SAFETY CONSIDERATIONS FOR BIOTECHNOLOGY

SCALE-UP OF MICRO-ORGANISMS AS BIOFERTILIZERS

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Foreword

This report is the latest in a series on safety considerations for biotechnology. It follows the OECD publication *Safety Considerations for Biotechnology: Scale-up of Crop Plants* (1993) which updated and extended the OECD's earlier work on *Recombinant DNA Safety Considerations* (1986) and "Good Developmental Principles (GDP): Guidance for the Design of Small-scale Field Research with Genetically Modified Plants and Micro-organisms in *Safety Considerations for Biotechnology 1992* (OECD, 1992), setting out safety guidelines for biotechnology applications in industry, agriculture and the environment. It is the outcome of work carried out over three years, from June 1991, and of many meetings of a "Preamble Subgroup", chaired by Mr. P. van der Meer of the Netherlands, and a "Micro-organisms Subgroup", co-chaired by Mr. D. Mahon of Canada and Mr. D. Harper of the United Kingdom, of Working Group III of the OECD Group of National Experts on Safety in Biotechnology (GNE). Working Group III was chaired by Mr. P. de Haan of the Netherlands.

The Preamble of this report sets this extended OECD activity in its overall context, which reflects the dynamic evolution of biotechnology. The section on micro-organisms (as biofertilizers) describes how the concept of "familiarity" – with the micro-organism, the introduced trait, the environment and with their interaction – may be applied to facilitate risk/safety analysis and to manage possible risks in the context of scaling up micro-organisms as biofertilizers towards commercial release.

This report was prepared by the OECD Directorate for Science, Technology and Industry, in collaboration with the Environment Directorate, and is published on the responsibility of the Secretary-General of the OECD.

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Executive summary

This project seeks to identify the principles and practices used to address the environmental safety considerations associated with scale-up in the testing and use of biofertilizers developed by the newer molecular techniques of biotechnology, based largely on *Rhizobium*. It should be noted that the general considerations are mostly drawn on the basis of experience and knowledge of *Rhizobia*.

The concept of familiarity, as defined in the "Preamble" (*Preamble to Reports on Scientific Considerations Pertaining to the Environmental Safety of the Scale-up of Organisms Developed by Biotechnology*) published in June 1993, is an essential aspect of risk/safety analysis and of risk management. In the theoretical framework related to the scale-up and use of biofertilizers, it can be applied to any element or stage in the system under consideration. This "Preamble" document is reproduced in each of the subsequent projects on safety and scale-up, including the present report.

Familiarity is the knowledge and experience that can be used in risk/safety analysis of biofertilizers, including those modified by the newer molecular techniques. It is part of the procedure used to identify potential adverse effects (hazard identification), to assign a level of risk (risk assessment), and to indicate appropriate methods for managing it (risk management).

Most effects of new traits in biofertilizers will be recognisable during small-scale field tests. However, some, including safety effects dependent on scale, may only become apparent during scale-up.

Potential adverse effects are identified on the basis of the biological properties/ characteristics of a new strain in a specific environment and an analysis of the associated risk. Perception of adverse effects may be based in part on information available for other organisms or environments (familiarity), or on research associated with the relevant micro-organisms or the specific environment.

The scientific principles derived from an analysis of several case studies and other relevant information provide a framework for evaluating the safety of scale-up for testing and using biofertilizers.

I. Preamble

1. Introduction

The introduction of new molecular technologies in the early 1970s initiated discussion on safety in biotechnology. When the power of moving genes between unrelated organisms with the new techniques was appreciated, the Asilomar conference was convened to explore the advisability of using the new rDNA technology and the conditions under which experiments should proceed. Uncertainty led to concerns that use of the new techniques might lead to increased risk from modified organisms.

This discussion resulted in a number of national and international recommendations, guidelines or regulations, and legislation. The 1986 OECD report *Recombinant DNA Safety Considerations* (the so called "Blue Book") was one of the first international scientific frameworks for the safe use of organisms derived from rDNA techniques in industry, agriculture and the environment. This book set out general principles for the safe development of rDNA organisms. The reason for focusing specifically on rDNA organisms in the Blue Book was that techniques were being used to produce organisms with novel genetic combinations, and there was limited or no experience with such organisms.

By the mid-1980s rDNA techniques were considered increasingly to represent an extension of conventional genetic procedures and rDNA organisms to present risks that are the same in kind as those posed by any other organism. There was also recognition that the same physical and biological laws control the behaviour of organisms, whether modified by conventional or by rDNA techniques.

However, the conventional and newer molecular techniques differ in two respects. Molecular techniques allow firstly a greater diversity of genes to be introduced into organisms and, secondly, in general, greater precision in the introduction of genetic material, yielding a more thoroughly characterised and potentially more predictable organism. As the characteristics of the organism depend on its genetic make-up, the view has been expressed that there may be a particular concern if there is a lack of experience with organisms having particular genetic combinations from different sources.

The OECD Group of National Experts on Safety in Biotechnology (GNE) has worked since April 1988 to update and develop further the particular principles set out in the Blue Book, aiming at the development of scientifically sound principles and practices for the application of organisms referred to in it.

The GNE began work in this area with the preparation of good developmental principles (GDP) for small-scale field trials of genetically modified plants and micro-organisms, published under the title *Safety Considerations for Biotechnology – 1992*. In the programme for further work, the GNE initiated similar activities on large-scale field trials.

At the meeting of June 1991, the GNE decided that a preamble should be prepared which places the different initiatives in a general overall context and which reflects the fact that biotechnology is a dynamic and rapidly evolving area.

The purpose of that "Preamble" and the reports that follow it are therefore to evaluate, interpret and apply the general principles for safety in biotechnology, thus reflecting current knowledge in order to offer a basis for further work in the area of introductions into the environment.

Scientific and technical methodologies for evaluating safety are likely to vary from one group of organisms to another and therefore subsequent reports will treat separately specific groups of organisms (*e.g.* crop plants, micro-organisms).

It is recognised that the safety of an organism is independent of the process of genetic modification *per se*. As stated in GDP, it is the characteristics of the organism, including new traits (however introduced), the environment and the application that determine the (likelihood of) risk of the introduction. The work of the GNE is carried out in the context of safety in modern biotechnology, but the principles for safety laid down in the subsequent paragraphs and reports apply to any organism.

2. General principles for safety in biotechnology

Safety in biotechnology is achieved by the appropriate application of risk/safety analysis and risk management.

2.1 Risk/safety analysis

Risk/safety analysis comprises:

- hazard identification and, if a hazard has been identified;
- risk assessment.

It is recognised that:

a) Risk/safety analysis is based on the characteristics of the organism, the introduced trait, the environment into which the organism is introduced, the interactions between these, and the intended application. Knowledge of and experience with any or all of these provides familiarity which plays an important role in risk/safety analysis. Further explanation and examples of familiarity can be found in detail in the subsequent reports. Familiarity is not synonymous with safety; rather, it means having enough information to be able to judge the safety of the introduction or to indicate ways of handling the risks. A relatively low

degree of familiarity may be compensated for by appropriate management practices. Familiarity can be increased as a result of a trial or experiment. This increased familiarity can then form a basis for future risk/safety analyses.

b) Risk/safety analysis is conducted prior to an intended action and is typically a routine and ongoing component of research, development and testing of new organisms, whether performed in a laboratory or a field setting. It can range from a routine *ad hoc* judgement by the researcher as an implicit part of good experimental practices, to adherence to a formalised assessment.

c) Risk/safety analysis is a scientific procedure which does not imply or exclude regulatory oversight or imply that every case will necessarily be reviewed by a national or other authority.

2.2 Risk management

In this report, the term risk management refers to the way appropriate methods are applied in order to minimise the scientifically identified risks. It does not encompass additional factors such as socio-political values, etc. In principle, appropriate management is based on and should be in proportion to the results of the risk/safety analysis.

All organisms are subject to natural biological, physical and chemical influences on their survival, reproduction and spread in the environment. Knowledge and experience of the effects of these natural influences on the organism, and of interactions between the organism and its environment can be used to manage the organism. Risk management encompasses all aspects of the management of the organism indirectly through management of the environment into which the organism is introduced, or directly, by management of the organism itself.

The practices used for managing the risk of different types of introductions depend on the type of organism and hence are dealt with in detail in the subsequent reports. Such practices may include the use of biological, chemical, physical, spatial, environmental, temporal or other isolation conditions, outside strict containment, to provide barriers to minimise the dissemination and impacts of organisms or their genetic material outside the area of application. For example, flowers or other reproductive structures may be removed in order to preclude gene transfer, and special decontamination treatments may be used to prevent persistence and dissemination.

Risk management to ensure safety is employed during the development and evaluation of an organism in a systematic and stepwise fashion, through an appropriate continuum, for example from the laboratory, through stages of field testing, to final (*e.g.* commercial) application.

The number and modalities of the stages to be visited in this continuum are not fixed, but depend on the outcome of the risk/safety analysis at the different stages. Progression through the appropriate developmental stages generally entails a reduction in control and possibly in monitoring, whilst often increasing scale in order to gain knowledge, or for functional purposes. Any particular developmental stage begins after incorporating information and experience from an earlier stage, or other appropriate information such as from monitoring, into that risk/safety analysis.

3. Operation of the concept of stepwise development and evaluation

3.1 Operational principles of risk/safety analysis and risk management

Several operational principles governing the stepwise development of organisms can be identified:

a) Progression through the continuum of developmental stages is based on information gathered from previous experiments, from other appropriate sources, or from empirical observations. Experiments will include observation and measurement of organisms and their impact as appropriate, in order to obtain data relevant to safety. A risk/safety analysis may indicate that: *i)* progression can proceed to a more advanced stage; *ii)* work should not proceed to another stage but that further work at the same stage is required, for example to accumulate data; or *iii)* further developmental work at an even earlier stage is required.

b) When appropriate risk/safety analysis and risk management are conducted, performance trials can be carried out at any developmental stage. Performance trials *per se* do not necessarily provide information relevant to the risk/safety analysis and risk management, but can be designed to do so.

3.2 Factors affecting the operation of the concept of stepwise development

As discussed above, progression through developmental stages is flexible and tailored to the particular situation. Factors that influence the operation of this stepwise concept include:

a) familiarity with the characteristics of the organism, the trait introduced, the interactions between these, and the intended application;

b) familiarity with the conditions and the environment into which the organisms are intended to be introduced;

c) familiarity with interactions among the organism, the trait and the environment.

3.3 Operation of the concept of stepwise development for specific groups of organisms

The principles set out above are of a general nature. Both the detailed design of the developmental stages and the information appropriate to proceeding with any particular stage may vary from one group of organisms to another.

As was indicated by the GNE, the scientific and technical methodologies for risk/safety analysis and risk management for specific groups of organisms will be dealt with separately in subsequent reports.

II. Scientific issues and principles pertaining to environmental safety on the scale-up of field trials of micro-organisms: biofertilizers

This project began as a follow-up to "Good Developmental Principles (GDP): Guidance for the Design of Small-scale Field Research with Genetically Modified Plants and Micro-organisms" in *Safety Considerations for Biotechnology 1992* (OECD, 1992). It identifies and addresses the environmental safety[1] issues associated with introductions of biofertilizer micro-organisms,[2] here called "biofertilizers", developed by the newer molecular techniques of biotechnology, *e.g.* recombinant DNA (rDNA) technology. The project is the second in a series of specific projects undertaken by the Group of National Experts on Safety in Biotechnology (GNE) on scale-up in the testing and use of organisms developed by such techniques. Each of these projects starts with the common statement of principles in the preceding, "Preamble" section.

There is considerable knowledge of and experience with the procedures for managing the introduction of certain biofertilizers developed by a wide range of methods. Biofertilizers containing traits introduced by the newer molecular techniques may initially raise specific concerns because of the possible effects of new genetic combinations (see Preamble, page 11, para. 4). However, as recognised in the preceding paragraph, "the same physical and biological laws control the behaviour of organisms, whether modified by conventional or rDNA techniques". Therefore, the knowledge and experience (familiarity) gained with traditional biofertilizers, as well as experience with biofertilizers developed by the newer molecular techniques, are applicable to this project.

Since scientific knowledge and experience in this area of research and development are increasing rapidly, the present report is intended to offer current information on the topic.

The term scale-up[3] is used, in preference to "large scale", to describe the continuum of research and development involving increasing scale from preliminary field testing to general use. The term is not intended to refer to a specific stage within a defined sequence of stages; rather, it refers to any of a number of advanced stages commonly used during biofertilizer development in order to gain information on performance or for functional purposes. Scale-up takes biofertilizers beyond small-scale introductions and results in an increased interaction with the environment.

1. Purpose and scope

This project addresses the scientific considerations pertaining to the environmental safety of scale-up for advanced testing and use of biofertilizers. It deals with the identification of potential adverse effects and with issues related to exposure, with a view to selecting appropriate management strategies for the scale-up of biofertilizers. These are discussed in terms of the major groups of biofertilizers, *e.g. Rhizobium* and mycorrhizal fungi.

The concept of familiarity as outlined in the "Preamble" is developed here for biofertilizers. Familiarity is useful for the development of principles and practices of environmental safety. The knowledge and experience available are used when conducting a risk/safety analysis and, if necessary, when developing appropriate strategies for risk management.

In view of the limited experience with biofertilizers developed by the newer molecular techniques, many of the principles formulated below have been derived from information generated in extensive experience (familiarity) with traditional biofertilizers. Detailed examples are provided in the case reviews, which, in turn, refer to the principles derived here.

2. Concept of familiarity

The concept of familiarity is dynamic rather than static. Its applicability improves with increasing experience, and it is flexible, that it may be applied to any level or element of the system under consideration. Lack of familiarity may make it less easy to predict potential adverse effects. Familiarity is based on knowledge of and experience with:

- the biofertilizer;
- the plant and its interaction with the micro-organism;
- the trait(s) or characteristic(s) introduced into the micro-organism;
- the environment into which the micro-organism is introduced.

Examples of other sources of familiarity may include experience either with similar micro-organisms or with the use of new micro-organisms in the same or similar environments. Much useful information can also be obtained from greenhouse studies, microcosm testing and small-scale field trials.

The sections that follow give a detailed explanation of the concept of familiarity in relation to micro-organisms, plant-microbe interactions, trait(s), and the environment.

2.1 Familiarity with micro-organisms

There is a long history of safe use of some biofertilizers, particularly *rhizobia* (Nuti *et al.*, 1993). The development of superior inoculum strains of biofertilizers, particularly

rhizobia, has been a goal for many years and recent progress on many fronts suggests that this goal may be achieved in the immediate future (Triplett and Sadowsky, 1992).

Historical information on biofertilizers, particularly *rhizobia*, should provide excellent points of comparison for strains of micro-organisms developed by newer molecular techniques. However, there is less experience with using some other micro-organisms as biofertilizers. The degree of familiarity therefore depends on the specific micro-organism used. However, experience gained with one group of micro-organisms can often be applied to some extent to related micro-organisms, or to those that occupy a similar ecological niche. Also, micro-organisms representing new genetic combinations for which little knowledge or experience may be available (relatively unfamiliar) when first developed will become increasingly familiar as they progress from laboratory to small-scale field trials.

2.2 Familiarity with plant-microbe interactions

The nutritional relationship formed between some biofertilizers and their host plants is relatively well understood. This is especially true for *rhizobia*-legume associations and for some interactions involving mycorrhizal fungi. However, even if the exact mechanism by which biofertilizers promote plant growth is not known, there may be considerable familiarity with the ecology of the plant-microbe interaction. In particular, the dynamics of rhizosphere colonisation and root infection have been investigated extensively.

Ideally, the degree of familiarity should be such that the response of plants to the introduction of certain biofertilizers can be more or less predicted, under known conditions. However, the relationships formed are complex and subject to significant perturbation if environmental or other conditions change. Furthermore, the use of co-inoculation (*e.g.* combined vesicular-arbuscular mycorrhizal and rhizobial inoculation) highlights the need for familiarity with interactions between micro-organisms as well as between micro-organisms and plants.

2.3 Familiarity with the trait

The introduction and expression of specific genes – such as *i)* marker genes (*e.g.* antibiotic resistance, enzyme activities); *ii)* functional genes; and *iii)* regulatory genes – may result in new phenotypic traits.

Familiarity with the trait can accrue from a number of sources, including: knowledge of the trait in the species of origin; knowledge of the genetic insert, including regulatory sequences; experimental data from basic ecological and genetic studies with the micro-organism; experience with the same trait as found in other micro-organisms.

2.4 Familiarity with the environment

Familiarity with the environment encompasses familiarity with the range of potential plant and animal hosts and their habitat ranges, including wild plants that may be

colonised by the biofertilizer. When testing micro-organisms, including biofertilizers, several points related to their native habitat should be considered. When a micro-organism is isolated from an area and returned to that area, without alteration, for testing as an inoculant, the level of risk should be minimal as long as the micro-organism is known not to be deleterious. When the micro-organism is used in a habitat where it is not indigenous, then it becomes more important to consider potential adverse effects. Similarly, if micro-organisms containing unfamiliar traits result from the use of the newer molecular techniques, additional safety measures may be warranted. This is especially true if there is reason to believe that the behaviour of micro-organisms developed by the newer molecular techniques is different from that of their unmodified progenitor.

2.5 Application of the concept of familiarity

The concept of familiarity is used in assessing risk. As the Preamble states, familiarity does not imply safety, it implies availability of information. This information is used to analyze potential adverse effects associated with a specific organism, with specific traits, and with identified target and non-target effects in a specific environment. The concept of familiarity helps in deciding whether sufficient information is available to determine that no hazard exists, or whether, if a hazard does exist, the exposure is insufficient to result in increased risk.

If there is insufficient information, *i.e.* too great a degree of unfamiliarity, on which to decide, or if it is considered that increased risk may result, risk management must address the relevant elements. The concept of familiarity can also be applied, if necessary, for selecting appropriate risk management strategies particularly as many risks associated with scale up of micro-organisms will not be specific to biofertilizers.

3. Risk/safety analysis and risk management

As the Preamble declares, safety in biotechnology is achieved by the appropriate application of risk/safety analysis and risk management. The concept of familiarity is a major factor in all phases of the evaluation, since it is used to identify potential adverse effects (*i.e.* hazard identification), to determine the level of risk associated with these adverse effects, and to adopt risk management strategies. The overall safety evaluation procedure can be outlined as follows:

a) risk/safety analysis:
- hazard identification;
- if a hazard is identified, risk assessment;

b) risk management.

3.1 Risk/safety analysis

This section considers the issue of environmental safety pertaining to the scale-up process for testing and using biofertilizers developed by the new molecular techniques and discusses potential adverse effects in the context of risk/safety analysis. Micro-organisms, including those being considered for biofertilizers, possess intrinsic biological properties that may need to be considered in order to identify potential adverse effects and to assess risk.

The level of risk is determined by considering any potential adverse effects and the magnitude of those adverse effects, and, by assessing the level of exposure, and the likely frequency of the adverse effects.

3.1.1 Exposure considerations

Exposure to the biofertilizer should be considered in the context of: *i)* survival, persistence, and dispersal; and *ii)* gene transfer. Such considerations are particularly important when a potential adverse effect has been identified.

3.1.1.1 Survival, persistence and dispersal

In general, micro-organisms introduced into soil tend to persist for long periods at low, relatively stable population densities. Strategies to encourage persistence at high population densities should generally be avoided, unless there is a history of safe use of the biofertilizer. Potential increases in risk from persistent high populations would have to be considered in relation to the perturbations that occur due to normal agricultural practices. Moreover, without a thorough understanding of the ecology of soil microbial populations the ability to measure such perturbations is limited.

The number of micro-organisms introduced and their potential for survival are important factors to consider before using biofertilizers in the environment. The scale of a field trial is both spatial and temporal; both aspects contribute to increased exposure and therefore to increased risk if an adverse effect has been identified. Laboratory and small-scale field trials can be used to generate information to be considered in relation to other safety factors.

The initial impact of field trials is governed by the number and sizes of the test site(s) and the amount of micro-organisms introduced. Since most micro-organisms occupy specific ecological niches, the population size will vary within the application site, depending on the availability of suitable microhabitats. For example, some micro-organisms establish on leaf surfaces, while others live within the soil near root systems of specific plants.

Once a biofertilizer has been introduced, its persistence may vary widely. After introduction, a micro-organism usually shows an initial rapid drop in numbers, since excessive amounts may have been applied and it may also not be adapted to the environment. Assessment of survival and persistence can be estimated by monitoring population levels. Factors that may affect survival include climatic and seasonal changes, host availability, edaphic factors and soil management practices.

To ensure minimal dispersal to non-target environment several factors relevant to the degree of dispersal of a biofertilizer should be considered. Some methods of application – for example, spray applications, with wind as the primary dispersal agent – will tend to result in greater above and below ground dispersal than seed inoculation. Water is the primary dispersal agent in the plant-soil system, so that the soil water regime is another key factor. Generally, micro-organisms will be more likely to be dispersed when applied to wet soils and when inoculation precedes significant rainfall or irrigation. Dispersal of inocula through soil drainage water and via soil animals may be important to consider for scale-up. Soil animals, particularly earthworms, nematodes and protozoa, are important dispersal agents, both directly, through gut transport, and indirectly, through water flow along earthworm channels. Other biological factors can include small mammals, birds, insects and molluscs. Physical factors, such as wind and the movement of farm equipment, can also contribute to the dispersal of biofertilizers.

3.1.1.2 Gene transfer

Gene transfer occurs between micro-organisms in the environment. Introduction of biofertilizers to the field may result in gene transfer which can occur through conjugation, transduction, and transformation. Conjugation is direct transfer of genetic material from one micro-organism to another; transduction is virus-mediated transfer of genetic materials, and transformation is direct uptake of DNA by micro-organisms.

Several conditions must be met if ecologically significant gene transfer is to occur. First, the population density of the recipient organism or the frequency of transfer must be sufficiently high to ensure that the transfer is statistically probable within given spatial and temporal constraints. Second, once the gene is transferred, it must be functional in the recipient organism. Third, the gene must persist in the gene pool of the recipient organism. Under most circumstances, it is unlikely that all these three conditions will be met.

Information on gene transfer obtained through laboratory or small-scale tests can be used to assess the risks associated with scale-up of biofertilizers. However, it is extremely difficult to predict the occurrence of gene transfer(s) in a complex soil environment and to determine frequency. In most cases, therefore, genetic constructs should be designed in ways that minimise the potential for gene transfer, in order to make it possible to predict minimal hazard and therefore minimal risk. It may be possible, in designing the genetic construct, to take an approach which would allow the detection of gene transfer in laboratory or small-scale tests. Additional care should be taken when gene transfer to indigenous micro-organisms can result in significant enhancement of their competitive advantage under natural or managed conditions.

3.1.2 Scale-dependent considerations

From the scientific standpoint, scale-up (see note 5) may be necessary to test the validity of assumptions made or conclusions reached during laboratory or greenhouse/ glasshouse experiments or small-scale field trials. Furthermore, scale-up may be an essential step in developing a commercial product.

Since failure to detect small differences or low-probability events may be due to the previous "small" size of the experiment, the development of biofertilizers for scale-up should take into account the sensitivity and limitations of methods used to identify any potential adverse effects. That is, by increasing exposure, scale-up may improve the ability to detect rare events or effects. If appropriate, monitoring can be performed to provide the data needed to answer certain questions raised in the risk/safety analysis.

3.1.3 Potential adverse effects

Identification of possible effects, based on familiarity with the micro-organism and the environment, will be key in determining whether there are potential adverse effects that will lead to increased risk. Not all effects are adverse, but those which, combined with increased exposure, result in increased risk, will require careful consideration from a management perspective prior to scale-up. The following section outlines effects in three areas: trait effects, target effects, and non-target effects.

3.1.3.1 Trait effects

Consideration of the specific and possibly unique trait(s) of the micro-organism that have led to its use as a biofertilizer may help determine the existence of potential adverse effects that could lead to increased risk in scale-up applications. In such cases appropriate risk management practices need to be applied (see section 3.2).

3.1.3.1.1 Functional genes

Examples of possible trait effects stemming from functional genes include: increased competitiveness in the environment; enhanced interaction with the target plant; increased resistance to adverse conditions; improved ability to produce substances that promote plant growth, etc.

The potential for adverse effects and risk associated with the introduction or modification of genes affecting specific traits in a biofertilizer should be assessed on a case-by-case basis, because the effects of the new traits depend on the complex genetic and environmental context in which they are expressed. In general, the potential for adverse effects and risk from a new trait may be minimised if:

- the new trait results from the modification of a gene originating in the biofertilizer rather than from the introduction of a gene from another organism;
- the new trait is essentially benign and results from the expression of genes whose function is well understood;
- the new trait reduces, or has minimal effects on, the survival, persistence or dispersal of the biofertilizer in the natural environment;
- the new trait reduces the number or range of organisms that the biofertilizer interacts with in the natural environment.

3.1.3.1.2 Selectable marker genes

A fairly wide variety of selectable marker genes are used in micro-organisms intended for release, either for the purpose of selection in the laboratory or for detection in the field. Antibiotic resistance genes (*e.g.* kanamycin) are the most commonly used selectable markers, but resistance to heavy metals (*e.g.* mercury or cadmium) or toxins are among the alternatives.

In the context of risk/safety analysis, different marker genes may lead to potential adverse effects. It is therefore important to assess risks associated with identified adverse effects on a case-by-case basis. Possible adverse effects include imparting selective advantage to the micro-organism in certain environments and transferring (directly or via other indigenous micro-organisms) antibiotic resistant genes to micro-organisms that are pathogenic to man or animals, and against which the antibiotic could be used therapeutically. More specifically, the transfer of resistance to clinically or veterinarily useful antibiotics to other organisms has been identified as an adverse effect of possibly great consequence. The use in the laboratory of marker genes already present in other micro-organisms indigenous to the specific environment is likely to minimise the risk posed by any identified adverse effect.

3.1.3.1.3 Non-selectable marker genes

Non-selectable marker genes are also useful for field detection of biofertilizers. The more commonly used genes include those coding for enzymes detectable in colorimetric assays (*xyl*E, *lac*ZY, *gus*) or by the emission of light (*lux*). However, in addition to inserted sequences, specific coding or "nonsense" sequences already present in the genome can be used as markers. With the limited experience gained to date, no specific adverse effect has been associated with the use of these non-selectable markers.

3.1.3.1.4 Regulatory gene effects

Consideration should also be given to modifications of regulatory sequences that affect the regulation of other genes. For example, sequences which may act on genes other than the gene it is specifically associated with should be assessed for their potential adverse effects.

3.1.3.2 Target effects

3.1.3.2.1 Establishment of competitive but poorly performing strains

In order to reveal any adverse effects of the trait and to confirm the intended function of the new strain, field trials designed to evaluate the performance of the crop plant with the introduced strain are necessary. This is a primary consideration and should be taken into account when developing superior strains.

Modification of traditional biofertilizers can lead to the development, during field testing, of competitive but poorly performing strains. Competition from these strains may

prevent effective use of subsequent inocula and displace elements of the indigenous microbial populations.

3.1.3.3 Non-target effects

3.1.3.3.1 Effects on indigenous microbial populations

Displacement of part of the indigenous community will generally occur when soil is effectively inoculated with an introduced biofertilizer. This displacement is usually short-term: only a small proportion of the introduced population persists in the long term; and the indigenous population effectively returns to its pre-inoculation level. The extent and duration of displacement will depend on the type of inoculum and the niche into which it is introduced.

Displacement effects should be considered in relation to functional microbial diversity, that is, process-level change. For example, displacement could affect key indigenous species involved in nutrient cycling, pesticide degradation, or other beneficial plant-microbe interactions. Process-level effects are most likely to occur and be significant only when there is little diversity in the original microbial community.

If familiarity with the biofertilizer, its host plant, or the target soils indicates that there are grounds for believing that introducing a biofertilizer might disrupt vital processes mediated by indigenous micro-organisms, then tests should be conducted on soil cores or the micro-organism to measure the effects of inoculation.

If these initial tests indicate that a vital process is affected by displacement of indigenous species, then changes in inoculum dosage, method of introduction, or co-inoculation and similar cultural manipulations can be explored to minimise the effects.

3.1.3.3.2 Increased growth by effects on non-target plants

In considering the effects of biofertilizers on non-target plants, there is the issue of whether the introduced micro-organism stimulates potentially weedy species, in addition to the target plant. This effect is considered normal in the use of conventional fertilizers. To date no enhanced weediness due to the use of biofertilizers has been reported in the literature.

Biofertilizers can be categorised into two groups: those directly associated with host plants and those that are free-living micro-organisms. For the first group, non-target plants are limited to those able to establish a symbiotic interaction. Free-living biofertilizers may potentially affect a wider range of non-target plants. Indeed, they may affect any plant that provides a suitable environment for the growth and establishment of the free-living micro-organism.

3.1.3.3.3 Potential pathogenicity and other harmful effects

This section discusses potential pathogenic and other harmful effects on plants, animals and humans.

a) Plants

Biofertilizers are normally placed in the environment to enhance plant growth and they are considered to consist of beneficial micro-organisms. However, an adverse effect may arise if a modification to the biofertilizer results in reduced plant growth due to an increased tendency to cause disease or to enhanced toxin production. Under certain environmental conditions, weak pathogenic interactions with their hosts have been reported for some mycorrhizal fungi (Amijee *et al.*, 1989). Viewed within the context of the overall level of familiarity with these biofertilizers, these isolated reports give little ground for concern.

More important, the introduction of new biofertilizers could have adverse effects on the intended host species as a result of symbiotic incompatibilities between specific genotypes of the host and the particular genotype of the biofertilizer micro-organism. Cultivar-specific differences in nodule formation and function are well known for *rhizobia* (Triplett and Sadowsky, 1992). Therefore it is important that, during scale-up, the biofertilizer is tested with a representative range of host genotypes (cultivars).

b) Animals

Before scale-up studies with a new biofertilizer are conducted, experts should be consulted and the relevant scientific literature studied to determine whether the micro-organism proposed for use, or any related pathogenic strain, has been implicated in adverse health effects on animal populations.

c) Humans

There are no documented cases of adverse health effects on humans due to the use of traditional biofertilizers. However, since the scale-up use of biofertilizers may increase frequency, duration, and level of exposure and possibly affect the route of exposure to the micro-organisms, the identity and characteristics of the micro-organism and the purity of the inoculum should be known, so that it can be distinguished from organisms known to be pathogenic or otherwise harmful to humans.

Any potential adverse health effects due to infectivity, toxicity, allergenicity and immunotoxicity of new biofertilizers should be identified and investigated. (Genetic) modification of biofertilizers to enhance their agronomic performance has the potential to change the interaction of these micro-organisms with humans, with consequent potential for adverse health effects. Modifications of biofertilizers that might reasonably lead to enhanced human infectivity, toxicity, allergenicity or immunotoxicity should be identified and investigated.

Occupational exposure is likely to be greater than the exposure of the general population. When considering potential adverse effects of working with microbial preparations for field release, particular consideration should be given to the microbial strain present in the preparation and to the presence of contaminating organisms.

In conducting risk/safety analysis for occupational exposure to new biofertilizers, attention should be paid to possible routes of exposure, including inhalation, ingestion or inoculation. The method of culture and application should be considered when assessing

safety issues that may arise from the use of the preparation and when deciding on the appropriate risk management strategy.

In terms of controlling exposure to the micro-organisms, the management of most human health and safety issues should be reasonably well understood. Personnel involved in the work should have adequate information and training. As a guide, management procedures would, in most cases, be similar to those currently used to protect personnel involved in the production/applications of similar microbial preparations.

3.1.3.3.4 Effects on mineral cycling

Micro-organisms play an essential role in biogeochemical cycles, such as those of carbon, nitrogen, phosphorus, sulphur and trace elements used as micronutrients by plants. Some biofertilizers might increase the supply of such elements within their area of application, particularly if their mode of action is to increase the availability of limiting elements required for plant growth. However, micro-organisms can also reduce the availability of trace elements by oxidation/reduction or immobilisation within microbial cells.

With the use of current biofertilizers, no adverse effects on mineral cycling have been reported. However, if new biofertilizers are intended to affect soil mineral cycling directly, the implications must be carefully considered.

3.1.4 Biofertilizer inoculant contaminants

Biofertilizers may contain contaminating organisms, some of which may cause adverse effects. Risk of introducing contaminants will be minimised if up-to-date standards in manufacturing and handling of biofertilizer preparations are adopted, and good manufacturing practices (GMP) are used.

3.1.5 Mixed inoculant biofertilizers

Biofertilizers may often contain more than one type of micro-organism in order to achieve either a greater response or to cover a broader spectrum. The effects of using mixed inoculant biofertilizers must be examined in the light of the complex interactions between micro-organisms and between plants and micro-organisms in the rhizosphere, as well as in the light of the possibility of synergistic effects (both beneficial and detrimental) of the co-inoculation. Such effects may be caused by a wide variety of interacting mechanisms, including changes in the persistence and dispersal of a co-inoculated micro-organism, as well as changes in the production of growth regulators. Some aspects of co-inoculation have been reviewed by Jarstfer and Sylvia (1993).

3.2 Risk management

Risk management refers to the way in which appropriate management strategies are applied in order to minimise risks from identified hazards during scale-up. Risk management prior to scale-up may include specifically designing the biofertilizer so as to reduce

risk. Management of the environment, during and after introduction of the biofertilizer, will play an important role in reducing the risks.

Many confinement procedures commonly used to manage risks associated with small-scale field tests of micro-organisms are inappropriate for managing risks that occur during scale-up. Confinement procedures, such as isolation of field plots, controlling irrigation water run-off, and cleaning farm machinery, become much less effective as field trials increase in scale. As testing increases in scale, confinement procedures become increasingly difficult to implement. Therefore, after small-scale tests are complete and resulting data indicate that scale-up with the organism is appropriate, it is better to use established or modified risk management practices rather than to rely on the confinement procedures normally used at small scale.

Fortunately considerable research has been conducted and experience has been gained, over the years, on managing both beneficial and pathogenic soil-borne micro-organisms (Agrios, 1988). It is generally considered that biofertilizers which are obligate symbionts, particularly those with a narrow host range, are less of a concern compared to biofertilizers with broader niches in the soil.

Cultural practices, such as crop rotation, tillage, and chemical treatments, have been used successfully as strategies to control or manage populations of soil-borne micro-organisms and therefore to manage risk. Risk can also be managed by selecting or modifying biofertilizers to meet specific performance standards, for example by using biofertilizers with reduced ability to associate with other host plants, winter over, or transfer genetic material. L-forms, for example, because they cannot survive at all in the environment in the absence of their host, may have particular advantages in the future in terms of safety.

Risks may be associated with increased exposure owing to a variety of mechanisms. For any given mechanism associated with a manageable risk, several management strategies may be appropriate, depending on the specific nature of the biofertilizer. Examples are provided below. They are neither exhaustive nor exclusive; they merely indicate that appropriate management strategies are available:

- survival and persistence:
  - using biofertilizers with short dormancy periods;
  - limiting the survival of introduced biofertilizers after they have carried out their intended function through the use of suicide genes or other methods;
  - rotating certain crops to minimise survival and persistence of biofertilizers;
  - improving monitoring to estimate survival and persistence;
- dispersal:
  - modifying application technology, for example by devising applications to seeds or soils as localised inocula rather than spraying;
  - improving monitoring to estimate dispersal;
- gene transfer:
  - practising genetic modifications on chromosomes rather than plasmids;
  - using transposable elements that are not subject to further transposition after genetic modification;

- using biofertilizers that are not subject to spontaneous uptake of genetic material;
- monitoring to estimate gene transfer;
- contaminants:
- designing methods of production to ensure that contaminants are not introduced in the preparation of the biofertilizer;
- improving monitoring to detect contaminants during the production process.

– contaminants,
- designing methods of production to ensure that contaminants are not introduced in the preparation of the biofertilizer;
- improving monitoring to detect contaminants during the production process.

4. Summary

The goal of this project is to identify the principles and practices used to address the environmental safety considerations associated with the scale-up of testing and use of biofertilizers developed by newer molecular techniques of biotechnology.

The concept of familiarity, as defined in the Preamble, is an essential aspect of risk/safety analysis and of risk management. In the theoretical framework of principles related to the scale-up and use of biofertilizers, it can be applied to any element or stage in the system under consideration.

Familiarity is the knowledge and experience that can be used in risk/safety analysis of biofertilizers modified by the newer molecular techniques. It is part of the procedure used to identify potential adverse effects (hazard identification), to assign a level of risk (risk assessment), and to indicate appropriate methods to manage it (risk management).

Most effects of new traits in biofertilizers will be recognisable during small-scale field tests, depending on the design of the tests and the effectiveness of the detection and monitoring methods available. However, some effects, including safety effects dependent on scale, may only become apparent during scale-up.

The identification of potential adverse effects is based on the biological properties/characteristics of the micro-organisms used as biofertilizer in a specific environment and on an analysis of the associated risk. It may be based on information available for other organisms or environments (familiarity), as well as on research associated with the specific micro-organism.

Specific and/or unique traits associated with functional, regulatory or marker genes that have led to the selection of the micro-organism as a biofertilizer should be considered in determining possible adverse effects that could lead to increased risk. The potential for adverse effects should be assessed on a case-by-case basis and may be minimised by appropriate selection and assessment of the trait.

Cultural practices have been used successfully as strategies to control or manage populations of soil-borne micro-organisms and therefore to manage risk. Risk management also can be accomplished by selecting or modifying biofertilizers to meet specific

performance standards, *e.g.* survival, persistence. Risk may be associated with increased exposure in a variety of ways. Risks due to exposure to biofertilizers can also be managed through appropriate strategies to control survival, persistence, dispersal and gene transfer.

There will be cases where, based on a risk/safety analysis, a biofertilizer with a new trait considered for scale-up in a defined environment will present no potential adverse effect. In such cases, the biofertilizer can be scaled-up and managed by conventional biofertilizer management practices.

The scientific principles derived from an analysis of several case studies and other relevant information provide a framework for evaluating the safety of scale-up for testing and use of biofertilizers.

5. Case reviews

The following reviews were produced during the elaboration of the principles outlined in the preceding sections. They are selective rather than exhaustive and are intended to illustrate the concept of familiarity by providing sufficient information for considering the general principles. They refer to the preceding sections on principles for which the information is particularly relevant.

5.1 Symbiotic nitrogen-fixing micro-organisms

5.1.1 Rhizobia

5.1.1.1 General description of rhizobial taxonomy

Rhizobium and *Bradyrhizobium* (hereafter, *rhizobia*) are rod-shaped, non-spore-forming gram-negative soil bacteria belonging to the family *Rhizobiaceae* (Jordan, 1984). Traditionally, this family also includes *Agrobacterium* and *Phyllobacterium*, but recently two new genera have been recognised: *Azorhizobium* and *Sinorhizobium* (Elkan, 1991).

Rhizobia are motile. *Bradyrhizobium* species have one polar or subpolar flagellum, whereas *Rhizobium* species have two to six peritrichous flagella. These bacteria are characteristically aerobic, anuclear micro-organisms containing one major bacterial chromosome. *Rhizobium* species also contain very large stable plasmids which can vary in number and size (200-1 500 kilobase pairs). Some of them are self-transmissible and able to co-transfer other plasmids that are not. This characteristic is very important in evolution and gene transfer. *Bradyrhizobium* strains generally do not have large plasmids.

Rhizobia are distinguished by their rate of growth in culture; the fast growing genus *Rhizobium* (generation time < 6 hours) is comprised of *R. leguminosarum*, *R. meliloti*, *R. loti* and *R. galegae*. The slower growing *Bradyrhizobium* (generation time > 6 hours) consists of a wide variety of strains nodulating various tropical legumes, mostly under *Bradyrhizobium* ssp., while some are under *B. japonicum*, which includes all strains capable of nodulating soybean. These bacteria are of agricultural interest primarily because of their ability to fix atmospheric nitrogen within nodules on the roots of

leguminous plants. *Rhizobia* that nodulate tree legumes have also found applications in agroforestry (Keyser and Turk, 1991). Only one non-legume plant has been found to form root nodules with *rhizobia*; Parasponia with *Rhizobium* (Rolfe and Gresshoff, 1988). Nitrogen fixation by stem-nodulating legumes has also been reported (Ladha, 1991).

The plant family *Leguminosae* comprises about 20 000 plant species taxonomically classified into 650 different genera. Many of these species are wild, and as yet only 15 per cent have been well studied (Elkan, 1991). Many important food crops belong to this family of plants, including: soybeans, peanuts, cow-peas, beans, peas, chick-peas, pigeon-peas and lentils. Important forage legumes include alfalfa, clover, sesbania and vetch.

The interaction between micro- and macro-symbiont is generally quite specific. As a consequence, *rhizobia* that fix nitrogen effectively on one leguminous species may be totally non-infective or ineffective on another. For example, *R. leguminosarum* specifically forms a symbiotic relationship with peas but not with alfalfa. However, some rhizobial species can nodulate a rather broad assortment of legume species [*e.g. Bradyrhizobium* spp., *Rhizobium* (Parasponia)] (Rolfe and Gresshoff, 1988).

In general, *rhizobia* fix nitrogen only in symbiotic association with legume roots. Infection of the host plant is initiated when the bacteria encounter specific signal substances secreted by the roots (Rolfe and Gresshoff, 1988). These substances induce the *rhizobia*to secrete specific compounds that cause division of root cortical cells, curling of root hair cells, and penetration of the cell walls of root epidermal cells. In most legumes, infections are initiated only in a developmentally restricted region of the root, usually the zone near the growing root tip where root hairs are just emerging. In some hosts, infections are initiated primarily at the base of the emergent lateral roots or at other points where the root surface permits entry through cracks. *Rhizobia* that penetrate a root cell wall continue to multiply and become surrounded by a tube of plant-cell wall material called the "infection thread". The infection thread grows inward and branches extensively, conveying a "linear colony" of *rhizobia* into plant cells throughout the root cortex. Extracellular signals from the *rhizobia* induce the root to de-differentiate, form a nodule meristem, then re-differentiate to form macroscopic nodules in which conditions are favourable for nitrogen fixation. Root nodule formation has been studied extensively.

5.1.1.2 Experience with uses of rhizobia in the agricultural environment

5.1.1.2.1 History of use

Rhizobia are used as natural or biofertilizers because of their ability to form symbiotic interactions with certain leguminous plants and convert atmospheric nitrogen into a form usable by the plant. Nitrogen is the dominant elemental requirement for most crops and is therefore very important for agricultural food production, both directly, for the production of edible legumes, and indirectly, for the production of animal feeds. The exploitation of *rhizobia* is generally recognised as a biotechnology research priority because of its fundamental importance to plant growth and food production.

Inefficient nitrogen-fixing strains or low populations of efficient strains pre-existing in the soil make it difficult for farmers to reap the benefits of these naturally occurring

micro-organisms. It is common practice today to add efficient strains of *rhizobia* to legume crops in order to establish high populations of bacteria and thereby enhance symbiosis.

In the past, *rhizobia* were applied to crops by soil transfer methods such as transferring soil from *rhizobia*-containing fields to new fields, or by taking soil from the roots and nodules of legumes and applying it directly to legume seeds. Then it was recognised that for microbial inoculation to be effective, it must be compatible with agricultural needs. This meant that the micro-organism had to be formulated with agents that facilitated packaging, maintained viability, and made the preparation easy to handle. At first, agar and sand were used as inoculant carriers, but these were later replaced by peat, which has become the industry standard.

Inoculants for agricultural uses come primarily from specialised private companies and have been available since 1896, when the first inoculant received a patent in the United States. In some countries (*e.g.* Australia, New Zealand, Canada, France), standards have been established to ensure that inoculant products will achieve optimum performance when applied in the field.

Two basic methods are used to inoculate agricultural crops with *rhizobia*. The first uses various types of seed treatments. The inoculant may be mixed directly with the seed, along with a "sticker" and a "sprinkle" of water or with larger amounts of water to make a slurry. Dry mixing is more popular because of its simplicity, but it unfortunately results in poor seed adhesion. Pre-inoculated seeds are also used to introduce some species of *rhizobia* into agricultural crops.

Inoculants in a granular or liquid form can also be applied directly to the soil. This can be preferable when agricultural chemicals used on the seed might be harmful to the *rhizobia*, when the soil is hot and dry (harmful to the bacteria), or when there are large populations of ineffective *rhizobia* and large amounts of inoculum are needed to inundate the rooting zone during stand establishment.

It has also been demonstrated that spraying a slurry of inoculant through the irrigation system over sowed, pre-inoculated seed is an effective combination application method (Ciafardini and Lombardo, 1991).

5.1.1.2.2 Basic research on the genetics of *rhizobia*

Rhizobia are very well characterised compared to most other micro-organisms used as biofertilizers; they consequently have high potential as micro-organisms that may be amenable to genetic improvement. Symbiotic nitrogen fixation is studied using a variety of *Rhizobium* and *Bradyrhizobium* species. Among the fast growers, *R. meliloti* and *R. leguminosarum* are the most studied; *B. japonicum* is the most studied of the slow-growing *rhizobia* (Glazebrook and Walker, 1991; Rolfe and Gresshoff, 1988; Caetano-Anolles and Gresshoff, 1991). A number of genetic, biochemical and molecular biological approaches have been used to study nitrogen fixation, including cloning techniques, immunological methods (antibodies), and mutation studies (transposon insertion, chemical or site-directed mutagenesis). Through these approaches, much information has been gathered about rhizobial genes, particularly those involved in the nodulation and nitro-

gen-fixation process and those regulating gene expression. In addition, restriction fragment length polymorphism, DNA fingerprinting, antibiotic selection, monoclonal antibodies, and polymerase chain reaction techniques have been used as means of identifying strains for taxonomic purposes and monitoring *rhizobia* that have been deliberately released into the environment.

5.1.1.3 General safety considerations and familiarity with nitrogen-fixing rhizobia

The use of rhizobial inoculants on agriculturally important legume crops has been an established farming practice for almost a century. Within this time, experience, research and technology has evolved to the point where production of commercial inoculants has become economically viable. Consequently, there is a high degree of familiarity for the use of these products.

Rhizobia have been the focus of strain improvement and use in agricultural practice for decades. Genetic improvement has focused on the production of high-quality inoculants able to form nodules on the roots of a range of species and varieties of host plants, to supply a large proportion of the host plant's nitrogen requirements, to dominate indigenous strains of bacteria, and to perform over a sufficient range of environmental conditions (Havelka *et al.*, 1982). Genetic improvement may also involve selection of the most desirable plant variety through legume breeding programmes. This allows the researcher to optimise the nitrogen-fixing capabilities of the host plant/ *rhizobia* inoculant combination. Mutagenesis techniques have also been employed to improve host plant performance and to develop improved strains of *rhizobia* (Glazebrook and Walker, 1991). More recently, molecular biology techniques, such as rDNA, have been used in research aimed at developing high quality inoculants.

The principal sources of *rhizobia* used as commercial inoculants are: *i)* existing cultures; *ii)* isolates from nodules collected in regions where the legume originated; and *iii)* genetically improved biovars. New strains are tested in the laboratory to authenticate them as true species. Agronomic properties of new strains are tested in small-scale, controlled and confined field experiments. Prior to field introduction, the issue of the safety of the new micro-organism (to the environment and to human health) must be addressed. For biofertilizer micro-organisms, such as *rhizobia*, scale-up may involve testing under an increased range of climatic conditions or geographic regions rather than over a larger area. In scale-up applications, these safety considerations become more important as applications move from a confined to an unconfined environment. Safety considerations will be discussed below.

5.1.1.4 Survival, persistence and dispersal

Spatial and temporal persistence is generally not a concern for *rhizobia*, since it has long been used agriculturally without reported adverse effects. Safety and economic considerations for genetically modified *rhizobia* include altered persistence, since persistence of higher numbers or for longer periods of time could lead to the displacement of beneficial soil micro-organisms (Bentjen *et al* ., 1989) and to later difficulties in replacing the more persistent strain with improved strains (see section 3.1.1.1). It is important to

consider the kinds of beneficial micro-organisms (such as other rhizobacteria that promote plant growth) that may be affected by rhizobial inoculants and to be aware of the growing information in the scientific literature on interactions between microbial populations.

Genetic modifications that affect the survival or competitiveness of introduced *rhizobia* may be valuable for enhancing the agricultural effectiveness of the inoculum strain and reducing the need for high inoculum doses, but they may also make it more difficult to introduce improved strains later if enhanced survival/competitiveness is coupled with enhanced persistence (see section 3.1.3.2.1).

In nature, movement of *rhizobia* by cell motility is limited; evidence suggests they are dispersed in soil primarily in association with plant root systems (Soby and Bergman, 1983; Caetano-Anolles *et al.*, 1988). Field studies with modified *rhizobia* in the United States and Canada indicate that the bacteria do not move more than several centimetres beyond the initial inoculation site. It is the method of application that raises greater concerns. Traditionally, *rhizobia* have been applied by inoculation of legume seeds or in furrow. Recently, cover inoculation (*rhizobia* sprayed onto fields) has been reported to achieve beneficial effects (Ciafardini and Lombardo, 1991). This method increases chances of dispersal, particularly in scale-up applications. Low levels of *rhizobia* can also be dispersed by blown soil, equipment and humans (*e.g.* on footwear and clothing). However, these organisms are primarily spread by tillage.

5.1.1.5 Gene transfer

The frequency of genetic exchange among symbiotic nitrogen-fixing bacteria in the environment is not well known. Studies of genetic diversity in *rhizobia* and other evidence suggest that genes located on symbiotic plasmids are not often transferred (Schofield *et al.*, 1987; Shantharam, 1990). There is little direct evidence of gene transfer, although conjugal transfer of a megaplasmid between *Rhizobium meliloti* has been reported in alfalfa nodules (Pretorius-Guth *et al.*, 1990).

Gene transfer has been identified as a concern more for traits such as antibiotic resistance than for traits associated with increased nitrogen fixation (see section 3.1.3.1.2). Transferred traits related to a micro-organism's nitrogen fixation phenotype are not likely to be expressed in, or confer a selective advantage to, most micro-organisms associated with soil or plants, with the possible exception of other closely related symbiotic nitrogen fixing bacteria.

The transfer and expression of genetic material which directly codes for nitrogen fixation may place an energy burden, and thus a selective disadvantage, on the recipient micro-organism. For example, transfer and expression of genes for the nitrogenase enzyme complex (*e.g. nif* genes) or genes for hydrogen uptake (*hup* genes) may increase nitrogen fixation capabilities while placing a drain on cellular sources of carbon and energy.

5.1.1.6 Trait effects

5.1.1.6.1 Antibiotic resistance

Past experience with intrinsic antibiotic resistance (IAR) in *Rhizobium* and *Bradyrhizobium* indicates that IAR is useful as a characteristic for comparing strains in culture (Antoun *et al.*, 1982) and, in natural populations (Glynn *et al.*, 1985), as a marker to monitor *rhizobia* in the environment. In addition, genes that confer antibiotic resistance have frequently been used to monitor introduced strains.

Transfer of R-factor plasmids containing antibiotic resistance occurs among *rhizobia* in culture (Kuykendall, 1979; Pilacinski and Schmidt, 1981). However, some field tests of modified *rhizobia* have involved antibiotic resistance markers located on the megaplasmids, which are not transferable (Sayre, 1990). In general, concerns about the transfer of antibiotic resistance in *rhizobia* will be similar to those for any micro-organism.

5.1.1.7 Target effects

Negative effects on the target legumes could be due to a variety of symbiotic incompatibilities that prevent the establishment of an effective symbiosis; these generally lead to effects such as decreased legume yield or nitrogen content (Rolfe and Gresshoff, 1988). There is no general agreement on how to measure the adverse effect that would cause concern during early tests of an inoculant strain. This is due to the fact that many factors – inoculant contaminants, the host plant genotype, the rhizobial inoculant, the inoculant cell numbers and their survival rate, the presence of indigenous *rhizobia*, and the soil environment – all affect yield.

Recognition of rhizobial strains by a host plant is related to the compatibility of that strain with the host cultivar. The largest variability in host preference for *Rhizobium* strains occurs between plant cultivars (Bromfield, 1984).

No direct correlation has been found between ability to compete and effectiveness. For example, in one study, an ineffective *R. leguminosarum* strain was found to be more competitive than an effective strain on the same root system (Johnson and Beringer, 1976). In another study, effective strains of *R. trifolii* always dominated the nodules in the presence of ineffective strains (Robinson, 1969). Competitive inoculant strains that fix nitrogen poorly are not desirable (see section 3.1.3.2.1).

The nodules established by an inoculant strain are often proportional to the number of rhizobial cells. However, competition among strains of *rhizobia* may not always depend primarily on the relative numbers of the competing bacterial strain. For example, competitiveness of *R. trifolii* strains was not influenced by inoculum level in studies performed in Australia (Brockwell *et al.*, 1982).

Many physico-chemical factors affect the ability of rhizobial strains to survive and consequently influence their success or failure in nodulating the host plant. These factors include soil temperature, soil acidity, nutrient levels, desiccation and flooding.

5.1.1.7.1 Establishment of competitive but poorly performing strains

Some of the factors contributing to the establishment of poorly performing but competitive strains are historical in nature and reflect agricultural practice. Legume crops such as alfalfa, soybean, clover, peanut (groundnut) and bean are grown repeatedly in the same fields, regardless of whether or not the indigenous *rhizobia* in those fields are capable of efficient nitrogen fixation. This practice favours the accumulation of *Rhizobium* strains, which are competitive with respect to forming nodules but indifferent, or even totally ineffective, with respect to fixing nitrogen. Many of the nodules formed by indigenous *rhizobia* on crops do not fix nitrogen efficiently. While advances have been made towards developing superior inoculum strains by isolating mutants or genetically superior strains, most of the nodules on plants in the field are still formed by indigenous *rhizobia*, not by inoculum strains. The failure of superior inoculum strains to nodulate field-grown plants is generally known as the competition problem. This problem arises because the indigenous *rhizobia* have roughly a thousand-fold competitive advantage over any inoculum strain added with the seed at the time of planting. The competitive advantage is due primarily to location. For most legumes, the only part of a root that is susceptible to infection by *rhizobia* is the small region near the growing tip where root hair cells form. These tips grow away from the inoculum bacteria towards the indigenous *rhizobia*, and, due to abrasion, inoculum bacteria cannot maintain an adequate population at the growing tips (Bhuvanesware *et al.*, 1988).

Another important facet of the competition problem is that most legume hosts strictly regulate the total number of nodules formed on the root system (Caetano-Anolles and Gresshoff, 1991). The formation of the first few nodules triggers a systemic suppression by the host of further nodule formation. As a consequence, most nodules on field-grown plants are located near the crown of the root system, on the oldest portions of roots. Such feedback regulation favours nodulation by the *rhizobia* that come into contact with the roots of the germinating seedling and significantly inhibits nodulation by latecomers.

5.1.1.7.2 Potential solutions to the competition problem and associated risks

One solution to the competition problem is to supply greater numbers of inoculum bacteria. However, the number of infections and nodules initiated on the roots increases in proportion to the logarithm of the number of *rhizobia* in contact with the root (*e.g.* Bhuvanesware *et al* ., 1988). Thus, a hundred-fold increase in the number of *rhizobia* leads to just a five-fold to six-fold increase in the number of nodules formed, not a 100-fold increase.

Increased emphasis is now being placed on enhancing the competitiveness of inoculum strains, so that they reach the infectible part of the root more quickly, in greater numbers, and in a more active state than the indigenous strains.

There are several new strategies for developing new strains or new delivery systems which ensure that the inoculum bacteria will form the nodules. Much of this work focuses on enhancing competitiveness. Some recent examples are:

- Making the strain resistant to some toxic agent that kills or inhibits most indigenous strains of *rhizobia*. This approach first used fungicides coated onto seeds with the inoculum bacteria. More recently, *R. leguminosarum* bv. *trifolii* strain T24 has been used successfully to suppress a range of potentially competing *rhizobia* by *in situ* secretion of a very specific peptide antibiotic (Triplett and Sadowsky, 1992). The antibiotic produced is a natural compound that is specifically targeted to a narrow range of bacterial species and is produced in small amounts by the inoculum strain at the site of action. Synthesis of the antibiotic and resistance to it do not seem to impair the efficiency of nitrogen fixation.
- Creating or taking advantage of gene-for-gene fits (lock and key) between a specific host plant and an inoculant strain. There is increasing evidence that single genes in both legumes and *rhizobia* determine ability to nodulate specific cultivars (Triplett and Sadowsky, 1992). Once understood, these genes could be modified and/or transferred (within a genus) to create inoculum strains that nodulate host genotypes resistant to nodulation by indigenous strains. A tight gene-for-gene fit does not preclude efficient nitrogen fixation. Other gene-for-gene strategies include:
  - transforming the host with genes required for synthesising a specific nutrient which only the inoculum strain can utilise, such as the opines of *Agrobacterium*;
  - inoculation with mixtures of micro-organisms that may act synergistically.
- Boosting the readiness of the inoculum strain to interact with its host. For example, flavonoids and related phenolic compounds from host legumes have been found to induce nodulating genes in *rhizobia*. These phenolics can be (carefully) added to the culture medium under appropriate manufacturing conditions during the formation of inocula in order to increase cellular potency in nodule formation. Similarly, Bhagwat and Keister (1992) have shown that certain genes in *Bradyrhizobium japonicum* are reduced by unknown substances in crude soil extracts in a strain-specific manner. The transfer of certain soil-inducible genes from a highly competitive strain of *B. japonicum* to a poorly competitive strain significantly enhanced nodulation by the recipient. In principle, the application of such genetic modifications to inoculum development would involve extending the range of the strains within a species or genus capable of responding quickly or fully to some natural stimulus.

For each of these strategies, safety considerations relative to potential ecological effects, *e.g.* production of antibiotics, extension of the cultivar range, and, especially, maintenance of nitrogen-fixation efficiency must be tested prior to scale-up.

5.1.1.8 Non-target effects

Safety considerations for non-target effects of genetically modified *rhizobia* are primarily related to the degree to which nitrogen fixation is enhanced in the field. For scale-up, testing considerations include potential effects on nitrogen cycling and impacts on non-target weedy plant species in the same cross-inoculation group. Selected or

genetically modified strains that can compete against other micro-organisms in the rhizosphere environment may raise concerns for the displacement of other beneficial micro-organisms. While these issues have been raised as safety considerations, no adverse effects relating to enhanced weediness and deleterious effects on microbial populations as a result of the use of *rhizobia* have been reported to date in the literature.

5.1.1.8.1 Increased growth of non-target plants

Effects on non-target plant species are related to increased growth of weedy legume species. Strains selected or genetically modified for enhanced nitrogen fixation that demonstrate, in the laboratory or greenhouse, a potential for significantly greater yield raise concerns for weedy species (Sayre, 1990). For example, *Rhizobium* not only nodulates alfalfa but also species of sweet clover, and increased biomass production of this potentially weedy legume may be a safety consideration. These concerns can be mitigated by: *i)* agricultural practices which prevent growth of sweet clover in most alfalfa stands where multiple harvesting is practised; *ii)* the desire for any crop legume to grow in single-harvest regions, since alfalfa does not grow well in this region anyway; *iii)* the presence of high moisture in many pasture fields. Further, adverse effects from dicoumeral is usually seen when sweet clover makes up 50 per cent of hay.

In general, potential infection of non-target plants by *rhizobia* is predicted on the basis of their ability to reach these plants in quantities large enough to effectively nodulate the plant. These concerns can be mitigated by evidence of the inability of the strain to nodulate sweet clover effectively or of poor survival and dissemination of the inoculant strain in the environment. Alternatively, yield increases of non-target plants due to association with the genetically improved strain may not exceed the range typically found with previous inoculants. Thus, it is important to consider the kinds of non-target organisms that may be affected and the probability of unusual or irreversible perturbations.

Biofertilizers, like chemical fertilizers, may stimulate the growth of other plants at the site. Whether this happens or not will depend on the ability of the organism to spread and persist, on whether close association with specific plant species is required, and on what improved properties for supplying nutrients are provided by the particular micro-organism.

5.1.1.8.2 Effects on indigenous microbial populations

The potential for long-term persistence of *rhizobia* inoculants raises concerns about the competitive displacement of beneficial micro-organisms. Enumeration of inoculant populations over time is an integral component of the field assessment of the success of inoculation (Brockwell, 1982). Generally, inoculant populations decrease rapidly after planting, although this depends on the form of the inoculum, methods of application, kind of seed, and storage conditions. Long-term establishment of *Bradyrhizobium* inoculants in the agricultural soil environment is dependent on a relatively low indigenous *Bradyrhizobia* population (see section 3.1.3.3.1).

Knowledge of rhizobial genetics and physiology has not advanced to the point of identifying genes that confer increased persistence in the environment. However, strains with improved ability to nodulate specific plants, or to nodulate a wider range of plants in a cross-inoculation group, may be more likely to raise issues related to competitive displacement. These issues will become more important as superior nodulating rhizobial inoculants are produced and competitiveness increases. Persistence of greater numbers or persistence for longer than expected periods of time could lead to displacement of beneficial soil micro-organisms (Bentjen *et al.*, 1989). Inoculant strains that have been selected or genetically improved solely to enhance nitrogen fixation would not be expected to raise concerns for competitive displacement of beneficial micro-organisms.

5.1.1.8.3 Potential pathogenicity for plants, animals and humans

a) Plants

For plants, pathogenicity is not of concern because *rhizobia* form specific, symbiotic relations solely with host legumes in the cross-inoculation group. While *rhizobia* infect their host plant, they are rarely considered either pathogenic or parasitic. Even though *Rhizobium* and *Bradyrhizobium* are related to *Agrobacterium tumefaciens* (a known plant pathogen which causes crown gall disease) and *Agrobacterium rhizogenes* (which causes hairy root disease), they are not themselves associated with any plant disease (Jordan, 1984).

Genetic modification of *rhizobia* should not raise concerns unless the host microbe is specifically modified to contain DNA sequences related to pathogenicity (*e.g.* Ti plasmid or part thereof from *A. tumefaciens*) or toxicity.

b) Animals and humans

For animals and humans, pathogenicity is not a concern for traditionally used *rhizobia* inoculants, because of their large-scale use, without reports of adverse effects on health, for almost a century. A search of the Medlars (medical) database back to 1966 reveals no reports of disease in humans or animals associated with *R. meliloti*. Similarly, searches of the bibliographic databases of CAB (Commonwealth Agricultural Bureau) or FSTA (Food Science and Technology Abstracts) discloses no reference to adverse effects.

As an example of potential toxicity, the cryIII δ-endotoxin gene from *Bacillus thuringiensis* has been cloned into *R. meliloti* and *R. leguminosarum* bv. *viceae* to control nodule-feeding insect larvae (Heron *et al.*, 1991). To the extent that disease-controlling micro-organisms may be considered as biopesticides, assessment of their safety may lie beyond the scope of this document.

Genetic improvement using certain traits that already exist in natural microbial populations should not be expected to pose additional health problems to animals or humans. If the new trait does not exist in the microbial population, the impact of introducing the modified micro-organism on a large scale must be considered.

c) Specific worker safety considerations

Rhizobia have been used in the laboratory for approximately 100 years without reported incidents of pathogenicity or other significant health effects on humans. Personnel in research laboratories frequently grow cultures of these bacteria to much higher titres than could be encountered during field application. On a larger scale, workers employed by inoculant companies are routinely exposed during industrial production to large numbers of *rhizobia* in fermenter batch cultures. The health record suggests that *rhizobia* used for this purpose do not pose a threat for humans. Few cases of allergenicity induced by exposure to large numbers of *rhizobia* during production have been reported. The potential for allergenicity can be mitigated by adequate containment of micro-organisms during production and/or by the use of face masks during handling. Consideration should be given to potential health hazards arising from the formulation, such as allergies to peat and/or to contaminating organisms in the carriers.

Large-scale production of genetically improved *rhizobia* is not expected to create additional health concerns, unless, for example, phenotypic traits are altered in a way that would affect toxin production or allergenicity.

5.1.1.8.4 Effects on nitrogen cycling

Concerns have arisen over effects on nitrogen cycling; they are based on the assumption that increased nitrogen production by the crop plant would result in higher nitrate concentrations in groundwater and surface water run-off (Tiedje *et al.*, 1989). They involve inoculant strains selected or genetically improved to enhance nitrogen fixation which demonstrate the potential for significantly greater yield (super nitrogen fixers).

However, it is unlikely that increased soil production due to rhizobial inoculants would result in nitrate concentrations in aquatic environments higher than those produced by the use of inorganic nitrogen fertilisers. Perturbations caused by a single application of nitrogen-fixing micro-organisms during a growing season would be minor compared with those due to other agricultural practices.

5.1.1.9 Inoculant contaminants

North American rhizobial products are often very contaminated. Contamination during large-scale production of legume inoculants may affect the ability of *rhizobia* to survive in the formulated product or may cause adverse effects in the environment. Good manufacturing processes (GMP) for handling micro-organisms can be used to prevent contamination. During inoculant production, consideration should be given to the presence of foreign micro-organisms, such as soil-borne pathogens, or of spontaneous mutants of the rhizobial inoculant which may appear with repeated transfers of the culture. It is not unusual for *rhizobia* to lose their efficacy during storage or, particularly, after successive serial transfers. Serial transfer from small to large fermenters for growth may also favour contamination. In general, the recommended inoculation level in the fermenter varies from 1 to 10 per cent by volume (Smith, 1987). At these levels, there is

some concern that cultures of slow-growing *rhizobia* will be contaminated by faster growing micro-organisms.

It has been suggested that the source of most contamination is not the process but the non-sterile peat carrier used to support *rhizobia* during storage. Although non-sterile peat is cost-efficient for large-scale production, use of peat-carriers pre-sterilised by gamma irradiation (Strijdom and van Rensburg, 1981) or by autoclaving (Somesgaran, 1985) generally results in higher rhizobial populations with fewer contaminants. If this system is used, it is essential to add the bacterial inoculum to the culture aseptically.

Peat carriers are the industry standard in North America but may not be available in many countries, particularly in the tropics. In such cases, the potential adverse effects of using substitute carriers should be carefully assessed. Attention should be given to the potential contamination problem as well as to the potential health hazards posed by the carriers themselves. For example, polyacrylamide, which is stringently regulated in Canada and is only commercially available for home use for non-food plants in small (< 5 g) quantities, has been used as a carrier (Dommergues *et al.*, 1979). Valid alternatives include the use of pre-sterilised vermiculite for small-sized legume seeds and, for large-sized legume seeds, liquid inoculants consisting of pure cultures in stabilized growth media.

5.1.1.10 Risk management considerations for *rhizobia*

Scale-up applications of modified *rhizobia* should distinguish between testing to establish commercial usefulness (*i.e.* efficacy) and testing to establish the safety of the micro-organism. Safety issues (*i.e.* adverse effects identified during the risk/safety analysis) should be addressed at the small-scale testing stage. Two risk management strategies are involved.

First, in developing commercial inocula, scale-up testing of modified *rhizobia* primarily addresses the efficacy issue. However, when the results (or the lack thereof) in small-scale testing indicate that potential hazards may still exist, scale-up trials should address this issue.

Second, given that the modified *rhizobia* have already been tested in the field, knowledge of and familiarity with the test micro-organism should have increased. Consequently, some conditions previously imposed to confine the micro-organism to the test site may be relaxed as scale is increased.

5.1.2 Frankia

5.1.2.1 General considerations for safety with Frankia *inoculants*

Frankia is a genus of actinomycete bacteria that forms nitrogen-fixing root nodules on a variety of woody plant species (Mullen *et al.*, 1992). During the past 15 years, methods have been developed to isolate *Frankia* strains from nodules and to culture them in defined laboratory media. This has made the preparation of commercial inoculants possible. However, there is as yet very little practical experience in working with *Frankia* inoculants on a large scale.

There are a number of reasons to believe that a significant inoculum industry may develop for *Frankia* in the future. Previous research has established that a wide variety of plant species can establish symbiotic associations with *Frankia* – over 160 species within six orders of angiosperms (Mullen *et al.*, 1992). A number of these angiosperm hosts, especially *Alnus* and *Casuarina*, are important to agroforestry and reclamation of disturbed land. *Frankia* nodules are generally quite efficient in nitrogen fixation, and they can function for many years. This allows the host plants to flourish in soils containing limited amounts of nitrogen. Both *Frankia* and its hosts are distributed throughout the world, in both temperate and tropical regions. It is believed that the *Frankia* symbiosis plays a significant role in global nitrogen cycling.

At present, no suitable methods for the genetic transformation of *Frankia* have been developed (Reddy *et al.*, 1992). Release of modified derivatives of *Frankia* for small-scale testing is therefore some years away. Over the next five to ten years any release of *Frankia* for large-scale testing is likely to involve wild-type strains or derivatives generated by random mutagenesis.

5.1.2.2 Survival, persistence and dispersal

Frankia, as an actinomycete, grows as long filaments and is non-motile. Its dispersal is therefore passive.

Many *Frankia* isolates can form spores, and this can greatly enhance their natural persistence, survival and dispersal. Usually, *Frankia* strains chosen as inoculants are likely to be spore formers, as this will help ensure long shelf-life and consistent host infection.

However, it is possible that inoculant strains will be selected or modified genetically in some manner to ensure that they do not persist from year to year. It is commercially desirable to sell inocula that fail to persist in the soil, so that new inocula must be purchased to infect newly planted hosts. Minimal persistence should be an important social and ecological objective as well. If the strains chosen as inoculants fail to persist from one growing season to the next, except in the host, then it remains relatively easy to introduce new and more useful inoculant strains without competing against previously introduced inocula. Also, the impact of inoculant strains on the ecology of other organisms, the concern of wide dispersal into new, non-target ecosystems, and the concern of unwanted genetic exchange between inoculant strains and the indigenous microflora are all likely to be minimal if the inoculants are chosen so that they do not persist from year to year outside the host. Strain persistence is thus a major concern for large-scale release of any microbial inoculant (see section 3.1.1.1).

5.1.2.3 Gene transfer

Exchange of genetic information in natural environments between *Frankia* isolates, and between *Frankia* and other micro-organisms, has not been studied. It is prudent to assume that some exchange of some genetic material does occur.

5.1.2.4 Target effects

There appear to be no reports of deleterious effects of *Frankia* on host plants. However, the infection of different host genotypes with specific *Frankia* isolates is expected to lead to symbiotic associations of varying degrees of compatibility and benefit. The symbioses generated by the infection of different host genotypes with a given *Frankia* strain are likely to range from highly compatible associations with considerable benefit for both partners to largely incompatible associations. The incompatibilities may result either in minimal infection or in substantial infection but little effective nitrogen fixation. Incompatible associations involving minimal infection are likely to have no adverse effects. Incompatible associations involving substantial infection but ineffective nodule function can have an adverse effect on the host. It may be possible to minimise such effects by increasing the specificity of inoculant strains so that they infect only the narrow range of host genotypes which form effective symbiosis (see section 3.1.3.2).

5.1.2.5 Non-target effects

Non-target effects of *Frankia* have been little explored. Most woody plant species develop important symbiotic associations with mycorrhizal fungi. On the basis of experience with *rhizobia*, it is likely that the association of host roots with *Frankia* will affect their association with mycorrhizal fungi, and vice versa. The nature and magnitude of these effects are relevant considerations in large-scale release.

5.1.2.6 Potential pathogenicity for plants, animals and humans

There are no reports of *Frankia* as a pathogen of plants, humans or animals. Given the long evolutionary selection of *Frankia* for specific and beneficial interactions with certain plants, it seems unlikely that large-scale culture of *Frankia* will reveal pathogenic traits in non-target organisms.

5.1.2.7 Inoculant contaminants

Frankia cultures grow slowly, with generation times of several days in liquid shake cultures. This slow growth rate and a filamentous growth habit will be major factors in determining how these bacteria will be cultured for large-scale testing or commercial use, and in ascertaining the nature of contamination in these cultures.

Frankia's slow growth rate greatly increases the risk that large-scale cultures will become contaminated with faster growing micro-organisms. There is no known culture medium that is selective for *Frankia*. Slow filamentous growth also makes it quite difficult and expensive to culture *Frankia* in large liquid fermentation vats, so that any large-scale production of *Frankia* inoculants is likely to involve solid-phase fermentation in small bags of sterilised carrier material. This approach promises to minimise both the risk of contamination and contaminant-generated risks to the environment and to workers. It may be useful to explore methods for detecting contaminant micro-organisms in small bags of inoculum (see section 3.1.4).

5.2 *Mycorrhiza-forming fungi*

5.2.1 *Background*

Several groups of fungi form symbiotic associations with roots of vascular plants: these are referred to as mycorrhizas (literally, "fungus-root"). The association is believed to be mutually beneficial and in some cases essential to the symbionts. The fungus utilises plant photosynthates as a carbon source, and the plant benefits from improved mineral nutrition and water relations. In some types of mycorrhizas, the physiological relationship between the symbionts has been incompletely studied, and the term mutualism should therefore be applied with care (Harley, 1969; Tinker, 1975; Smith, 1980; Harley and Smith, 1983).

Two main types of mycorrhizas are recognised; endomycorrhizas and ectomycorrhizas. Of the endomycorrhizas, vesicular-arbuscular (VA) mycorrhizas are the most important group, and substantial efforts have been made to produce commercial inocula of the fungi which produce them.

VA mycorrhizas are ubiquitous in nature, colonising roots of most vascular plants in most geographical locations (Nicolson, 1967; Gerdemann, 1968; Trappe, 1987). They associate with roots of arable crops, but are more effective in plants lacking extensive root systems, for example *Allium* spp. (Baylis, 1970; St. John, 1980). Several genera of zygomycete fungi form VA mycorrhizas, and their taxonomy has been extensively studied (Hall, 1984). The main feature of VA mycorrhizas is the internal colonisation of the root cortex by fungal hyphae which form characteristic structures known as arbuscules and vesicles. It is by means of this intimate relationship that nutrient exchange between plant and fungus can occur (Woolhouse, 1975). The main benefits to plants are enhanced phosphorus uptake and, probably, improved water relations (Sanders *et al.*, 1975; Nelson and Safir, 1982).

Ectomycorrhizas are also widespread and form prolonged associations with roots of forest trees. The fungi involved are mostly basidiomycetes (mushrooms and toadstools). Instead of penetrating the root cortical cells, ectomycorrhizas tend to form a net of fungal mycelium around young root tips (Marks and Foster, 1973). Unlike VA mycorrhizas, ectomycorrhizas can often be discerned and typed with the naked eye. The principal benefit to plants is improved nutrient uptake and water relations (Marks and Kozlowski, 1973; Harley and Smith, 1983).

5.2.2 *Use as biofertilizers*

Mycorrhizas are common in managed (agriculture and silviculture) as well as unmanaged terrestrial ecosystems (Jeffries, 1987). Under most conditions, susceptible plants will tend to be naturally infected with at least one strain of a mycorrhizal fungus. Consequently, strategies for introducing inocula of these fungi have tended to concentrate on using selected strains thought to improve early plant growth and survival.

VA mycorrhizal inoculants have mainly been used on a small scale on marginal soils such as acid upland pastures (Hayman and Mosse, 1979; Newbould and Rangeley, 1984), open cast mine (stripmine) reclamation sites (Allen and Allen, 1980), and semi-

arid environments (Lee and Wani, 1991). However, there has been some recent success in developing commercial inoculants of mixed strains, mainly for use on high-value ornamental and vegetable crops (Stribley, 1989), particularly in Japan.

A major factor restricting the more widespread use of VA mycorrhizas as inoculants is the difficulty of culturing them. No successful method has been found for culturing large volumes of the fungi *in vitro*. Solving the problems of culturability and improving performance in promoting plant growth are the main issues for strain selection and development programmes. Because the classical genetics of mycorrhizal fungi are poorly understood, the genetic basis for controlling beneficial traits has yet to be elucidated.

Similar problems exist for ectomycorrhizal fungi. Inoculating plants usually only results in improved seedling establishment and initial survival after transplantation from nurseries to forest plantation sites. As plants mature, different species of indigenous fungi form ectomycorrhizas, and any improvement from inoculation is short-lived (Marks and Foster, 1967). Methods for producing large yields of ectomycorrhizal fungi exist, but the cost of producing effective inocula is often greater than the benefits. Consequently, except in marginal ecosystems, ectomycorrhizal inoculants are rarely used extensively. Several research programmes target the problem of culturability of effective inocula and results appear promising.

Recombinant DNA technology has been used to transform fungal species that are well characterised, cultured in defined media, and whose protoplasts are easily obtained; examples are *Aspergillus*, *Neurospora* and *Saccharomyces* (Hirsch, 1986). Recently, several laboratories have begun to use molecular techniques to identify ectomycorrhizas (Egger and Fortin, 1990; Lobuglio *et al.*, 1991), and similar techniques might be applied to VA mycorrhizal fungi in the future.

If methods are developed to culture adequate amounts of mycorrhizal fungi and to obtain pure fungal material, it might be possible to transform mycorrhizal fungi to the benefit of plant growth. This would entail developing compatible vector systems or other techniques for inserting DNA into the fungi; it should be remembered that mycorrhizal fungi tend to be multinucleate, a characteristic which may give rise to instability. However, even small-scale use of modified mycorrhizal fungi is probably some years away.

5.2.3 General safety considerations with mycorrhizal fungal inoculants

Commercial use of mycorrhizal fungal inoculants is fairly recent, but there is a high degree of familiarity with these inoculants, as researchers have studied mycorrhizas for some time.

Most effort is concentrated on strain selection and development to enhance plant growth or to increase the ability of plants to survive. The major safety concerns are similar to those described in section 5.1.1 for rhizobial inoculants, namely:

- inoculant purity;
- establishment of competitive but poorly performing strains;
- displacement of indigenous microbial populations;
- potential pathogenicity for plants, animals and humans.

There may be human health and safety concerns, in particular allergenic reactions, associated with the use of mycorrhizal inoculants impregnated into inorganic carrier materials such as pumice, montmorillonite, vermiculite, etc.

5.2.4 Survival, persistence and dispersal

Mycorrhizal fungi can survive and persist in soils for long periods as active mycelia or in resting structures such as rhizomes, root fragments, spores and sporocarps.

VA mycorrhizal fungi probably rely on hyphal growth through soil and on movement of colonised root fragments for dispersal, since no spore dispersal mechanism has been identified. Ectomycorrhizal fungi, on the other hand, produce abundant basidiospores from above-ground fruit bodies, and a range of dispersal agents are likely to be involved. However, the infectivity of spores of most mycorrhizal fungi is variable.

Despite their ability to survive, introduced inocula, either of ectomycorrhizal or of VA mycorrhizal fungi, are frequently displaced by indigenous strains. The mechanism is poorly understood. In forest trees, different fungi form successions of mycorrhizas on different parts of the root system as it grows. Inoculated VA mycorrhizal strains are usually replaced rapidly as developing roots become colonised with indigenous strains better adapted to local conditions (Abbott and Robson, 1982).

It is possible that wider applications of mycorrhizal inoculants could lead to displacement of indigenous strains, although this seems unlikely given current inoculation practices. A breakthrough in production of low-cost inoculum might, however, change the situation.

5.2.5 Gene transfer

Information on mycorrhizal genetics is scarce partly because of difficulties in culturing the fungi, although several laboratories are now actively involved in doing so. Significant gene exchange between VA mycorrhizal fungi has probably occurred in the course of their evolution, since their low fungal-host specificity. Opportunities for gene exchange between different VA mycorrhizal fungi, and with their host plants, appear to be high, especially as the symbioses involve close and extensive membrane-membrane contact with associated nutrient exchange (Gianinazzi *et al.*, 1983).

It is difficult to foresee novel negative consequences of gene exchange associated with the use of non-genetically modified inocula, since any such exchange is likely to have occurred already. The introduction, by genetic modification, of a novel gene into one symbiont might present a new concern, but for the foreseeable future, this is only likely to occur from a modified plant to the fungus. Extensive links between mycorrhizal fungi on different plants do occur (Heap and Newman, 1980), but the impact of a recombination event on the genotypes of members of a community is far from understood.

t effects

c traits in mycorrhizal fungi have not yet been identified, so it is difficult to ciated risks.

et effects

ussed in section 5.2.1, it is unclear whether VA mycorrhizal associations are mutually beneficial. Mycotrophy may have deleterious as well as beneficial ost plant growth (Safir, 1980). As with rhizobial infections, mycotrophy can growth when plants are carbon-limited (Trappe, 1987).

indirect effects on target plants may include altered resistance to disease. In , infection by viruses and leaf-infecting biotrophic fungi increases in the VA mycorrhizas, whereas infection by necrotrophic root-infecting fungi Daft and Okusanya, 1973; Schonbeck and Dehne, 1979; Graham and Menge, araj, 1984). These are probably indirect effects of altered mineral nutrition partitioning in host plants resulting from infection by VA mycorrhizal fungi.

-target effects

Increased growth of non-target weedy plants

oad host range of VA mycorrhizal fungi suggests that the fungus is likely to d hence promote growth of most plants, including non-target weeds, in the the area to which inocula are applied. The consequences are debatable, since likely to be infected by indigenous VA mycorrhizal fungi. Evidence for yphal links has suggested a possible advantage for shaded-out plants (New-).

trast, it is unlikely that ectomycorrhizal fungi in silviculture have any noticea- on growth of non-target weeds. It is possible that indirect effects on soil ay have some unexpected results.

2 Effects on indigenous microbial populations

rrhizal fungi may have some indirect effects on other parasites and may digenous organisms for a short period; long-term effects have not been studied.

3 Potential pathogenicity for plants, animals and humans

than the potential deleterious effects on plant growth already discussed, there ious pathenogenic hazards for other organisms.

4 Effects on nutrient cycling

ugh mycorrhizal plants often benefit from improved mineral nutrition, this is consequence of the extension of the root system by the development of phae which absorb nutrients, particularly phosphorus, beyond the soil deple-

tion zone immediately around the root (Sanders and Tinker, 1973). The mechanism has been discussed in depth (Tinker, 1978; Abbott and Robson, 1984), and it is unlikely that such scavenging has much direct effect on phosphorus cycling, although doubts have been expressed, particularly in relation to iron phosphates (Bolan *et al.*, 1984). There may, however, be indirect effects on uptake of other nutrients, such as improved nitrogen fixation (Mosse *et al.*, 1976).

5.2.9 Inoculant contaminants

Production of inocula of mycorrhizal fungi is presently based on solid-phase culture. This mode of production takes considerable time and space and often results in contamination of the inoculum by other fungi, particularly saprophytes and nematodes.

Production of VA mycorrhizal inocula requires growth of the fungus on live plants for some weeks. If root fragments from these plants are then incorporated in the inoculum, they can serve as a bridge for spreading plant pests and diseases. Processing raw inocula reduces the residual root material, but carry-over of material such as spores from contaminating organisms and nematode cysts can be a problem. Moving into liquid culture may alleviate many of these problems but may also create new ones.

5.2.10 Risk management considerations for mycorrhizas

If adverse effects have been identified in the risk/safety analyses, development during scale-up use of mycorrhizal inoculants should be based on studies undertaken in the glasshouse and in small-scale field trials, during which risk/safety issues as well as the effectiveness of the inoculum are considered and identified. Results of such studies should then be confirmed during scale-up, when confinement is likely to be reduced and certain risks might become more apparent.

Usually, as many small-scale studies on mycorrhizal inoculation have shown, it is difficult to obtain high levels of root colonisation from the inoculum because of competition from indigenous mycorrhizal fungi. Further, contaminants present in the inoculum may decrease colonisation, and routine testing of inocula should therefore be carried out.

The risk/safety analysis should identify potential risks. In many cases, it is likely to indicate minimal risk from the use of mycorrhizal inoculants as biofertilizers. However, unexpected risks might arise if mycorrhizal fungi are used on a large scale in different environments or if the fungus is genetically modified. Therefore, it is imperative always to carry out a risk/safety analysis.

Should the risk/safety analysis identify any potential risk, then appropriate management practices must be applied to ensure that the risk is controlled. For example, if lateral spread from the mycorrhizal inoculant beyond the release site should be minimised, then border rows of unsusceptible plants such as *Cruciferae* or *Chenopodiaceae* spp. (Tester *et al.*, 1986) could be grown around the release area. Similarly, planting an unsusceptible crop following the application of the inoculant to a susceptible host could minimise its establishment.

In extreme cases, if eradication of the mycorrhizal inoculant is necessary, then systematic fungicides or soil disinfectants such as formalin could be applied to prevent survival of any fungi. Such practices should only be used if the risk is very high.

5.3 Phosphate solubilising fungi

5.3.1 Background

Phosphorus is an essential plant nutrient. It limits crop growth in many soils because it is unavailable in a soluble form. Phosphate solubilisation in the soil – by bacteria (Taha *et al.*, 1969), fungi (Khan and Bhatnagar, 1977) and actinomycetes (Rao *et al.*, 1982) – offers potential agricultural benefits. A wide variety of micro-organisms exhibit phosphate solubilisation properties. This discussion will focus on a phosphate-solubilising biofertilizer which is currently marketed only in North America. The active organism is a strain of *Penicillium bilaii* (previously, *P. bilaji*).

Fungal spores of *P. bilaii* mixed with a food colour dye or spores contained within the pores of a porous granule-type carrier are used as the inoculant. The fungus acts by colonising target crop roots and then secreting organic acids near the rhizosphere, thereby making possible solubilisation and uptake of normally insoluble inorganic phosphate compounds (Kucey *et al.*, 1989). Increased phosphorus uptake has been reported in wheat and field beans inoculated with *P. bilaii* in Canada; no adverse ecological effects were noted (Kucey, 1987). The role of these micro-organisms in the release of inorganic phosphorus in soils has been described (Kucey *et al.*, 1989).

5.3.2 General safety considerations

Safety considerations related to the inoculation of crop plants with *P. bilaii* are similar to those for the introduction of any micro-organism into an agriculture environment. *P. bilaii* is a naturally occurring soil fungus. Strains containing marker genes or antibiotic resistance have not been developed or used, so concerns about gene transfer from the introduced fungus to indigenous fungi are minimal at present.

5.3.2.1 Competition with indigenous micro-organisms

Studies of survival and persistence in the soil following inoculation with the fungal strain have been conducted. They show an increase of *P. bilaii*-like organisms in the rhizosphere. The organism population decreases with distance from the inoculated crop plant row, and *P. bilaii* populations return to background levels following crop harvest. Further studies have shown that the *P. bilaii* fungus is able to coexist with V.A.A. mycorrhizas and with *rhizobia*. Studies also suggest that antibiotics are not produced by the *P. bilaii*.

5.3.2.2 Plant pathogenicity

The *P. bilaii* fungus does not invade plant tissue and does not grow out well from a point of inoculation into surrounding soil, nor does it produce toxins or toxic metabolites.

atory and field studies show that inoculation with *P. bilaii* does not reduce or delay crop germination or emergence.

.3.2.3 Mineral cycling

Early Russian research on phosphate-solubilising organisms indicated negative s on soil organic matter due to solubilisation of organically bound phosphorus. s have shown that *P. bilaii* solubilises only inorganic phosphorus.

Plant-associated and free-living micro-organisms as future biofertilizers

Background

As research continues to produce more information about the numerous micro-isms that are found in close association with plants, there will be new opportunities micro-organisms as biofertilizers. Much of this research is being driven by ces in both ecology and molecular biology. Molecular ecologists are employing echnologies to better understand the dynamics of microbial populations and what ns to these populations when attempts are made to introduce a beneficial specie as a biofertilizer micro-organism. In addition, plant scientists and microbiologists refully studying interactions between plants and micro-organisms at the molecular This work should result in numerous new candidates for microbial biofertilizers. section describes several groups of micro-organisms that are currently being investi-for future biofertilizer applications.

Plants support a great diversity of plant-associated micro-organisms. These include ytes that occur in close association with leaves and shoots (the phyllosphere) and (the rhizosphere) as well as endophytes that occur within the plant. Considerable is currently being devoted to utilising these organisms directly as biofertilizers or as ery systems for specific gene products that stimulate plant growth.

5.4.1.1 Endophytes

Fungal and bacterial endophytes also have certain characteristics that can be ited for biofertilizer purposes. The fungus *Acremonium* sp. offers an example of the mercial use of endophytes (Siegel and Schardi, 1991). Fescue containing this endo-is more resistant to drought. The mode of action is not clear but may be tied to the ol of soil-borne pests such as plant-parasitic nematodes. Unfortunately, this fungus produces a number of toxic compounds that induce toxicosis in livestock. Modified is are being developed that reduce or eliminate toxicosis-causing compounds.

Since endophytes exist in such close association with plants, they are ideal candi-for delivering gene products that stimulate plant growth. Development of a modified rial endophyte that expresses the *Bacillus thuringiensis* delta endotoxin, which is tive in controlling lepidopteran pests of corn is under way. Although this endophyte ed for pest control, it demonstrates the type of biological delivery system that may be to promote plant growth.

5.4.2 *Safety considerations for scale-up*

General safety considerations and potential effects of these plant-associated and free-living micro-organisms at scale-up are the same as those for other types of microbial biofertilizers. Commercial use and experimentation with many of these organisms have not revealed safety considerations unique to this group of organisms. Obligate symbionts with a narrow host range should pose fewer environmental concerns by reason of this specificity. Because of a shortage of carbon in most soils, free-living bacteria may also present fewer environmental concerns.

5.5 *Algal biofertilizers*

5.5.1 *Background*

The soil algae comprise four main groups – the blue greens (cyanobacteria), greens (chlorophyta), yellow greens (xanthophyta) and diatoms (bacillariophyta).

Because of their autotrophic metabolism, which requires only sunlight, carbon dioxide, water and inorganic nutrients, algae have an important role as primary colonisers of bare rock surfaces and as primary colonisers in the absence of plants. The capacity of the blue greens to photosynthesise and fix atmospheric nitrogen is not only of great ecological significance, it also identifies them as potentially promising biofertilizer micro-organisms. Production of extracellular polysaccharrides, particularly by the palmelloid-forming green algae such as *Chlamydomonas* and *Asterococcus*, can also be employed for soil conditioning (Metting *et al.*, 1988) although this is generally not defined as biofertilization.

As biofertilizers providing fixed nitrogen to the soil-plant system, the most important blue greens are the filamentous genera possessing specialised sites (heterocysts) of nitrogenase activity. The use of the flamentous blue greens, such as the genera *Anabaena*, *Nostoc*, and *Sesbania*, for their biofertilizer properties is often referred to as algalisation and is practised in Southeast Asia and India (Venkataraman, 1981).

Estimates of nitrogen fixation rates associated with algalisation of 25-30 kg N $ha^{-1}y^{-1}$ (Venkataraman, 1981) are probably broadly realistic, although good data are scarce. Benefits other than nitrogen inputs from algalisation include increases of organic matter content (particularly in relation to soil conditioning) and available phosphorus (Roger and Watanabe, 1986; Watanabe and Liu, 1992).

The main constraints on the use of algae as biofertilizers relate to moisture, pH and sunlight. Most algae are sensitive to water stress and prefer alkaline conditions. Successful use of algae as biofertilizers is generally restricted to the flooded soils of tropical rice paddy systems.

5.5.2 *Safety considerations for scale-up*

There is little immediate scope for the widespread commercial use of algalisation. Roger and Watanabe (1986) have concluded that free-living blue greens have less potential than the *Anabaena-Azolla* and legume symbioses. Furthermore, field testing of

non-genetically modified algal inoculants to date suggests that there are no major safety considerations, although there is evidence that *Cyanobacteria*, specifically *Anabaena*, produce hepatotoxins and neurotoxins that are toxic to humans and animals. Scale-up may be facilitated in the future by genetic modifications such as the incorporation of genes for herbicide resistance and/or overproduction of compatible cell solutes, as well as incorporation of genes for systems that facilitate maintenance of derepression of algal nitrogenase in the presence of nitrogen fertilizers. Safety considerations include the stability and fate of the foreign genes and possible impacts on nutrient cycling (see section 3.1.3.3.4). In addition, since algal inoculation may be associated with irrigation, dispersal from the site of application may be a concern.

Experience suggests that algal inoculants establish poorly, because they are dominated by indigenous strains. It is necessary to understand competition between inoculated and indigenous algae in order to decide whether to proceed to scale-up and what the further development of safety considerations should be.

Notes

1. Environmental safety includes safety of the work environment and therefore issues of human/ worker safety.
2. Biofertilizers are defined as beneficial living microbial preparations, *e.g.* bacterial, fungal or algal, which directly enhance or support plant growth, for example by supplying substances such as plant growth regulators or minerals.
3. Scale-up includes unconfined (other than by natural barriers and cultural practices) performance evaluations, advanced testing, demonstration trials beyond basic and preliminary field research in which special confinement measures can be applied.

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SAFETY CONSIDERATIONS FOR BIOTECHNOLOGY

SCALE-UP OF MICRO-ORGANISMS AS BIOFERTILIZERS

The OECD produces the most authoritative set of internationally agreed-upon safety guidelines presently available in biotechnology. Since the publication of *Recombinant DNA Safety Considerations* in 1986 and *Safety Considerations for Biotechnology* 1992, the OECD has continued this important work in specific sectors. *Safety Considerations for Biotechnology: Scale-up of Crop Plants* 1993 updated and extended the earlier work, discussing scientific concepts and principles pertaining to the environmental safety of the scale-up of crop plants developed by biotechnology.

This publication again addresses the question of the environmental safety of the scale-up of micro-organisms developed by biotechnology for use as biofertilisers. It describes how the concept of "familiarity", with micro-organisms as biofertilizers, the introduced trait, the environment, and their interactions, will help facilitate risk/safety analysis and manage possible risks in the context of scaling up modified micro-organisms towards commercial release.

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