern that in insect-, virus-, bacteria- or fungus-resistant or herbicide-resistant plants, the pest organisms that are to be combated might soon overcome the genetically conferred resistance mechanisms via adjustment or resistance development. Resistance generally refers to the ability of an organism, whether a plant, bacterium, fungus or animal, unlike other organisms of the same species, to tolerate a certain outside influence. It can basically be assumed that all pathogens have a potential for building up resistances. The important parameters for the development of resistances are the genotype of the respective organism and the intensity of selection pressure. In agricultural cultivation, there have been problems due to anti-resistance capacities in a number of pathogens and surrounding weeds. In the meantime, resistances of many pathogens against certain insecticides have come to be known (GEORGHIOU, 1990). The development of resistances against *Bacillus thuringiensis* toxins was identified as one of the greatest agronomic risks that *Bt*-transgenic plants carry. However, developments of resistances differ in speed and probability. In an attempt to delay them, in the USA for instance the cultivation of *Bt*-maize and *Bt*-cotton was tied to resistance management requirements (MELLON & RISSLER, 1998; NATIONAL CORN GROWER REPORT, 1999).

[7] The transgenic plants also contained the glufosonate resistance gene *bar* and the kanamycin resistance gene *npt II.*

2.5.2 Resistance Management in *Bt*-Maize

It can basically be assumed that all insects have the potential for developing resistances. The speed at which they develop (and if they develop) in *Bt*-plants depends on the following factors (GOULD et al., 1998; RAPS et al., 1998):

- the resistance mechanism
- the inheritance pattern of resistance
- the biology and population dynamics of the pathogens concerned
- the selection pressure exerted by the cultivation of *Bt*-plants.

The **inheritance pattern** of resistance is a decisive factor in the resistance development of pathogens. Important parameters are the transmission mechanism (dominant, intermediary, recessive) and the initial frequency of the resistance allele. Concerning European Corn Borer (ECB) there are as of yet no experimental data on the transmission pattern and the resistance allele frequencies in the field. Thus, the development of resistance management is based on theoretical models. Risk assessment has so far assumed that potential *Bt*-resistance in European Corn Borers is transmitted recessively and that it occurs in populations at low allele frequency (see also KLÖPFFER et al., 1999).

More recent laboratory investigations by HUANG et al. (1999), however, indicate that there are European Corn Borer populations which transmit their resistance in a part-dominant manner. Also, low initial allele frequencies cannot always be assumed. Experiments with a different butterfly species show that frequency may be considerably higher than originally assumed. The selection of resistant *Heliothis virescens*, a pest of cotton, showed an allele frequency that was up to 1000 times higher, that is 1.5×10^{-3}, than it had thus far been assumed in the models (GOULD et al., 1997; KLÖPFFER et al., 1999).

The **biology and mobility** of the pathogen also determine the dispersal of resistance genes in a pest colonization. Adult *Ostrinia nubilalis* butterflies have a relatively high degree of mobility. If resistance is transmitted recessively, the cultivation of non-transgenic maize refugia theoretically creates favourable conditions for delaying the development of resistance for a while. However, this is only the case if the development of European Corn Borer larvae takes place simultaneously on transgenic and non-transgenic maize (RAPS et al., 1998). In a laboratory experiment it was shown that in the cotton pest *Pectinophora gossipela* ("Pink Bollworm") the development of resistant populations was delayed by five to six days (LIU et al., 1999). It remains to be investigated if these results are significant for field conditions. In case the resistant and non-resistant populations become sexually mature at different times, they will not copulate and the dispersal of resistance genes in a population would be accelerated.

The **selection pressure** that transgenic plants exert depends on the *Bt*-expression level and pattern in transgenic plants and on the size of the cultivated area (ANDOW & HUTCHINSON, 1998; RAPS et al., 1998). The degree of *Bt*-concentration in the plants is decisive for the survival of European Corn Borer larvae. To make sure that the lowest possible number of heterocygous caterpillars survive, the toxin level should not be below a certain (high) level. This guarantees that as few partly-resistant European Corn Borer individuals as possible survive. If they then have the possibility to copulate with non-resistant individuals in the refugium, resistance development is delayed.

So far insects treated with *Bt*-injections have hardly shown any resistance, because the *Bt*-preparations contain several protoxin variations that due to their susceptibility to ultraviolet light are rather short-lived. In transgenic *Bt*-plants, by contrast, the *Bt*-toxin is expressed in an active form and over a long period of time. Under these circumstances the selection pressure is much higher than with *Bt*-spore preparations (BERNHARDT et al., 1991). The US Environmental Protection Agency has therefore decided to prescribe resistance management plans. The above parameters have contributed to the specific design of the plans, which focus on "high toxin expression" along with the cultivation of "refugia" (MELLON & RISSLER, 1998).

In the early stage, when the share of *Bt*-maize is still low as compared to conventional maize, the „refugium/high-dose“ strategy (ANDOW and HUTCHINSON, 1998) has proved successful. The companies Novartis, Mycogen and DeKalb (now Monsanto) have cultivated, maize varieties, the expression level of which declines quite early in the growing period. This is problematic particularly in view of the findings made by HUANG et al. (1999). A “moderate” expression level could not suffice to kill partly-resistant European Corn Borers.

Recommendations as to the minimum size of **untreated** refugia range from 4 % to 50 % of the total cultivated area (according to GOULD et al., 19997; STEIN and LOTSTEIN, 1996, 4 % are recommended by the Environmental Protection Agency,). ANDOW and HUTCHINSON (1998) recommend a minimum size of 25 %. The share of *Bt*-maize in a region may however exceed 50 % only if the European Corn Borer can also attack alternative host plants and thus be available to “alternative refugia” (KLÖPFFER et al., 1999).

The Novartis *Bt*-176 maize can only be cultivated according to the refugium/high-dose strategy in regions in which only one pyralid moth generation develops. This maize offers only little protection against the second pyralid moth generation, because the expression level of CrylA(b) sharply declines in the course of the growing period and the toxin is not synthesized in the cob. For *Bt*-176 maize ANDOW and HUTCHINSON (1998) thus recommend that in regions with several pyralid moth generations the size of the refugium be 50 % refugium (**without** insecticide treatment).

More specifically, in 1999 the EPA decided to pass regulations for three different maize lines, according to which on a certain section of the total cultivated area *Bt*-maize must not be planted. The size of the *Bt*-maize-free area depends on whether or not these areas have been treated with pesticides. In particular, the following ratios were prescribed: for the *Bt*-maize by Monsanto 20 % treated or 10 % untreated, for Novartis (popcorn) 40 % treated, 20–30 % untreated and for AgrEvo (PGS) 40 % sprayed, 25 % unsprayed. In the light of the above, more recent results it is doubtful if these measures are sufficient to prevent fast resistance development.

The refugium strategy as a whole will have to be questioned if the findings by LIU et al. (1999) also hold true for European Corn Borers in open environments and if the development of resistant and non-resistant European Corn Borers does not take place simultaneously.

2.5.3 Herbicide Resistance

Whether the herbicide resistance strategy is fit for practice will depend on whether weeds develop resistances against these herbicides. In conventional cultivation it was shown that weeds have a potential for becoming resistant to herbicides (ECKELKAMP et al., 1997a). A great number of plant cultures have already been made insensitive to the herbicides Liberty, Roundup, Bromoxinyl and sulfonyl urea by means of genetic engineering. If genetically modified plants prove successful in agricultural practice, these herbicides will be used on vast areas in the future. Every herbicide that is used on a large scale is expected to trigger surrounding weeds to develop resistances on account of high selection pressure. In 1996 it was first reported that a glyphosate resistant grass (*Lolium rigidum*) in Australia could no longer be eliminated by Roundup. This case attracted attention, since until then resistance development against glyphosate had been considered unlikely (WEBER, 1997). In transgenic plants surrounding weeds do not only develop resistances in the typical way. Genetically modified herbicide-resistant plants can transmit their transgenes also to related cultivated and wild plants and weeds, which in turn become resistant against the respective herbicides. If they disperse, these plants may cause problems as weeds in the most varied agricultural crops that are treated with the respective complementary herbicides (see also chapter 2.4). The problems caused by resistant surrounding weeds as a consequence of the cultivation of transgenic herbicide-resistant plants may surpass by far the problems with herbicides on account of the possibility of gene transfer and the expected increase in the use of a few complementary herbicides (increased selection pressure) (see also chapter 2.4.1.3).

2.5.4 Development of new Virus Variations

In virus-resistant plants the development of new virus variations and/or new courses of infection are considered to be a risk. Here too the effects on cultivated plants dominate, yet also wild plants are infected by viruses. Their impact on the population dynamics of plants is, however, hardly clarified (RAYBOULD et al., 1998).

There is a variety of different strategies to make plants more resistant to viruses by means of genetic engineering. In most cases the plants are transformed with viral genome sections. 94 % of the viruses are RNA viruses, of which complementary DNA sequences (cDNA) are cloned (MATTHEWS, 1991): Mostly they are genes that code for viral coat proteins. Still, also viral replication and transport protein genes as well as satellite sequences are integrated into the plant genome.[8] In addition, antisense protein-coding viral sequences are cloned.[9] Investigations of the utilization of proteases, protease inhibitors and ribozymes are rather recent.[10] Another new approach is the transformation with non-viral nucleic acid sequences, which code for animal antibodies, interferones or 2'–5' oligo-andenylate synthetase (JÄGER & WEBER, 1993, HENRY et al., 1995; ECKELKAMP et al., 1997b; WEBER et al., 1998).

At the centre of risk research on virus-resistant transgenic plants is the agronomically relevant question if genetic strategies to confer virus-resistance can change the dispersal and evolution of viruses and if plant viruses or highly harmful viral plant diseases and epidemics are provoked in the process. Safety research must investigate if the processes that carry a risk actually take place in transgenic plants and, if so, if they occur at a higher frequency than in nature, or if new combinations are possible that have not existed in nature so far.

In general, three main risks associated with transgenic plants that have been transformed with viral nucleic acid sequences are disussed:

1. **Recombinations** can take place between the viral genetic information cloned in plants and infectious viruses. RNA recombinations are basically possible and also occur in mixed virus infections. RNA recombinations may produce viruses with genetically embodied increased fitness and pathogenesis as well as modified host range.

   It is largely unknown how many times recombinations take place in plant viruses (GIBBS et al., 1997). Apparently different virus types, as well as different viruses of the same type differ in terms of intraspecific recombination rate (REVERS et al., 1996, CANDERESSE et al., 1997). Maybe differing recombination frequencies that have been found reflect actual differences in the attained evolutionary balance of the viruses. In many cases, however, they may be hidden on account of missing systematic investigations.

   LOMMEL & YOUNG (1991) were the first to show that RNA plant viruses can recombine with the mRNA of viral sequences cloned in plants. Their experiments carried out with red clover necrotic mosaic dianthovirus (RCNMVI) were similar to the later experiments by GREENE & ALLISON (1994). In both cases, the transgenic plants were infected with disabled viruses that were made capable of systematically infecting the plants only by recombining with the cloned wild type sequence. Experiments with a strong selection pressure made it possible to detect recombinations of the DNA virus cauliflower mosaic caulimovirus (CaMV) with genome sections of this virus cloned in plants (GAL et al., 1992; SCHOELZ & WINTERMANTEL, 1993). MAISS et al. (1997) identified the recombination of potyviruses with transgenically expressed potyviral sequences in plants under selection pressure.

[8] Satellite RNAs occur in some RNA viruses in addition to the virus genome. They code for no functions essential to the respective virus, but may influence weight and symptoms of the course of the infection. Satellite RNAs in turn require a helper virus for their replication, wrapping and transmission, with which there is however no significant degree of sequence homology.

[9] Antisense clonings lead protein biosynthesis to run in the opposite direction, from the end of the coding sequence to its beginning. The simultaneous synthesis of the respective protein in the normal sense can thus be inhibited.

[10] Ribozymes are RNA molecules with RNA splitting enzymatic activity.

WINTERMANTEL & SCHOELZ (1996) showed, however, that even under moderate or low selection pressure virus combinants can be formed in transgenic plants. They worked with *Nicotiana bigelovii* plants that had been transformed with the gene VI of a CaMV strain, which enables CaMV to systemically infect these and other host plants. The transgenic plants were infected with another CaMV strain, which is also capable of systemically infecting them. There was no obvious selection pressure that would enhance recombinations or recombinants. Still, in 13 % of the tested plants virus recombinations were found, which besides were more virulent than the wild type virus they descend from. A similarly high recombination frequency was found in a chimeric CaMV that can systemically infect the transgenic plants, because it is complemented by the recombinant gene product. This experimental design is characterized by only moderate selection pressure.

Two of the recombinants isolated by SCHOELZ & WINTERMANTEL (1993) had an enlarged host area in relation to the parent organism. One of the recombinations caused reduced symptoms in turnip rape. Still, the authors basically consider it possible that recombined viruses cause worse symptoms than those viruses that originally infected the transgenic plants. By means of recombinations in transgenic *Nicotiana benthamiana* plants that expressed the CCMV coat protein gene, mutants of the cowpea chlorotic mottle bromovirus (CCMV) with a deletion in the coat protein gene were able to systemically infect these very plants. Three out of seven recombinants caused modified symptoms in cow pea plants in relation to the wild type.

In coat protein transgenic plants it is possible that recombination of infectious viruses with the cloned sequences gives rise to genetically embodied modifications of the course of the infection and the vector- and host plant spectrum. Furthermore, recombinations may cause high-fitness viruses to grow in plants that express viral sequences.

2. Moreover, in plants that were transformed with a viral coat protein gene **heterologous encapsidations** may occur. In this case the nucleic acid of a virus whose propagation is at least not totally inhibited by the cloned coat protein is wholly or partly wrapped by recombinant CP. Heterologous encapsidations also occur in mixed infections with various viruses. They may change viral qualities that are determined or influenced by the coat protein, such as the vector or the host range and the course of the disease.

   Heterologous encapsidations were more often found between viruses of the same virus type than between viruses of different types. They also occur between non-related viruses. Heterologous encapsidations appear to be spread differently in different virus types and to occur in unequal specificity. In potyviruses, for instance, heterologous encapsidations between viruses of this type occur relatively easily. Luteo and nepoviruses show a low degree of specificity in terms of the wrapped RNA (COOPER et al., 1994; MAISS et al., 1994). These findings, however, are influenced by differences in the intensity of the research on different viruses and virus types.

   Heterologous encapsidations were also found in CP-transgenic plants, that is between RNA and coat proteins of viruses of the same virus type in the case of potyviruses (FARINELLI et al., 1992; LECOQ et al., 1993; MAISS et al., 1994) as well as between viruses of different types. The cucumber mosaic cucumovirus (CMV) in tobacco plants is thus wrapped by transgenically expressed CP of the alfalfa mosaic virus (AMV).

   Heterologous encapsidation may help a transmission-deficient virus to transmit by means of vectors.[11] BOURDIN & LECOQ (1991) assume that heterologous encapsidations may for instance explain the transmission and maintenance of non-transmissible virus isolates in the environment. What is of more ecological importance, the authors say, is probably the change of vector, which may open another host range to the virus. The effects are confined to one growing season, since they are not genetically embodied.

[11] transmission: vector transmission

3. **Synergisms** may occur between the cloned gene or gene product and the infected viruses. Plants into whose genome viral genetic information has been integrated are mostly resistant to the virus from which the cloned sequence stems and to related viruses, but not to non-related viruses. It has as yet not been investigated to what extent the plants show synergisms with heterologous viruses (PALUKAITIS & KAPLAN, 1997): In some cases, such effects were observed in the transformation with intact viral replicase and transport protein genes (TASCHNER et al., 1991, COOPER et al., 1995). Viral synergisms are also known to occur in mixed infections of non-transgenic plants. If they occur in transgenic plants, the effects are, unlike with recombinations, not genetically embodied.

2.5.5 Non-target-Effects

Apart from the damage to target organisms, the cultivation of transgenic plants may directly or indirectly effect organisms that have not originally been targeted. In this study non-target effects refer to effects that transgenic plants have on organisms they are not directed at. Non-target effects also refer to effects that occur as side-effects of transformations and that are not linked to the recombinant gene product (see also ch. 4.2.4). Effects on non-target organisms due to modified cultivation methods shall be discussed in chapter 2.6.

According to RAPS et al. (1998) the following non-target effects are to be taken into consideration:

- **effects on non-target pests**
  Non-target pests may be damaged sublethally by transgenic plants and thus be displaced for a short time. If the organisms concerned form resistances in the following period and suddenly propagate, they may do severe harm to the crops concerned.
- **effects on useful plants and natural enemies of pest organisms**
  An essential element of integrated or ecological agriculture is the maintenance and enhancement of the balance between pests and their enemies or useful plants. It is therefore important to investigate the impact the cultivation of transgenic plants has on these organisms. The cultivation of transgenic plants may also indirectly lead to changes in the original flora and fauna and thus cause changes in the insect fauna living in combinations.

Field experiments studying the effects of transgenic plants on non-target organisms have so far been carried out by companies that intend to place transgenic plants on the market (e.g. CIBA-APPLICATION, 1994). The research the authors have carried out has shown that there have been no effects of transgenic plants on non-target organisms, which finding was also confirmed in a personal communication by Angelika Hilbeck (FAL Zurich, 1999), who is doing experimental research work in this field.

Still, investigations in laboratories or greenhouses pointed to possible direct or indirect damage of useful plants caused by transgenic insect-resistant plants:

PHAM-DELEGUE (1997) found that feeding sugar solutions with purified proteinase-inhibitor (PI) proteins may shorten the life span of bees and interfere with their learning behaviour. PICARD-NIZOU et al. (1995, 1997) investigated into the possible damage that may be done to bees by transgenic rape plants, each of which expressed chitinase (from beans), β-1,3 glucanase (from tomatoes) or a proteinase inhibitor (Cp II:cowpea trypsin inhibitor). The subject of the experiment was the effect of isolated recombinant proteins. These experiments found no acute toxic effect in the three proteins. The behaviour of the bees in their search for food also remained unimpaired. Still, β-1,3 glucanase and CpTI from transgenic rape plants had a negative impact on the learning behaviour of adult bees (PICARD-NIZOU et al., 1997).

BIRCH et al. (1997) found lectin-expressing potatoes to be harmful to ladybirds (*Adalia bipunctata*). Lectin-expression caused the aphid population to decrease by 50 %. Ladybirds that had lived on aphids surviving on lectin potatoes were found to be less fertile. The life span of female ladybirds was half of that of the control animals that had eaten aphids of non-transgenic potatoes.

Laboratory experiments with transgenic *Bt*-maize showed a harmful effect on a useful insect species. Green lacewing larvae (*Chrysoperla carnea*) died at an increased rate compared to the controls after being fed with European Corn Borers, which had been killed after the consumption of *Bt*-maize. What was most surprising was the fact that the green lacewing larvae also died after being fed African cotton worm if the latter had eaten *Bt*-maize before, although the cotton worm itself is not attacked by the *Bt*-toxin (HILBECK et al., 1998a). HILBECK et al. (1998b) found in further experiments that green lacewing larvae also die if fed synthetic food containing *Bt*-toxins. *Bt*-toxins can thus have a direct effect on green lacewings. When classic *Bt*-spores were applied, only minor effects of *Bt*-toxins on useful organisms were found.

The *Bt*-toxin (CrylAb) expressed in *Bt*-maize is specific to lepidopterans (butterflies). It thus affects not only European Corn Borers specifically, but also other butterfly caterpillars. Since maize cultures are only infected by European Corn Borers, it was assumed for a long time that *Bt*-maize would practically not do any harm to other butterflies. Recent findings published in the scientific magazine "*Nature*" suggest, however, that *Bt*-maize could also become dangerous to other butterflies in the vicinity of *Bt*-maize fields. If maize pollen containing *Bt*-toxin are blown by the wind to adjacent milkweed, they may seriously threaten the larvae of the monarch butterfly living there. In laboratory feeding experiments carried out in the USA only half of the butterfly caterpillars survived, if the leaves of their host plant were covered with *Bt*-maize pollen (HANSEN & OBRICKI, 1999; LOSEY et al., 1999). Similar findings were made by researchers of the Iowa State University, who for their feeding experiments had collected pollen-covered milk weed in *Bt*-maize fields. In nature milk weed is the only food for caterpillars of the monarch butterfly. In the USA it is frequently found in the neighbourhood of maize fields. Maize pollen containing *Bt*, which are scattered by the wind, can thus be harmful to monarch caterpillars which feed on milk weed plants in the neighbourhood of *Bt*-maize fields. This finding was surprising in that until then it had been assumed that only butterfly larvae could be damaged, which as the European Corn Borer directly feed on transgenic *Bt*-maize plants.

The findings by BIRCH et al. (1997) and HILBECK et al. (1998 a,b) suggest that not only direct effects of *Bt* maize plants have to be expected, but also effects that skip some links of the food chain. RAPS et al. (1998) have found that the studies by HILBECK et al. (1998 a,b) demonstrate that "modified application of pesticides may cause side-effects and that when transgenic plants are released the environmental safety of substances, even of well-known ones, must be re-investigated."

2.5.6 Effects on Decomposers

The activity of decomposers living in the soil is considered an important parameter when assessing soil quality (RAPS et al., 1998). Safety experiments on the release of transgenic plants are carried out to identify their effects on these organisms. To examine the effect of *Bt*-maize on rainworms, laboratory experiments with the compost worm *Eisenia foetida* were carried out and revealed no acute toxicity of *Bt*-maize (CIBA APPLICATION, 1994). In laboratory experiments, however, effects on springtails were found, which play a vital role in the decomposition of litter. Highly concentrated (LD_{50} 250 mg leaf protein per kg soil) *Bt*-maize δ endotoxin had a toxic effect on the spring tail species *Folsomia candida*. The EPA (1995) assumes that the δ endotoxin quantities from *Bt*-176 maize are not sufficient to enrich the soil to such an extent. There have been no long-term studies on the sublethal effect of *Bt* toxin (EPA, Bt Fact Sheet 1995).

Findings published in December 1999 by the research team of Strotzky (SAXENA et al., 1999) cast doubts on this assessment. The scientists found that with the exudate of the roots, *Bt*-toxin is steadily emitted into the soil and that this toxin is absorbed by soil particles and thus remains stable for a long time. In said experiment the toxin could be detected in the soil for 243 days and also kept its toxic potential. Together with toxin molecules released from decomposing plant material quite enormous toxin concentrations may build up in the soil.

DONEGAN et al. (1997) in their laboratory decomposition experiments also discovered that springtails were impaired by transgenic proteinase-inhibitor expressing tobacco plants. Compared to the control the number of spring tails was strongly reduced; the nematode density, on the contrary, was significantly increased.

2.5.7 Effects on Soil Microorganisms

Microorganisms play a vital role in geochemical substance cycles and are also responsible for the fertility of the soil. There is however little knowledge about which microorganism populations interact and how in order to cause these effects. It is estimated that only 1 per cent of microorganisms occurring in the soil is known (TORSVIK et al., 1990). It can thus not be assessed how changes in the composition of populations affect soil fertility.

In spite of, or because of, this lack of knowledge, it is essential to investigate the effects of transgenic plants on microbial soil life. Investigations by DONEGAN et al. (1995) show that it is not sufficient to study only the effect of recombinant gene products. They showed that *Bt*-cotton leaves incorporated into the soil changed the composition of soil microorganism colonizations. The effect was not caused by the delta endotoxin itself, but due to a side-effect of the genetic modification (DONEGAN et al., 1995). The transformation of plants can thus cause unanticipated changes in the metabolism of plants, which in turn can have an impact on soil life.

2.6 Effects of changes in management methods

Considerations of effects caused potentially by changes in management methods suggest the following possibilities:

The broad-action pesticides applied so far might as a „side effect“ have acted also upon secondary pathogens in some cultures. Once this form of pest control is stopped because the methods of genetic engineering are applied, conditions are favourable for these pathogens to propagate.

The selective control of one pathogen that has been dominant so far opens up niches for other pathogens.

The application of herbicides may produce changes of the weed flora and thus of the insect flora living in combination with it. This applies especially to total herbicides.

Some data such as the infestation with a pest to date unknown in the course of a release of transgenic insect-resistant trees suggest that the above considerations have to be taken seriously into account (see chapter 2.8.1.5). This is necessary also when considering increased infestation rates under certain conditions, requiring the application of larger quantities of pesticides in certain regions, which has been reported in the case of cotton (see chapter 2.7.4).

2.7 Plants, case-specific

2.7.1 Exemplary Case Description and Discussion of the Experiments of CRAWLEY et al. (1993) and HAILS et al. (1997) in the Light of Anticipated Effects

The experiments carried out and analysed by CRAWLEY et al. (1993) and HAILS et al. (1997) leave open many questions concerning the dispersal of rape, or they provide no definite answers.

2.7.1.1 Comparability with the Conditions under Cultivation

The fundamental drawback of the experimental pattern chosen by CRAWLEY et al. (1993) is the absence of any selection pressure on the transgenic plants that might be comparable to the conditions under cultivation. Since the experiments were carried out without the application of herbicides or kanamycin their aim was to detect unintended and unpredictable increases in fitness, which may be a side effect of genetic modification. If herbicide-resistant plants were cultivated, the application of the complementary total herbicide would bring about a selective advantage for the transgenic plants which could increase their potential for dispersal considerably (WEBER, 1995; PARKER & BARTSCH, 1996).

2.7.1.2 Suitability of the Selected Plants with Regard to the Issues Being Studied and the Generalisation of Results

Summer rape lines of seeds sown in the spring were studied although the cultivation of winter rape is more common in Europe (DIETZ-PFEILSTETTER et al., 1999). It is therefore uncertain whether the results apply to winter rape. Seeds produced from a high degree of inbreeding were used. Of the seeds of the transgenic lines, only some were truly transgenic and inconsistencies were found as far as the zygotes of the transgene were concerned.[12] It is therefore uncertain whether the lines studied can be compared with plants intended for cultivation where inbreeding is avoided because of inbreeding depression. One also aims to achieve a high degree of purity of the seeds as well as a close to 100 % expression of the transgenic trait. When considering the topic under discussion (influence of the transformation on dispersal), lines produced from inbreeding that are inconsistent as regards the zygotes of the transgene seem unsuitable. This problem is only partly resolved through comparison with the non-transgenic control rape because plants produced from inbreeding would probably fare "better", i.e. resemble the wild-growing plant more in terms of fitness, in comparison with *Sinapis arvensis L*.[13]

CRAWLEY et al. (1993) as well as HAILS et al. (1997) do not discuss the reasons why deep-buried seeds do not exhibit secondary dormancy, a phenomenon observed with rape seeds (DIETZ-PFEILSTÄTTER et al., 1999). It is possible that this phenomenon varies with different plants and that the lines studied were not representative of plants that can exhibit secondary dormancy.

[12] According to CRAWLEY et al. (1993), 65 % of the kanamycin- and glufosinate-resistant line were homozygous transgenes, 27 % heterozygous transgenes and 8 % no transgenes. HAILS et al. (1997) state that of the same plants only 65 % are transgenes (62 % homozygous and 3 % heterozygous) referring to identical information from SCHEFFLER et al. (1993). HAILS et al. (1997) also think it likely that the transgenic lines studied by them vary with respect to the zygotes of the transgenes, which may have an influence on the fitness of the plants.

[13] Besides, the control plants are not described in detail in the publications of CRAWLEY et al. (1993) and HAILS et al. (1997). According to BERGELSON et al. (1996), double backcrossed non-segregated control lines have to be used as control plants with the original line to find out whether potential losses in fitness result from pleiotropic effects of resistant genes. Up to 1996 such control plants had not been used when testing the fitness of transgenic plants in field experiments. It can therefore be assumed that CRAWLEY et al. (1993) had not used them either for his comparison purposes.

2.7.1.3 Significance of Mean Values

Another question is whether mean values provide an appropriate basis for estimating the environmental impact of organisms. On the areas under cultivation, CRAWLEY et al. (1993) observed a population growth during the year of sowing in all the rape lines studied in two out of three years.[14] On the areas that are not under cultivation, however, a decrease in population was observed during the year of sowing in all rape lines studied in all three years. From this the authors conclude that there is a lack of invasiveness. In undisturbed places however a few rape plants grew vigorously and produced rather large quantities of seeds. This means that individuals of the lines studied seemed able to develop spontaneously an enhanced ability to establish themselves or that the ability to establish themselves varies in these lines. As regards the triggering off of ecological effects, individuals more vigorous than average can have greater significance than the fact that the **mean** values of the lines studied on uncultivated areas do not indicate any population growth in the course of one year. Some plants with increased fitness may establish themselves in the long term (see also WEBER, 1995).

In some cases it seems unjustified to set mean values. After two years of experiments, CRAWLEY et al. (1993) included the survival rates of the seeds of the two transgenic lines in one single value. This does not do justice to the conditions under commercial cultivation, the assessment of which should have been the purpose of these experiments. It was not until HAILS et al. (1997) that the results were discussed separately for the two lines. But they still derived a mean value from the survival rates of the first and of the second year. With both rates related to the number of seeds buried at the beginning of the first year of the experiment, it seems inappropriate that they were used for the calculation of a mean value, which (as it was related to both years) thus also included the reductions in viability of the first year.

Another aspect was not considered. After two years, fewer seeds (or even none) of the two transgenic lines (0.1 % of the kanamycin-resistant line) had the capacity for germination than in the control line (0.5 %). However, survival rates in the second year were higher in both the control and the kanamycin-resistant transgenic line, with a more distinct increase in the transgenic line.[15] Here it would have been necessary to continue the experiments to find out whether in later years or under improved environmental conditions, more seeds (in absolute terms) of the same transgenic line would have been able to survive than in the control line. This phenomenon, too, is probably caused by a few individual seeds whose capacity for persistence differs from the mean value.

2.7.2 Soya

Since 1996 large areas in the USA and meanwhile also in Argentine have been planted with Roundup® Ready soya beans. The gene, which was integrated in the DNA of soya plants, codes for the EPSPS (= 5-enolpyruvylshikimat-3-phosphate synthase) enzyme and makes the plant resistant to Roundup® Ready, a herbicide containing the active agent glyphosate. A comparative study of the material composition of transgenic soya beans presented in the course of the US notification procedure showed a changed fat and carbohydrate content and an increased proportion of fatty acid (PADGETTE et al., 1996). The differences were significant. This indicates pleiotropic or position effects. Experiments where some cows were fed transgenic soya and others conventional soya resulted in changes in the milk fat content of the cows fed with transgenic soya beans (HAMMOND et al., 1996). The study mentioned above revealed other facts as yet unclear. The test group was extremely small (only 5-6 cows per

[14] In the second year rape plants succumb to competition and become overgrown with weeds if the fields are no longer managed.

[15] Survival rate refers here to the proportion of viable seeds at the end of the year compared to the viable seeds at the beginning of the year.

test group!), which raises considerable doubts about the statistical significance of the results. Also, the data derived from the results leave open a number of questions, as it seems that some remarkable differences found when comparing the individual transgenic lines with conventional soya were not considered. It should be mentioned that the plants of the transgenic line that were compared and fed to the animals in the course of the experiments had not been treated with Roundup® Ready. Of glyphosate however, the main ingredient of the herbicide, it is known that it has a considerable effect on the health of mammals even at sublethal doses, causing a slight weight gain, reducing the libido and lowering the ejaculate volume (YOUSEF et al., 1995; COX, 1995). Recent tests suggest also carcinogenic effects in humans (HARDELL & ERIKSSON 1999). In transgenic soya plants it was found that also the phytohormone balance had changed. LAPPE et al. (1999) found that the levels of various phytohormones were up to 14 % lower compared to non-transgenic control groups. PADGETTE et al. (1996) found differences in the fibre contents of transgenic and non-transgenic soya plants, but a significance test was not carried out. More recent tests showed that growth and yield of transgenic soya beans decreased in comparison to control groups at temperatures above 45 ºC near the ground. Also, the plants were bursting, which led to crop failures of up to 40 %. This was explained by an up to 20 % increase in the lignin content of the transgenic plants (COGHLAN, 1999). Pleiotropic or position effects can also be assumed here.

2.7.3 Cotton

Transgenic cotton is cultivated in two transgenic varieties. One of these varieties is designed to develop a resistance to insects through the expression of gene sequences coding for Bt toxins, the other (similar to soya, see above) to develop a resistance to Roundup®. The commercial cultivation of the modified plants has caused some major problems. In 1997, deformities and shedding of the capsules of transgenic glyphosate-resistant cotton were observed in the US state of Mississippi, causing severe losses on about 200 farms (HAGEDORN, 1997). The cultivation of Bt cotton was also not entirely successful. Target organisms developed a resistance, and partial resistance caused susceptibilities in insects considered unproblematic up to then (SADRAS, 1998). The fear that the cultivation of Bt transgenic cotton promotes resistance in target organisms seems to be true (PRAKASH, 1997). Hopes of reducing the use of pesticides by cultivating Bt transgenic cotton are not realistic according to latest reports. On the contrary, data suggest that when cultivating these plants even larger quantities of pesticides have to be used (USDA, 1999). Although for example in 1997 fewer insecticides were used for Bt target organisms (as expected) on the areas of Mississippi Portal and Southern Seaboard than with conventional cotton, these savings were negated by the use of other insecticides acting on pests resistant to Bt.

2.7.4 Further Examples

In Canada, release experiments with transgenic plants that had been designed to develop a resistance to abiotic stress through genetic engineering were carried out as early as in 1992 (MCKERSIE et al., 1996). Clover (*Medicago sativa* L.) had been transformed with a manganese-superoxide-dismutase gene from *Nicotiana plumbiginofolia* to enhance winter hardiness[16]. Each of the release experiments was carried out with two transgenic lines where the recombinant protein was transferred either to the chloroplasts or to the mitochondria. All transgenic plants survived two subsequent winters better than the non-transgenic control plants. The increased survival rate resulted in increased yields. Yields of the transgenic plants increased

[16] Winter hardiness means resistance to a combination of influences such as frost, ice, drying up, flooding and disease (MCKERSIE et al., 1997).

during the season before the first winter already. Such increases in fitness without any winter stress had not been found in preliminary experiments under controlled conditions. It is possible that transgenic plants are more tolerant when it comes to replanting and in the event of water shortages since superoxide-dismutase is assumed to increase the tolerance of "oxidative stress", which is caused by frost, shortages of oxygen supply and dry conditions (MCKERSIE et al., 1997).

BERGELSON et al. (1996) used the model plant *Arabidopsis thaliana* to study the effect of a herbicide-resistance gene on the fitness. As fitness indicator, the seed production of the plants in the field was used. The authors compared transgenic plants containing the csr-1 gene (the gene that confers resistance to the herbicide chlorsulfuron) with *Arabidopsis* plants containing the gene as a result of mutation and with non-segregated control lines that had been backcrossed twice with the original herbicide-sensitive plant. It became clear that the considerable decrease in fitness (about 34 to 40 %) had been caused by pleiotropic effects of the specific csr-1 gene. The fitness decreases without application of the herbicide, and this decrease is not caused by the addition of an extra gene *per se*. The experiments thus show that transgenes do not necessarily mean a "genetic burden".

Field experiments with non-transgenic plants have shown that the seed production is only one out of many factors deciding over the dispersal success of plants (BERGELSON, 1994). In these experiments, plants were exposed to inter-specific competition. Here the (non-transgenic) *Arabidopsis* plants containing the csr-1 gene fared as well as the control plants lacking the gene although the latter produced considerably more seeds. The limiting factor in these experiments was the space available to the plants and buds rather than the quantity of seeds.

2.8 Trees

There has been growing activity in the field of transgenic trees in recent years. Until 1998, 116 release experiments with 24 different tree species had been carried out in 17 countries (see table 2). More recent data are not known as the OECD list has not been updated since 1998 (OWUSU, 1999). It seems that the growing awareness of the problems of transgenic organisms in the food sector has caused economy and research to step up their activities also in other fields. Among those are also trees. The objectives of the genetic engineering of trees are:

- Lignin modification to improve further processing for the paper industry
- Improved growth
- Herbicide tolerance
- Pest resistance
- Product harmonisation
- Sterility.

Tab. 2: Release experiments with transgenic trees (table from OWUSU, 1999)

Tree species	Year of first release
Aspen (Populus tremula)	1988
Black walnut *(Juglans nigra)*	1989
Poplar *(Populus spec.)*	1989
Papaya *(Carica papaya)*	1991
Apple *(Malus domestica)*	1991
Sweet chestnut *(Castanea sativa)*	1992
Plum *(Prunus domestica)*	1992
Red river gum *(Eucalyptus camaldulensis)*	1993
Black spruce *(Picea mariana)*	1993
Sweet gum tree *(Liquidambar styraciflua)*	1994
Black poplar *(Populus nigra)*	1995
Silver birch *(Betula pendula)*	1996
Castanea dentata	1996
Orange *(Citrus spp.)*	1996
Southern blue gum *(Eucalyptus globulus)*	1996
Norway spruce *(Picea abies)*	1996
Pine *(Pinus spp.)*	1996
Scots pine *(Pinus sylvestris)*	1996
Acacia mangium *(A. mangium)*	1997
Monterey pine *(Pinus radiata)*	1997
Teak *(Tectona grandis)*	1997
Flooded gum *(Eucalyptus grandis)*	1998
Olive *(Olea europea)*	1998
Populus deltoides	1998
Quaking aspen *(Populus tremuloides)*	1998
Wild cherry *(Prunus avium)*	1998

2.8.1 Observed effects in release experiments

2.8.1.1 Stability of Expressions

The stability of expressions, a common problem with transgenic organisms, is a special problem in trees because of their longevity. Instabilities were found in field experiments with trees even after a relatively short time. This is known from greenhouse experiments (e.g. FLADUNG et al., 1997a; FLADUNG, 1999). Because of the fact that trees can reach an age of several hundred years, it is particularly difficult to know what the consequences of such effects will be. The loss of relevant sequences or, where the sequences are still present, a lack of expression causes such reversions (FLADUNG & KUMAR, 1999). Such phenomena have also been observed in field experiments. In a field experiment with *Populus*, FLADUNG & MUHS (1999) found two reversions that clearly resulted from two different causes. The reasons are still being examined. The uncertainties about the stability of expression (especially over longer periods of time) mean that any plans to avoid dispersal through a genetically engineered sterility of plants are highly questionable (see below).

2.8.1.2 Pleiotropic and Position Effects

Pleiotropic and position effects were found to be relatively common in trees under field conditions. It is known from greenhouse experiments that poplars endowed with a 35S-rolC promoter can flower earlier than the non-transgenic control groups (FLADUNG et al., 1999a). This might be due to the significant change in phytohormone levels observed in these transgenic poplars (FLADUNG et al., 1997b), which are closely associated with the flowering of plants in general. This risk was pointed out to the notification authorities before carrying out a release experiment with transgenic poplars in Schleswig-Holstein (FLADUNG, personal message). In the course of a release experiment started in 1996, female flower buds were found on a plant after 3 years. Under natural conditions, it takes about 8 years for poplars to flower. The flowers were removed and destroyed after they had been discovered during a routine check. The risk of a potential dispersal of transgenic trees that would arise with male flowers is immense because of the wide range of possible hybridizations of poplars (see below). Further pleiotropic effects are mentioned in the chapters on resistance (see below).

2.8.1.3 Mycorrhiza

Mycorrhiza, i.e. the symbiotic association between the roots of a plant and a fungus, is of great importance in the life of plants and especially for trees. The benefits for the tree resulting from this relationship include improved nutrient absorption because of the larger root surfaces, improved water supply and an improved tolerance of heavy metals. In return, the fungus is supplied with various nutrients by the host organism. The question of horizontal gene transfer (hGT) and how much of it takes place here is still a subject under investigation although e.g. HOFMANN et al. (1994) have been able to prove it with *Aspergillus niger*. In this greenhouse experiment hygromycin-resistant transgenic plants (including *Brassica napus*, *B. nigra*, *Datura innoxia*) were cultivated with *A. niger* as a mycorrhizal partner and it was found that hGT had taken place with the resistance gene and other DNA sequences. Similar effects were found in the parasitic fungus *Pasmodiophora brassicae* by BRYNGELSSON et al. (1988) who also mentioned the potential for dispersal due to the various possibilities for hybridization in fungi. When the poplars of the release experiment in Schleswig-Holstein were first examined to study their effects on mycorrhiza, significant differences were found in the mycorrhiza of transgenic and non-transgenic plants (FLADUNG et al., 1999b). Data as to whether hGT had taken place are currently not available as they are still under investigation.

2.8.1.4 Hybridization

The possibilities for hybridization and thus the risk of unintended dispersal of genetically modified gene sequences have to be considered separately for each individual tree species. Of *Populus, Eucalyptus* and *Pinus* (genera intensively used in genetic engineering) it is known that they have great potential for hybridization. Of *Populus* for example about 30 species with numerous hybrids are known in the northern temperate regions. With *Eucalyptus*, the situation is similar in Australia and south-east Asia, with species and hybridization numbers being much larger than those of the genus *Populus*. Accordingly, the risk of outcrossing seems to be high. In a model experiment in the US, incidents of outcrossing were found in the surroundings of 2 industrial (non-transgenic!) *Populus trichocarpa* X *P. deltoides* hybrid plantations. 3.8 % of wild-growing plants nearby were crosses between mother/wild plant and father/hybrid with the same growth and establishment rates as homozygous wild plants (DIFAZIO et al., 1999). With Eucalyptus, the situation could be similar since the reproduction of these plants occurs through pollen (as with *Populus* and *Pinus*), which means that they spread over larger distances (SOUTHERTON et al., 1999). Methods that have been suggested to prevent such phenomena, such as sterility conferred via transgenes, should be viewed with scepticism given the uncertainties mentioned above about the stability of transgenic expression.

2.8.1.5 Insect Resistance

In China, clones of *Populus nigra* were tested in a field experiment for their insect resistance conferred through genetic engineering and for other effects. After the transformation, the trees in the greenhouse were divided into 3 different groups:

- No resistance to target organisms despite successful gene transfer, habit and growth unchanged.
- Resistant, changes in leaf habit, disturbance of chlorophyll biosynthesis (yellowish leaves), reduced growth.
- Resistant to target organisms, improved growth compared to non-transgenic control groups.

Trees of the third group were tested in the field. It was noteworthy that after 2 years, these plants were found to have been damaged by insects that had up to then been regarded as irrelevant. It seemed that resistance to target organisms had caused a higher susceptibility to other phytophagous animals or had opened up possibilities for secondary pests. Leaves on the trees were found to be larger and bark structures had changed. This shows that genetic modification may lead to changes of various other traits that could not have been foreseen (EWALD & HAN, 1999). A previous field experiment in China with insect-resistant *Populus nigra* had shown that the genetic modification had also affected the leaf morphology. The cause of this is still not fully understood (WANG et al., 1996).

2.8.1.6 Virus Resistance

In a field experiment with transgenic plum trees (*Prunus domestica*) that express a viral coat protein, MALINOWSKI et al. (1998) found that the resistance had been overcome by PPV-S viruses. The general risks of the coat protein strategy (see also chapter 2.5) seem to constitute a special risk in trees because of their longevity.

A note on pest resistance: it is often not possible to make comparisons (not even within one and the same species) because the formation of ecotypes of host organisms and pest populations of a wide genetic variability may produce a wide range of differences (PERRY et al., 1996; HE, 1998; KELLEY et al., 1999). This has to be considered in “traditional” tree plantations as well as when planting transgenic trees, especially where the latter have undergone additional cloning and are thus genetically largely identical.

2.8.1.7 Lignin Modifications

Release experiments with trees modified to produce changes in their lignin synthesis have lately been taking place in China and France. Results regarding potential effects are not yet available. Nevertheless, the procedure will be briefly described here. The delignification of wood is done in the paper industry using a sulfide and/or sulfate process, which requires a large economic effort and entails considerable environmental pressures. Therefore ways are being sought to produce genetically modified trees where the modifications in the wood structure facilitate further processing in the paper industry. The manipulations are still at the experimental stage. Modifications of various steps in the synthesis where the metabolic path is highly complex and some of it not understood are being worked on (BOUDET et al., 1998). Lignin plays an important part in the trees’ defence against pathogens and many questions are still open here as well (FINK, 1999). Modifications in the lignin synthesis or delignification might correlate with an increased susceptibility to pathogens. Modifications in the lignin metabolism also have an influence on other secondary plant components such as individual plant hormones or phenolic components and their content (BOUDET et al., 1998). Consequently, one cannot rule out the possibility of changes in growth and flower development.

2.8.1.8 Summary

Because of the longevity of trees there are great uncertainties regarding ecological risk analysis. Also, trees are highly adaptable as far as their interactions with specific environmental conditions are concerned. This means that genetically identical trees (e.g. clones) may differ significantly as regards their phenotype depending on their habitat. Effects observed in one habitat therefore only sometimes apply to another habitat. This refers to both biotic and abiotic environmental factors. The uncertainties about expression described above indicate that more research is required, as to the high potential for hybridization in genetically modified tree species and the considerable lack of knowledge about natural gene transfer. When looking at historical analyses of the risk potentials of neophytes, another factor of uncertainty arises because of the fact that there may be decades or centuries between the introduction and the spontaneous dispersal of such organisms, especially trees (KOWARIK, 1999).

2.9 Animals

2.9.1 General Research in the Light of Anticipated Effects

Currently much work is being done in the field of the genetic engineering of animals. The genetic modifications are carried out to achieve different aims, depending on the animal species. Modifications in domestic cattle are intended to make the animals resistant to disease, produce drugs in their milk and/or provide organs for xenotransplantation. Research here however is in its early days. Much more within reach is the commercial use (see e.g. IDEL, 1998) of transgenic fish whose modifications are aimed at a more rapid growth, an increase in the final weight or an increased frost resistance. The fish species modified worldwide so far include salmon, trout, carp, catfish, Medaka, zebra fish, loach, pike, swordtail and gold fish (HANKELN & SCHMIDT, 1997). Another important subject area in the field of genetic engineering is the production of transgenic insects (especially mosquitos) that transmit diseases such as malaria or dengue fever to humans (BAYMANN & TAPPESER, 1996). Attempts are being made to endow useful animals (insects and other arthropods) with genes that confer tolerance of commonly used pesticides so as to reduce non-target effects of chemical pest control (HANKELN & SCHMIDT, 1997).

Compared to plants, there have been few releases with animals so far. Corresponding to the ways of research described in greater detail in chapter 2.3, the current state of knowledge on potential effects of the release of transgenic animals will be outlined in this chapter. Against this background, only those release experiments will be analysed that indicate the possibility of environmental damage in the event of future releases.

2.10 Animals, Case-Specific

As at the present time an uncontrolled dispersal is unlikely with domestic cattle, the case examples refer mostly to release experiments with transgenic fish.

2.10.1 Transgenic Fish

Continuous over-fishing in the world's seas and the concern for future supplies of the earth's population have stepped up research and development in the field of transgenic fish in recent years. One also speaks of the "blue revolution" in this context (MARTINEZ, 1997). Apart

from fish, crustaceans, mussels, sea urchins and algae are modified. Here only the fish will be described because greatest advances have been achieved with their genetic modification. Fish are also regarded as the first genetically modified animals that could be placed on the market to be consumed by humans (HEW & FLETCHER, 1997). The objectives of the genetic modification of fish are the following:

- Increased and improved growth
- Increased cold tolerance
- Sterility
- Pollutant tolerance
- Disease resistance
- Increased meat quality (colour, fat proportion, taste).

The greatest advances have been achieved with modifications aimed at increased growth by integrating growth hormone genes, and with modifications of the cold tolerance that can be obtained by transferring gene sequences coding for anti-frost proteins (AFP). It is also expected that more knowledge about genetic regulation mechanisms in general will be obtained. Fish species that are genetically modified are listed in the following table (part of which according to PIKER et al., 1998).

Tab. 3: Transgenic fish species

English name	Scientific name	Natural place of origin	Distribution, region
Atlantic salmon	*Salmo salar*	North Atlantic	North Europe, Chile, North America
Rainbow trout	*Oncorhynchus mykiss (Salmo gaidneri)*	Eastern North Pacific	Worldwide
Pacific salmon	*Onchorynchus spp.*	North Pacific	
Pike	*Esox lucius*	Eurasia, North Amerika	Freshwater worldwide
Zebra fish	*Brachydanio rerio*	East-Indian Subcontinent	Worldwide
Carp, including breeding forms	*Cyprinus carpio*	Eurasia	Worldwide
Gold fish	*Carassius auratus*	East Asia	Worldwide
Grass carp	*Ctenopharyngodon idella*	China	Eurasia
European weather loach	*Misgurnus fossilis*	Europe	Europe
	Ictalurus punctatus	North America	North and Central America
	Clarias gariepinus	Africa	Africa
Alaska Pollack, Mintai	*Theragra chalcogramma*	North Pacific	
Medaka	*Oryzias latipes*	Japan	
	Xiphophorus spp.	Mediterranean, Mexico	in aquaria worldwide
	Sparus aurata	Mediterranean, Red Sea	Mediterranean, Red Sea
	Rhabdosargus globiceps	South Africa	
Akadei	*Chrysophris (Pagrus) major*	Japan	Israel
Tilapia	*Oreochromis sp.*	Africa, Southwest Asia	Worldwide
	Cichlasoma nigrofasciatum	Guatemala	Worldwide

The effects of the commercial use and release of transgenic representatives of these species (i.e. their introduction into breeding ponds or net pond aquaculture) have not been publicised. However, laboratory experiments give some clues about ecological risks, as does the experimental keeping in closed breeding pools.

2.10.1.1 Pleiotropic Effects

The various genetic modifications of fish sometimes entail major pleiotropic effects. Sharp increases in the growth of transgenic salmon and *Misgurnus fossilis (Schlammpeitzger)* caused severe deformities of the head and other parts of the body and changes in the fat deposits. The growth hormone balance changes altogether as a result of genetic engineering (DUNHAM, 1999). The larger gill surfaces lead to an increase in oxygen absorption (STEVEN & SUTTERLIN, 1999). It was found that an enlargement of the gill surfaces by a factor of 1.24 increased the oxygen absorption of transgenic salmon by a factor of 1.6 as compared to the control groups. Experiments with *Tilapia*, where modifications were also aimed at the growth hormones, showed that, compared to the control groups, changes in their amino acid and cholesterol composition had occurred (MARTINEZ et al., 1999). In transgenic carps, an increased protein content was found compared to the control groups as well as a reduced fat and water content in the meat, a significantly different amino acid composition (CHATAKONDI et al., 1995) and changes in the metabolising of food (FU et al., 1998). Similar effects were observed in transgenic wels (DUNHAM, 1996a).

A list of observed deformities of the body has been drawn up by PANDIAN et al. (1999).
It includes:

- Tumours
- Changes in fin and vertebra shape
- Skull deformities
- Abnormal gill growth
- Missing body segments
- Atrophies of nape and tail.

FARRELL et al. (1997) mention a severely reduced ability to swim in transgenic salmon. Modifications affecting the growth hormones in general seem to produce unforeseeable effects on the entire ontogenesis of fish (OSTENDFELD et al., 1998). GUILLE N et al. (1999) found that the feeding and social behaviour of transgenic *Tilapia* differed from that of wild forms of the same species. Changes in behaviour were also found in transgenic rainbow trout. It was observed that after simulated attacks of herons, transgenic trout were quicker to return to the zones close to the water surface, started feeding sooner and ate more food in general (JÖNSSON et al., 1996). DEVLIN et al. (1994, 1995) achieved a 100 to 600 % growth increase in the first generation of Atlantic salmon expressing a foreign growth hormone gene. In their offspring, the gene expression was sustained in only 2.2 % to 18.9 % of the animals. These animals had an abnormal green colour and various deformities of their bodies were observed later in their first year. After a year, the deformities became more pronounced and the animals died. It appears that apart from pleiotropisms, there are also problems with the stability of expressions (see below).

2.10.1.2 Stability of Expressions

With transgenic fish, the stability of the gene expressions is a problem that is far from being solved. According to PANDIAN et al. (1999), the stable and sustained expression of a gene conferring increased growth in the F_1 generation is not documented in any scientific publication to date. Not even the gene expression as such is fully confirmed. Experiments where in-

creased growth is achieved often entail low survival rates, a high frequency of deformities and reduced reproduction rates (see above). Apart from that, the phenomenon of mosaicism (i.e. cells both with and without the gene sequences transferred are found within a modified individual) can be observed in all transgenic fish. With this phenomenon, the number of target gene sequences varies from cell to cell, from organ to organ and from individual to individual. Examples of this phenomonon have been found especially where growth hormone genes had been transferred in catfish, zebra fish and carp.

2.10.1.3 Further Risk Aspects

The scenarios predicted for potential ecological effects range from extremely optimistic views of the benefits derived from transgenic fish for both the threatened aquatic fauna and the world population (SUTTERLIN et al., 1996) to computer models suggesting that one single transgenic fish may lead to the extinction of a complete fish population (MUIR & HOWARD, 1999). It is a fact that, with the conventional forms of commercial fish farming, individuals and their offspring often escape and that this is difficult to avoid. This escape of fish has already affected wild populations and may reduce their stocks (HINDAR et al., 1991) as well as have a negative impact on aquatic biocoenoses in general (HINDAR, 1993). Given the other effects described above, changes in the food range of transgenic fish have to be expected in terms of both quality and quantity as the extreme growth of the individuals will entail changes in their eating habits. Also, modifications of frost resistance enable expansions of potentially usable habitats, which may have an impact on species adapted naturally to cold waters. Competitiveness and sexual selection also change because of the often above-average size of transgenic fish. Studies of potential effects on predators and other members of aquatic biocoenoses are as yet not available. With fish as with plants, methods of conferring sterility by genetic modification to avoid potential outcrossing have to be viewed with scepticism. To date, neither “traditional” ways nor the methods of genetic engineering have been able to produce 100 % sterility. With catfish it was found that crosses between transgenic individuals and non-transgenic individuals were happening without any problems (DUNHAM, 1996b). An unintended transfer of the modified gene sequences into natural populations can therefore not be ruled out. A detailed summary of the potential risks arising from the deliberate release of aquatic organisms can be found in PIKER et al. (1998).

3 RESEARCH METHODS

To elaborate the topic of the present study, the following ways of research were used:

- Analysis of release-related biosafety research projects on the deliberate release of transgenic organisms presented at national and international conferences and published in conference papers [e.g. conference on "The Biosafety Results of Field Releases of Transgenic Organisms" (CASPAR & LANDSMANN, 1992; JONES, 1994; MATSUI, 1997), technical discussion held in Hannover with the title "The State of Safety Research Regarding the Release of Genetically Modified Plants" (*Stand der Sicherheitsforschung zur Freisetzung gentechnisch veränderter Pflanzen) (BUNDESGESUNDHEITSBLATT 41*, 1998) or the *BMBF* Workshop "Release-related Biosafety Research with Genetically Modified Plants and Microorganisms" (*Freisetzungsbegleitende Sicherheitsforschung mit gentechnisch veränderten Pflanzen und Mikroorganismen*) (BRAUNSCHWEIG, 1999)]. Where the research results published indicated an environmental effect of GMOs, special investigations were made to follow the further progress of the studies. Either the relevant research groups were asked directly, or the research was done in databases and in the Internet using authors' names and appropriate keywords.
- Analysis of publications where up-to-date knowledge of releases of transgenic plants is summarised (e.g. FÖRSTER, 1998; SCHÜTTE et al., 1998a,b).
- Research in databases (Current Contents, Medline, Biosys) and in the internet using appropriate keywords and authors' names.
- Enquiries at the company groups Monsanto, Novartis and AgrEvo to find out about past and present research projects on the topic under discussion and about the results achieved so far.
- Enqiries at European research institutes such as BBA/Germany, INRA/France, John Innes Institute/United Kingdom, CCRO/Netherlands[17].
- Direct questions addressed to research groups who carried out research projects under the US research programme entitled "USDA's Biotechnology Risk Assessment Research" (Documented under: http//nbiap.biochem.vt.edu/brag/cris. html).
- Enquiries at the Union of Concerned Scientists (USA)
- Enquiries at research institutes or institutions in the countries of the south e.g. CSIRO, CIMMYT, IRRI, ISAAA.[18]

Successful ways of research:

- Databases with appropriate keywords
- Analyses of conference papers
- Mailing lists (costly and time-consuming)
- Communication via e-mail for specific enquiries about a release that had been carried out. As a rule, unpublished results are unobtainable!
- Enquiries at authorities and companies were usually unsuccessful!

[17] CCRO: Coordination Commission Risk Assessment Research; BBA: Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, Germany; INRA: Institut National de la Recherche Agronomique, France.

[18] CSIRO: Commonwealth Scientific and Industrial Research Organisation; CIMMYT: International Maize and Wheat Improvement Center, Mexico; IRRI: International Rice Research Institute, Manila, Philippines; ISAAA: International Service for the Aquisition of Agro-Biotech Applications.

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