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Identification of Data Needs

NERI Technical Report, No. 303

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Data sheet

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Abstract: This report suggests a structured way to identify the type of data needed to perform a sound ecological risk assessment for genetically modified higher plants (GMHP). The identified data types are intended to support the evaluation of the following risks: risk of invasion and establishment of the modified plant in natural habitats; risk of introgression of the inserted traits to other plant species; and the risk of adverse effects to non-target organisms. The guidance paper considers only aspects of ecological risk assessment. Possible risks to human health, livestock and weediness of the transgenic crop plants as well as biogeochemical and environmental impacts ascribed to altered management should be considered separately.

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Preface

This publication is a first version of a manual identifying the data needs for ecological risk assessment of genetically modified higher plants (GMHP). It is the intention of the authors to stimulate further discussion of what data are needed in order to conduct a proper ecological risk assessment of GM plants when application for placing on the market is made. It is our hope that both the scientific community, the biotechnological industry and the regulatory bodies will participate in the process of improving the present "draft", so that it can develop into a useful tool for both the industry as well as the national regulatory bodies. Furthermore, we hope that these efforts will improve the transparency of risk assessment and harmonisation of the requirements for data.

The report suggests a structured way to identify the data need for risk assessment of GMHPs. It does not discuss the actual risk assessment procedures and the risk evaluation, which must proceed the data collection.

The report use the terminology "ecological risk assessment" rather than "environmental risk assessment" because at present this work does not include bio-geochemical effects and environmental impacts from altered management of the fields, e.g. possible changes in for example leaching of pesticides or nitrogen, etc.

Furthermore, we have abstained from suggesting number of species to test for specific issues because different risk assessment procedures have been developed which add a safety factor accounting for uncertainties in the extrapolation from limited laboratory studies to the species rich field environment. The relationship between the size of the safety factor and the number of species is therefore an issue of the risk assessment. Some of the issues raised in this report overlap with data needs to the assessment of agricultural risks or health risks.

The work was overseen by a steering committee, which consisted of Gitte Silberg Poulsen, Jan Grundtvig Højland, and Hans Erik Svart from the Ministry of Environment and Energy, National Forest and Nature Agency and was performed within the framework of the project "Biotechnology: elements in environmental risk assessment of genetically modified plants".

December 1999
Christian Kjær

1 Introduction

Aim of report

In ecological risk assessment of transgenic plants, information on a wide range of subjects is needed for an effective and reliable assessment procedure. The information obtained from literature, field trials, laboratory and greenhouse tests have to be interpreted in a structured and well-defined manner. This guidance paper intends to assist the risk assessment procedure, as a working tool that stipulates the specific type of information needed in relation to the biotechnologically inserted or modified trait. Furthermore, reference to relevant test methods must be made for each type of information required. The present guidelines cover the need for information relevant to ecological risk assessment, raised in EU directive 90/220/EEC, including new issues raised in the amendment to the directive.

Risks covered

This report suggests a structured way to identify the type of data needed to perform a sound ecological risk assessment for genetically modified higher plants (GMHP). The identified data types are intended to support the evaluation of the following risks: risk of invasion and establishment of the modified plant in natural habitats; risk of introgression of the inserted or modified traits to other plant species; and the risk of adverse effects on non-target organisms. The guidance paper considers only aspects of ecological risk assessment. Possible risks to human health, livestock and weediness of the transgenic crop plants as well as biogeochemical and environmental impacts ascribed to altered management should be considered separately.

Guideline structure

At present, no accepted test guideline exists for the issues mentioned above. However, it is agreed that a tiered approach is desirable for a standardised and effective treatment (Illueca, 1996; Rissler and Melon, 1993; Strandberg *et al.*, 1998) and hierarchical systems are generally used in risk assessment (Suter, 1993; US-EPA, 1998; van Leeuwen and Hermens, 1995). The present guideline suggests a five-tiered approach with complexity of problems and required tests increasing from Tier to Tier. The guidance paper is structured so that first a summary of the Tier structure is presented (Chapter 2), hereafter chapter 3 gives a more detailed description of each Tier, and then test details are presented (chapter 4). Finally, chapter 5 describes the framework for establishing monitoring programmes.

2 Summary of the Tier structure

Tier I. General information on the receiver plant and the inserted trait.

Tier II. Evaluation of general information and identification of additional data needs of the receiver plant
- A. Identification of capacity for reproduction of the GMHP
- B. Identification of possible effects on non-target organisms
- C. Detection of potential hybridisation, and assessment of the selective force acting on the inserted gene in a natural plant population

Tier III. Laboratory measurements and small scale trials on the GMHP
- A. Test of changes in critical fitness components for the establishment of GMHP's in natural habitats
- B. Assessment of effects on non-target organisms
- C. Measurement of the rate of hybridisation and estimation of the probability of introgression

Tier IV. Field assessments
- A. Identification of habitats/ecosystems that may be invaded by the GMHP and field tests for invasiveness
- B. Measures of effects on non-target organisms in the field

Tier V. Investigation/calculations of regional and landscape effects.

Tier progression

Tier I and II are both obligatory, and the design of Tier II experiments should be based on Tier I data. The progression from one Tier to the next depends on the results in the former Tiers. If a preceeding Tier recommends further investigation progression to the next Tier is obligatory. If the plant is able to complete a full life cycle under normal environmental conditions in the region of potential use within the EU it is necessary to proceed to Tier IIIA. Contrary, if the GMHP is unable to reproduce and survive for example winter conditions the Tier IIIA tests are not necessary, and so forth for the other issues raised.

In any new case, it is obligatory to establish a monitoring program when the test programme has been carried through according to the tiered structure.

Statistical considerations

Power analysis

The criterion for procedence to the next Tier depends on differences between the transgenic plant and the receiver plant. Such a comparison require that data are of a certain quality standard, because otherwise such a procedure would penalise good studies with a low variability. Therefore, the type of statistical tests needed and the number of replicates required for an adequate significance level (normally $P < 0.05$) must be carefully analysed when the trials are planned. This will require that a statistical power analysis is carried out. Statistical power is defined as 1-b, where b is the probability of not rejecting the null hypothesis when it is false. It must be tested that the power of the conducted experiments is above a specified value depending on the demands of the type of trial. A value of at least 80% is suggested. The power can be estimated before the experiment is performed if the design and variance is known. The power of a test will increase with an increased number of replicates. In some cases additional pretrials are needed to estimate the level of statistical variation. For methods, see standard statistics textbooks, e.g.Sokal and Rohlf (1995) and

Effect size

Cohen (1988). The effect size is defined as the size of change in the test parameter in relation to the expected value, incorporating variation, i.e. the difference between the null and the alternative hypothesis. An effect size of at least 0.25 will normally be required for detection of effects. Definitions of effect sizes for different statistical tests can be found in Cohen (1988).

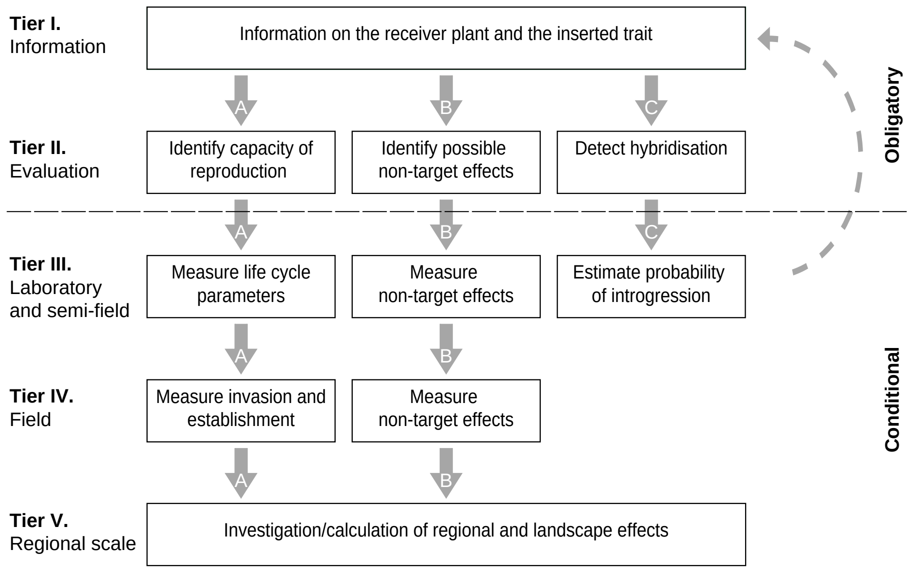

Figure 1. Schematic presentation of the Tier structure. The full line arrows indicate the flow through the Tier structure. The broken arrow from Tier IIIC to Tier I indicates a new assessment procedure for formed hybrids. Tier I and II are obligatory, which mean that they must always be performed, whereas the higher Tiers (III-VI) are conditional. This means that they are only necessary if an evaluation at a preceeding level indicates this positively.

3 Description of the specific content of each tier

3.1 Tier I: General information on the receiver plant and the inserted trait

Receiver plant

The general information collected at Tier I is used both as background for decisions in risk assessment at Tier II to V and as scientific base for the choice and adjustment of relevant tests and procedures, as well as in the planning of monitoring programmes. The necessary information encompasses taxonomy, evolutionary history, morphology and life history traits, pollination, gene-transfer, hybridisation, recruitment and vegetative reproduction. Also information on history of cultivation in relation to the regional aspects have to be included. Much of the required basic information can be obtained from relevant literature, e.g. Monographs from OECD, from national environmental agencies and other sources, e.g. ecological databases (BIDS Ecoflora, etc.) and scientific research literature. In each case exact reference to consulted literature must be made.

Inserted trait

A genetic and molecular description of function of insert (biochemical, physiological and morphological changes) should be included. Information on the method used for transformation is not essential. However, a detailed list and description of additional inserted traits (e.g. markers, herbicide resistance and promoters) must be included. The description of the function of the insert must be concise and extensive. This includes the expression of the modified trait: under which circumstances and in which parts of the plant (leaves, flowers, roots, cuticle, etc.) it is expressed. Any information on synergistic effects between inserted genes and the new genetic background (receiver plant) must be given.

3.2 Tier II: Evaluation of general information and identification of additional data needs of the receiver plant

3.2.1 A. Identification of the capacity for reproduction of the GMHP

The ability of the GMHP to reproduce under the climatic and environmental conditions in the release area is a prerequisite for invasion and establishment in natural habitats. An assessment must be made based on available information and if necessary additional data on basic biology of the receiver plant and the transgenic insert may be asked for. If the analysis make probable that the GMHP has the potential to sexually reproduce or propagate vegetatively and establish in natural habitats in the region of the release, the assessment proceeds to Tier IIIA.

3.2.2 B. Identification of possible effects on non-target organisms

A number of species are potentially affected due to compounds produced in the transgenic plants as a result of the inserted trait or due to an altered performance of the GMHP compared to the receiver plant. At this level it must be found and argued which species groups are likely to be exposed to new plant compounds or altered performance of the transgenic plant. Relevant species groups of non-target organisms comprise other plant species, pollinators, detrivores, herbivores and predators. If non-target effects can not be excluded proceed to Tier IIIB.

3.2.3 C. Detection of potential hybridisation, and assessment of the selective force acting on the inserted gene in a natural plant population

If the GMHP hybridise with a naturally occurring plant species, the inserted gene may change the competitive ability of the natural plant and change the community structure in natural ecosystems. Detection of potential hybridisation with any naturally occurring plant is therefore needed. This can be done by a literature study or if no data exist by simple hybridisation experiments with plants from closely related taxa. Furthermore, in order to predict whether the inserted gene will be introgressed into the naturally occurring plants, it is necessary to describe and assess the direction of the selective forces operating on the inserted gene in the natural plant population. If hybridisation is possible and selection is positive, i.e. a plant that has the inserted gene has a fitness advantage over an otherwise similar plant without the inserted gene, then further investigations are needed (Tier IIIC).

Additionally, it is necessary to take into account whether the transgene by horizontal gene transfer can be introgressed into another organism and the effect of such an introgression. The event is highly unlikely but it may happen e.g. Hoffmann *et al*. (1994) and Dröge *et al*. (1998). There may be unwanted consequences of horizontal gene transfer to another organism. Such consequences may be assessed verbally if no data are available.

3.3 Tier III: Laboratory and semi-field measurements

3.3.1 A. Test of changes in critical fitness components for the establishment of GMHP's in natural habitats

If the plant is able to produce viable propagules that may reach natural or semi-natural plant communities then the fitness of the plant needs to be tested. The question to be answered is: Is the fitness of the GMHP changed compared to the fitness of the receiver plant? If possible this needs to be tested in a full-life-cycle experiment under relevant experimental conditions in small scale field trials, in the greenhouse or in controlled environment chambers. The life cycle of a GMHP normally involves the following stages and processes: germination, seedling survival and growth, adult plant survival and growth, flowering, pollination and gene-flow (see 3.2.3 Tier IIC), seed set (production), seed dispersal (see 3.4.1 Tier IVA), seed bank and for some species vegetative reproduction. Any changes to the main

Critical phase

stages and critical phases will affect the fate of the population in terms of fitness and the consequent risk of invasion of natural habitats. If the receiver plant possesses particular critical phases, then the experiments needs to pay specific attention to changes in these stages e.g. germinability, seedling survival, seedling persistence, growth rate, number and quality of seeds produced, survival of propagules in the soil.

Environmental test conditions

If full life-cycle experiments are impossible, as may be the case with perennial species and especially trees and shrubs, tests of critical stages should preferently be supplemented with growth modelling by use of the short term tests results. Relevant experimental conditions both refer to the environmental conditions in the area of release and to the stress component, which may have been removed by the inserted trait (e.g. herbivory, plant pathogens and drought). A range of loads of the relevant stress-types should be tested in order to identify the type of environmental conditions giving "no fitness advantages" to conditions eventually resulting in "improved fitness". Likewise the growth stage most susceptible to the stressor needs to be found. If fitness is improved at a level occurring in any recipient environment proceed to Tier IVA

3.3.2 B. Assessment of effects on non-target organisms

When assessing effects on non-target organisms upon the release of a genetically modified plant, it is very important that the tests are performed as ecologically relevant as possible. This includes choice of species, exposure conditions, and end-point chosen.

Direct effects

Indirect effects

Choice of relevant tests

Non-target effects include effects on all organisms in the environment surrounding the GMHP both in cultivated and in natural or semi-natural environments, if the GMHP or the transgene may be dispersed to these. Effects may be direct or indirect. Direct effects encompass toxic effects on other plants, herbivores, pollinators, detrivores and microorganisms. This may result in a changed in species diversity in the invaded community. Another type of direct effects is e.g., the deprivation of pollinator food sources in GM plants modified to be pollen sterile. Indirect effects include food-chain effects in terms of the removal of food for higher trophic levels and effects through a changed management regime, e.g. changed herbicide use which gives a more efficient weed control in the field. An effective weed control directly results in a decrease in the floristic diversity in the agricultural land but indirectly it may also have effects on higher trophic levels, e.g. herbivorous insects and birds. Which tests that are relevant depends on both the mode of action and expression of the trait (in roots, leaves, flowers, seeds or in the whole plant) and on the distribution and species specificity of toxic compounds. For an inserted trait which is designed to affect other organisms (i.e. plant pathogen resistance, insect resistance, increased allelopathic activity) the specificity of the trait should be established, because these species may be important in other ecosystems than the agro-ecosystem.

Tests must be performed for all the different functional groups of non-target organisms, i.e. detrivores, predators, etc. If the test species perform differently in trials with transgenic plants these species groups have to be tested at Tier IVB.

3.3.3 C. Measurement of the rate of hybridisation and estimation of the probability of introgression

To evaluate a possible ecological effect of an inserted gene being introgressed into a natural population it is important to estimate the probability of introgression. Such a probability estimate can be obtained from measurements of hybridisation rates, assumed selective advantage of inserted gene, and fitness measurements of parent plants, hybrid plants, and plants from the first and second back-cross generations.

Probability of introgression

If hybrids are formed and it is likely that these hybrids are able to survive the consequences should be discussed. This discussion must include considerations on invasiveness in new ecosystems and possible effects on other organisms (Tier IIIB).

3.4 Tier IV: Field assessments

3.4.1 A. Identification of areas that may be invaded by the GMHP and field tests for invasiveness

If the GMHP has shown improved fitness in the Tier IIIA tests then the plant needs to be tested in full-life-cycle experiments under relevant field conditions. The question to be answered is: Does the GMHP perform better than the non-modified comparable cultivar under natural conditions? Selection of field localities which are relevant for the experiments are primarily dependent on the plant species and on the inserted trait as well. If the inserted trait results in changes of the biotic or abiotic stress on the plant through e.g. insect, pathogen or drought resistance, then the field localities should cover the range in stress levels resulting in improved fitness found in Tier IIIA. If many habitats exist which fulfil this criteria then habitats where the receiver plant is all ready present, are preferred. The experiments must pay special attention to changes in the most susceptible life stage.

Habitats to test

If full-life-cycle experiments are impossible as may be the case with some perennial species and especially with trees and shrubs, tests of critical stages need to be supplemented by modelling of e.g., seed dispersal, habitat invasion and reproductive success using representative data and estimation on life-cycle parameters.

3.4.2 B. Measures of effects on non-target organisms in the field

In order to evaluate the significance of the results obtained in Tier IIIB comparative tests of the population development for those taxa, which have proved sensitive in Tier IIIB, must be made. The sampling must be adjusted to the species of interest and supplementary also other species of the same functional group, i.e. detrivores, predators etc.). Non-target effects on vegetation are tested in the field and may be conducted as a joined design with tests of invasiveness included (Tier IVA).

3.5 Tier V: Investigation/calculations of regional and landscape effects

If the use of transgenic plants on large adjoining areas can be foreseen possible spatial and regional effects should be discussed. Whenever possible this should be done in a quantitative sense by examining the sensitivity of the conclusions in a spatial or regional model. Tier III and Tier IV data are used in spatial modelling of fitness components and competitive effects on other species. The integration of mathematical modelling and field experiment allows approximate estimates of the probability that the GMHP will invade local natural habitats as well as ecological effect on a regional scale.

4 Specific tests

4.1 Tier I: General information on the receiver plant and the inserted trait

No specific tests are required but a number of data on the receiver-plant and the transgenic plant should be available including the following issues:

Receiver plant

Receiver plant:
- Taxonomy
- Evolutionary history and centre of origin
- History and geographic area of cultivation
- Morphology and life-history traits
- Pollination biology
- Propagation and vegetative reproduction
- List of organisms interacting with the receiver plant
- List of known hybridisation partners
- Natural habitats

Trangenic plant

Transgenic plant and the genetic construct:
- A genetic and molecular description of function of insert
  - Inserted elements, markers, location and copies in the genome
  - Phenotypic characteristics, new traits or traits not expressed (biochemical, physiological and morphological changes)
- Genetic stability of the GM plant
  - Mendelian inheritance, translocation, etc.
- Identification and detection of transgene insert
- History of previous releases in the EEC and outside the EEC
  - Sites, duration, post release monitoring, conclusions

4.2 Tier II: Evaluation of general information and identification of additional data needs of the receiver plant

4.2.1 A. Identification of capacity for reproduction of the GMHP in natural habitats

If the GMHP has the capacity to reproduce sexually by seeds or spread by vegetative growth it has the potential to survive and propagate in cultivated or in natural habitats. This is the normal condition for species that are cultivated as seed crops (e.g. grain crops, canola). The extent of reproductive success depends on plant use and cultivation practices. Some GMHP crops, which are cultivated for chemical constituents or biomass production (e.g. sugar beets), will normally be harvested before seed set. However, the possibility of gene flow from single volunteers through pollen or seeds still exists.

Other GMHP crop species, which are unable to reproduce sexually, can propagate vegetatively through tillers (e.g. grasses), tubers (e.g. potato) or rhizomes. If there is any chance that the plant will reproduce under climatic or environmental conditions which it is likely to meet in a range of years (e.g. 10 years), proceed to Tier IIIA, where the critical conditions are assessed (and further in field tests at Tier IVA). Ecological data from conventional field trials under optimal conditions will be sufficient at this Tier - extended trials, including different environmental conditions, will be asked for at Tier IIIA.

4.2.2 B. Identification of possible effects on non-target organisms

Test species must be selected on theoretical basis by assessing the species groups likely to be exposed to the transgene plant, plant products or changed agricultural practices. This mean that tests are selected on the basis of the type of inserted trait, which parts of the plant express the gene and under which circumstances. A full argumentation must be given for those of the following functional groups which are not tested for non-target effects: pollinators, detrivores, herbivores and predators.

4.2.3 C. Detection of potential hybridisation, and assessment of the selective force acting on the inserted gene in a natural plant population

Information on possible hybridisation between the receiver plant and a natural occurring plant may be obtained from the literature, or if there is no prior information, from different types of experimental pollination trials (e.g., method M13 or M18 in Kjellsson *et al*.(1997). Hybridisation may occur between the GMHP and a number of plant species present in natural habitats. It is therefore important that hybridisation data are obtained from all of the naturally occurring species that are likely to hybridise with the receiver plant.

The assessment of the selective forces of the introgressed transgene in different habitats and environmental conditions may be argued verbally using traditional adaptive explanations and existing data.

4.3 Tier III: Laboratory and semi-field measurements

4.3.1 A. Test of changes in critical fitness components for the establishment of GMHP's in natural habitats

If viable propagules are produced by the GMHP then the fitness of the plant needs to be tested in full life-cycle experiments. Test data for the GMHP, which is mandatory for all cases and traits at this level, include: growth rate, total biomass and reproductive output (i.e. seed production, viability and germination). These data are essential components to assess changes in competitive interactions with natural vegetation and establishment of persistent populations. The tests at this level should be conducted under controlled conditions either in laboratory, greenhouse, controlled environment chamber or as small-scale semi-field experiments. Semi-field tests are preferred as wild plant species generally perform badly under greenhouse conditions (Parker and Kareiva, 1996). The critical components for establishment of the GMHP in natural habitats will depend on the genetic background (receiver plant and species), the inserted trait and the environmental conditions in exposed habitats (see IVA).

Two-species competition experiments

The fitness of the GMHP and the receiver plant should be tested in two-species competition experiments with a number of natural plant species at a range of proportions and densities. If available a related species (from the same genus or family) with known aggressiveness should be run parallel to the GMHP and the receiver plant. For plants secreting allelopatic substances the natural plants chosen for the competition experiment should cover target species as well as species expected to be non-target.

Relevant competition design

A range of relevant test designs for studies of competitive interactions are available (e.g. M1, M28, M29, M33, M60, M61, M79 in (Kjellsson and Simonsen, 1994)). A number of measures related to the critical components: plant growth, survival, flowering, seed set and vegetative reproduction, are listed in Table 1.

Table 1. Critical life cycle components of a GMHP, relevant parameters and available measures.

Critical component	Parameter	Examples of relevant types of measures*
Plant growth	Growth rate	M24, M58 in K&S-94
	Leaf area	M35, M36 in K&S-94
	Total plant biomass	M12 in K&S-94
	Dry weight allocation	M3, M6, M13 in K&S-94
Plant survival	Survival rate	M25, M43, M76, M78 in K&S-94
Flowering and seed set	Flower production	-
	Sexual reproductive effort	M70 in K&S-94
	Seed production	M67 in K&S-94; M68 in K&Al-97
Vegetative reproduction	Dry weight allocation	M13 in K&S-94
	Ramet demography	M55 in K&S-94
	Vegetative reproductive effort	M82 in K&S-94

*References: (Kjellsson and Simonsen, 1994) (K&S-94), (Kjellsson *et al*., 1997) (K&Al-97).

Test conditions

The tests should be performed under relevant experimental conditions which primarily refers to the specific inserted trait e.g. herbivore tolerance, pathogen resistance or drought tolerance. An analysis must be made of how the transformation could affect life-cycle components. An example would be a plant made resistant to fungal infection of the seeds. In this case test data on seed survival in the soil (seed bank) are required. Furthermore, tests should be performed at a range of environmental and biological stress levels in order to identify levels for significant changes in critical components. The life cycle component most susceptible to the stressor also needs to be identified.

Critical components

A range of suitable test methods is available for detection of changes to additional major critical components, which need special types of test conditions. For seed germination, changes in germination and seed viability can be detected by method no. M17, M18, M19, M65, and M78 in Kjellsson and Simonsen (1994) and by M68 in Kjellsson *et al*. (1997). For the seed bank stage, changes in seed survival rate and dormancy can be detected by method no. M19, M20, M25, M65 in

Kjellsson and Simonsen (1994). Issues concerning pollination, gene flow and seed dispersal are not included here, but treated separately under tests for hybridisation (Tier IIIC) and habitat invasion (Tier IVA). For example, increased starch content in potato tubers may affect the tolerance to frost and tuber survival (vegetative reproduction). Consequently, this becomes a critical component which must be tested for. If the performance of the GMHP is improved in the critical stage additional data on plant establishment are needed (Tier IVA).

4.3.2 B. Assessment of effects on non-target organisms

GMHP's may cause effects on non-target organisms, and therefore the test subjects presented below should all be carried out unless it on scientific and logical grounds can be argued that the inserted trait pose no threat to the specific species group.

Test conditions

When assessing effects on non-target organisms upon the release of a genetically modified plant, it is very important that the tests are performed as ecologically relevant as possible. This includes choice of species, regional differences (e.g., climate), type of habitats, exposure conditions, and end-point chosen. If the test species respond differently to transgenic plants than to receiver plants Tier IV testing is necessary. Details for different groups of non-target test organisms are presented below

Pollinators

When the inserted trait cause the plant to produce potentially toxic compounds, or if flower characteristics are changed, i.e. colour, flowering period, pollen production etc. then effects on pollinators has to be measured. A test of effects on honeybees (*Apis melliferae*) is obligatory because of the importance of honeybees as pollinators of both wild and crop species and because standardised test protocols testing for effects of conventional pesticides exists for this pollinator. These tests include exposure through nectar and pollen. Basic guidelines for such an acute test on bees can for example be found in the BBA-guideline (Stute, 1991). Larval survival must also be assessed. Other species groups than bees might be tested concurrently and for plant species that are not pollinated primarily by honeybees a test must be made with the most relevant pollinator(s). There exist no agreed guidelines for test of effects on bee larvae.

Detrivores

The detrivore and soil community may be affected if the decaying plant material or the fine roots and root exudates are toxic to the detrivores. A significant change in the composition of this community most probably indicates altered soil fertility. If such effects are widespread, the use of such plants may reduce soil fertility and mineralisation in the agricultural soils as well as in natural ecosystems if the t5ransgene is dispersed. A number of laboratory test procedures are available for for soil inhabiting species, and among these also detrivores (Jepson *et al*., 1994; Løkke and Van Gestel, 1998). The tests must be conducted with plant debris containing the active substance(s), and the tests should be conducted with increasing amounts of plant material added. Timing of the test depends on the degradation process and must be optimised to the point of highest effect. Tests with actively growing plants should also be performed at different planting densities. Effects measures for collembola species, earthworms and for effects on microbial processes are obligatory. For earthworms both reproduction and survival curves must be produced.

Herbivores

Vertebrates

A range of wildlife species forage in the crop depending on the type of crop. Some bird species forage on crops especially in the spring, where they consume seeds and seedlings (e.g. skylarks and partridges for Western Europe). Other wildlife species utilise the crop species later in the season; examples hereof are mammalian species like brown hare and roe deer. Similarly, these species may be exposed after the growing season due to seed spillage**.** Some of these species may be affected if the inserted trait causes the plant to produce toxic compounds. It should therefore be tested if any of these organism groups are affected by eating the genetically modified plants. Feeding and reproduction tests should be performed simultaneously on herbivorous birds as a worst case approximation. Worst case is no-choice feeding on the plant material based on data of peak occurrence, both in term of plant parts as well as timing of potentially toxic compound in the plant (This information may be drawn from the General information given in Tier I). A test of the oral toxicity of GMHP's has to be designed according to the ecology of the species and the mode of action of the compound.

Invertebrates

If a GMHP cause adverse effects to herbivorous arthropods this may elicit effects on higher levels of the food chain if the survival and reproduction of particular herbivorous insects are reduced. Therefore, screening tests for effects on folivorous and root herbivorous species most be carried out.

Predators

Toxic effects

It is inevitable that predatory and parasitic insects in the field may experience a loss of food if insect resistant crops are efficient. However, secondary toxic effects caused by ingesting injured or dead prey (e.g., target organisms that have been eating the insect resistant GMHP) are undesired because such impacts may result in loss of biodiversity as well as loss of beneficial organisms in agriculture. There are indications that such secondary effects to insects can occur for plants producing *Bt*-toxins (Hilbeck *et al*., 1998a; Hilbeck *et al*., 1998b). Other traits than insect resistance may have this effect. It is therefore necessary to test if these organisms are toxicologically affected by eating the dead or living primary consumers of the GMHP. A range of suitable test systems is listed by Jepson *et al*. (1994).

Food chain effects

Birds foraging on insects in an insect resistant GM crop may not be directly toxicologically affected by the use of the GM plants, but they may experience a significant reduction in the amount of insect food present in the crop. An effect on food availability will occur due to the reduction of pest species in insect resistant crops. Together with the reductions in plant dwelling insects most likely also a reduction in the predaceous fauna will be found. A thorough discussion of the probability that such indirect effects occur should be included in the assessment report also for other traits than insect resistance. For crop systems, the calculations will then include assessments of the relative amounts of species and specimens dwelling on crop and weed plants respectively, as well as measures of the functional response of predatory insects. Food web effects are relevant to predatory insects, entomophagous birds and small mammals.

Measurement of the specificity of the inserted trait.

It is necessary to establish the specificity of the active trait to other organisms than the primary target species. The choice of species must be made so that it covers a large taxonomic distance and a large number of species. It is suggested that at least 10 different species are screened. The screening tests should be made with GMHPs expressing the peak concentration of the active compound, according to the information given in Tier I. If the tests are performed with purified compounds at least two times the maximum occurrence in the GMHP plant must be used as discriminatory dose because the activity may alter under field conditions. Also comparative studies of the potency of the compound in purified form and incorporated in the GMHP must be done.

When choosing test species a range of consideration should be made: They must be ecological relevant to the species of concern, the exposure should be realistic, the width of the taxonomic tree should be as large as possible and finally different feeding guilds should be represented, e.g. for insect resistance: sap sucking, leaf chewing, root eating etc. Specific requirements depends on species and exposure route of the active compound, but reference can be made to a range of existing test guidelines for ISO, OECD, ASTM and US-EPA.

Vegetation

Non-target effects on plants and vegetation may be found both in cultivated areas and in the surrounding natural and semi-natural environments. The necessary tests depend on the mode of action of the inserted trait and the distribution of potential active substances. Negative effects may occur directly through the growth vigour and competitive ability of the GMHP when invading natural plant communities or through the release of toxic compounds. Both processes may result in a change in species diversity in the invaded community. Effects may also be indirect e.g. through a changed pesticide use, which gives a more efficient weed control in the field. A decrease in the floristic diversity in the agricultural land may also indirectly have effects on higher trophic levels e.g. on herbivore insects and birds. Toxic effects can be tested in the greenhouse or in a controlled environment chamber, however, changes in exposed plant communities are assessed in conjunction with the test of the invasiveness of the transgenic plant (Tier IVA).

4.3.3 C. Measurements of the rate of hybridisation and estimate of the probability of introgression

Hybridisation

If hybridisation and introgression of the transgene into a natural plant population is biologically possible, it is important to estimate the probability by which the gene is introduced into the natural population and the probability by which the gene is fixed in a finite natural plant population. Pollination by wind or insects is normally required for gene transfer between the GMHP and wild relatives or other cultivars. Analysis of pollinator preferences and foraging behaviour, which influences gene flow to wild relatives, can be made for relevant habitats before GMHP release (methods M57, M58 and M62 in Kjellsson et al. (1997)). Tests of mating system and chance of hybridisation can be performed in the greenhouse by different methods of experimental pollination see, e.g., methods M2, M12 and M13 in Kjellsson et al. (1997).

The probability that an advantageous allele crosses a species barrier has been examined theoretically by Piálek and Barton (1997). They found that, dependent upon the fitness of heterozygotes, a strong species barrier might delay the spread of an advantageous allele significantly, and if gene flow is restricted below a critical value, spread across the species barrier is prevented.

Gene flow

In order to estimate the amount of gene flow of a neutral gene across a species barrier it is necessary to estimate the relative fitness (Method II M14 in Kjellsson *et al*. (1997)) of the two parent species (the receiver plant and the natural relative species), the F1 hybrid, and a number of back-cross generations to the natural species, preferably in a realistic ecological scenario where the different types are competing with each other.

Necessary estimates

The following probabilities in the introgression process have to be estimated.

I. Hybridisation probabilities.
   A. The probability that the GMHP hybridise with natural species under normal growing conditions
   B. The probability that a F1 hybrid successfully backcross to the natural species
   C. The probability of the backcross generations to further backcross to the natural species
II. Competition among different crossing types.
   A. The fitness of the F1 hybrids and the different backcross generations when grown in competition with each other
III. Selective advantage of transgene.
   A. The selective advantage of the transgene in the F1 hybrids and the different backcross generations
IV. The probability that a transgene "survives" the different backcross generations until it can be regarded as being introduced in a naturally occurring population.
V. The probability that the transgene is fixed in the natural population?

If the transgene is introgressed into a natural population, then the risks associated with this event should be asessed, possibly by used of the suggested tier structure.

Alternatively, and assuming that the two parent species can be considered to be in equilibrium with respect to gene-flow, it is possible to estimate gene flow indirectly by examination of the genetic variation within and between the two parent species (Method II M19 and M21 in Kjellsson *et al*. (1997)), or by a phylogenetic approach.

4.4 Tier IV: Field assessments

4.4.1 A. Identification of areas that may be invaded by the GMHP and field tests for invasiveness

Field localities

If the GMHP has improved fitness in greenhouse tests, smal scale test in semi-field or controlled environment chamber tests, then the fitness of the plant needs to be tested in full life-cycle experiments in the field. The type of inserted trait and the particular plant species will determine which environmental conditions and field localities that are necessary for the experiments. If the inserted trait results in changes in the biotic or abiotic stress on the plant then the field localities should cover the range in stress levels resulting in improved fitness found Tier III experiments. Furthermore, they should include a range of vegetation cover, disturbance levels, soil fertility and humidity.

Experimental set-up

The relevant localities for the field tests must be selected from those where the GMHP is most likely to invade and have a negative impact, based on information of both species and inserted trait. Generally, habitats where the non-modified crop species occur are likely targets for invasion, including roadsides, wastelands, cultivated and semi-natural grasslands. The experimental set up for study of invasion should be a block or a split plot design with the number of replicates of each treatment determined by power analysis and mechanically disturbance of the surface needs to be included as a treatment in the experiment. The PROSAMO design e.g. (Crawley *et al.*, 1993) or equivalent designs e.g. (Parker and Kareiva, 1996) should be used for tests of invasiveness. When performing field studies of GMHP invasion with transplant experiments, any effects to the invaded habitats should also be monitored. For GM plants with identified potential for seed escape (Tier IIIA) separate tests for seed dispersal and survival in seed bank are required (methods presented in Kjellsson and Simonsen, (1994)).

4.4.2 B. Non-target organisms

Framework for experimental set-up

Field effects on non-target vegetation is not covered here but assessed under Tier IVA (see above). For the other non-target organisms, no specific tests can be given as it depends on the particular effects, which have been identified at Tier IIIB and on the specific organism. However, some general guidance for design and procedures for field experiments is provided. Experiments should be optimised for the species that were affected on a lower Tier-level; additionally other organisms with the same functional role in the ecosystem should be sampled. If the GMHP is a crop species, the experiments must be made as repeated sampling in a GMHP-crop over the season, unless the species life cycle suggests otherwise. For non-crop GMHP species testing should be conducted in relevant habitats for the area of release. The final design has to be agreed upon by the competent authority.

For those species, which the laboratory or greenhouse studies have identified as possibly affected, field test can be conducted to examine whether these effects pose a problem in the environment. The set-up of the experiments depends on the species which have proved susceptible in the Tier IIIC testing. If more species are sampled the analyses should be kept separate. The test are essentially performed in a random block design or a split plot design. For mobile test species care should be take either to reduce emigration and immigration or to measure the rate of these processes.

Test conditions

The soil in the chosen fields should preferentially cover a range of organic matter content. The report of the test should include a description of the site, plot layout, soil type (texture, organic matter content, pH), crop and weather conditions during the experiment (regular/continuous measurements of soil moisture, temperature, and precipitation). Exposure conditions of the susceptible species should be made as realistic as possible in a worst case approximation, i.e. highest density of GM plants expected etc. Care must be taken not to employ unrealistic high degradation rates of the plant material (i.e. not to add the active compounds in a dried, fragmented or purified form). In the following paragraphs comments are made for specific test groups if relevant.

Honeybees and other pollinators

The field test of effects on bees or other pollinators should be done with plants in the flowering stage. The test may be done as a tent experiment to ensure that the honeybee actually forage on the transgenic plant. Procedures for such tests are given in the guidelines developed for pesticide testing (Stute, 1991). An alternative is whole-field test with the bee-hive in the immediate proximity of the fields.

Earthworms

Some earthworm species have a high dispersive ability, such as *Lumbricus terrestris* which overnight may move several tens of meters on the soil surface (Mather and Christensen, 1988). Therefore, plot sizes of 25 x 25 m should be enclosed with barriers, unless whole field designs are employed. In order to get high efficiency of sampling, a large volume of soil should be hand sorted. Both earthworm density and the biomass of the single species should be measured. Biomass of the collected earthworms is determined on dry or fresh weight basis of worms with emptied gut. If the earthworm density is high, single species analyses are preferred; otherwise all species are pooled. Under all circumstances, changes in the species composition should be accounted for.

Microarthropods

Microarthropods are a diverse group of animals, which all live in the soil environment and represent a wide range of ecological functions. They are therefore relevant test species. Effects on this organism group should be measured at community level because community structure tests have the advantage of integrating both direct and indirect effects and it includes many species with different ecological functions. The microarthropods should be sampled in soil cores and extracted from the soil by means of a high gradient extractor (MacFayden, 1961; Petersen, 1978). Soil samples are collected regularly through the growing season and also after harvest.

Herbivores

Herbivorous birds and/or mammals

Measures of field effects on warm blooded wildlife can be made from a range of methods. In choosing among the available test methods, considerations should be made to the relevance, statistical power, selection of field site and the behaviour/biology of the test species.

The selection of design and methods of course must be targeted to the trait under study. Some guidance on these issues can be found in US-EPA: Public draft: Ecological Effects Test Guidelines: "Field testing for terrestrial wildlife" (OPPTS 850.2500).

Arthropod herbivores

In order to measure possible effects on herbivorous arthropods of the introduction of a GMHP a number of confounding variables should be measured concurrent with population measures of the species of concern. These variables encompass predation rate, immi- and emigration rate, and other explanatory variable prominent to the herbivore.

Predatory arthropods

Predatory arthropods that feed on pest species can play an important role in the balance of the agroecosystem and are therefore important as test species. Furthermore, they may regulate herbivore populations in natural habitats as well. If secondary effects on predators are observed in Tier IIIB it is necessary to determine the extent of the problem in the field. For transgenic crop species, the agroecosystem is used as a worst case approximation, i.e. the habitat with the highest expected density of plants expressing the new trait. Prior to the test, sampling of the arthropods must be conducted to assess the within-field variation in density. Sampling techniques (D-vac, ground search, and pit fall traps) must be chosen to optimise the catch in relation to the limitation of each type of sampling (i.e. D-vac can not sample large items, pitfall traps are merely an activity measure dependent of the environment, etc.). Furthermore, the plots must either be fenced or sufficiently large to reduce migration, which could bias the results.

4.5 Tier V: Investigation/calculations of regional and landscape effects

If the validity of the conclusions made at the previous Tier can be questioned due to the omission of spatial or regional effects, then possible spatial and regional effects should be discussed. Whenever possible this should be done in a quantitative sense by examining the sensitivity of the conclusions in a spatial or regional model (e.g. stochastic metapopulation model). The model tool that should be used depends highly on the effects under study and no general recommendations can be made, but the fast growing expertise on spatial and regional modelling in the scientific community may be consulted.

5 Establishment of monitoring programmes for cultivated fields and natural habitats

Aim of monitoring

A monitoring programme is obligatory when a new GMHP is placed on the market. At this stage major hazards to the environment are unlikely. However, all hazards that may occur in a complex natural environment cannot possibly be foreseen. Any adverse effects of the GMHP are most likely initially to occur in the area of cultivation and its surroundings. Therefore, it becomes important to monitor for specific effects in fields with GM crops and to survey the surroundings for major unforeseen changes. Monitoring must be made to assurthat unaccepted occurrence and dispersal of the GMHP and potential adverse effects on the environment does not take place. In addition monitoring is the only way to evaluate the ecological risk assessment and to assess long term effects and effects associated with large scale use. The monitoring period for new cases should depend on both cultivation regime and crop rotation schedules. A minimum of three rotation cycles and at least a 10 year monitoring period will normally be required. The programme may provide results that give early warning of emerging problems and allow the relevant control and management measures to be taken.

Baseline information

In order to be able to detect environmental changes it is necessary to obtain relevant baseline information before the GMHP is marketed. This includes selection of relevant reference sites in different natural and cultivated habitats and acquiring data of e.g. population density of relevant target and non-target organism groups. Furthermore, information is needed on spatial and year-to-year variation in occurrence, cultivation practice of the GMHP and the conventional crop and occurrence of hybridisation partners. The problems involved in detection of trends and change in natural ecosystems (e.g., space-time analysis) needs to be carefully considered before the onset of the monitoring programme (Edwards, 1998). Also, the use of proper tools for statistical power analysis of monitoring designs must be emphasised (Thomas and Krebs, 1997).

If unintended gene flow is indicated by the monitoring procedures, identification of the suspected transgene should be performed by DNA analyses (PCR and RFLP) or other adequate techniques (Kjellsson *et al.*, 1997).

5.1 Provisional suggestions for guidelines for a monitoring program

It is suggested that the monitoring plan include three subprograms, which considers: 1. Dispersal of the GMHP or the transgene; 2. Effects to the environment (e.g. changes in vegetation and organisms including non-target species in agricultural fields) and 3. Surveillance for unexpected effects which has not been anticipated in the ecological risk assessment. Monitoring for dispersal need not be activated for GMHPs, which have no potential for reproduction by seeds or vegetatively under the present environmental conditions. Effects to organism groups which could possibly be affected (e.g., through food chains or changes management practice) should be monitored for each case. If any significant effects are detected from monitoring or surveillance, the results has to be carefully analysed to exclude major environmental effects from other sources than the GMHP (e.g., deposition, use of pesticides and other changed agricultural practices). Further information is available in (Kjellsson and Strandberg, 2000, in prep).

5.1.1 Choice of monitoring habitats in cases where monitoring outside farmland is recommended

The choice of monitoring habitat should in each case be based on an ecological assessment considering the properties of the receiver species, the properties of the inserted trait, characteristics of the GMHP growth area with respect to soil type and climate. The distance to relevant ecosystems and hybridisation partners (identified in the ecological risk assessment) should also be taken into consideration. Disturbed sites may be likely targets for monitoring purposes, because they are widespread, they are often found in connection with farmland, and they offer the possibility of establishment. Types of sites, where primary invasion of GMHP may be expected, vary depending on plant species and exposure such as seed spillage. Monitoring of invasion in these areas should also be included in the procedures. Inclusion of areas not identified in the ecological risk assessment should be covered by the general surveillance.

5.1.2 Basic ecological characteristics of monitoring habitats to be determined

Before monitoring it is important to characterise the monitoring habitat. It would be an advantage if this could take place over a range of years and in different areas in order to determine the variation in time and space. Concerning the aspect of time this will usually not be possible. The ecological characterisation of monitoring habitats includes:

a) Baseline determination, characteristics of habitat, i.e. species composition, soil and variation in time.
b) Species, organism groups and ecosystem structures to be monitored

5.1.3 The monitoring plan: objectives, data collection, evaluation, reporting and decision making

According to the directive a detailed plan for the activities must be made before monitoring is started. This includes:

a) Definition of objectives and selection of suitable methods which include the determination of monitoring intervals and total period. Furthermore, the responsibility for data collection and reporting must be placed before monitoring is started.
b) Data collection must be made using standard protocols and baseline information. Data analysis is made to detect statistically significant effects.

c) An evaluation of the power of chosen monitoring procedures and analytic methods must be made.
d) The relevant information must be reported to both official bodies and the public.
e) Finally, the monitoring results should lead to decisions at different levels: reassessment of the conditions for the release and the marketing permission; adjustment of monitoring procedures and objectives; and management measures may need to be taken to reduce any foreseeable adverse effects.

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