

Detection of Living Modified Organisms (LMOs) and the Need for Capacity Building

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Abstract: The paper emphasizes on the need of implementing the Biosafety Protocol for the developing countries and suggests to establish capacity building measures. It identifies various ways to detect GMOs and discuss their relevance in the context of developing countries. The paper has elaborated the concept of transgene and provides an overview of GMO regulations across various countries.

Keywords: GMOs/LMOs, Detection, Biosafety, Capacity Building

Introduction to Genetically Modified Organisms (GMOs)

With the advent of modern biotechnology, specifically genetic engineering, it has become possible to transfer a specific gene, called a transgene, from one organism to another across or within species boundaries through a process called transformation. Genetic engineering has the potential to produce improved varieties in terms of quality and yield traits, such as pest and disease resistance, more quickly than traditional breeding. Genetically engineered (GE) crops are referred to as GM (genetically modified), GMO and GE. Transgenic organisms able to replicate (seeds or living organisms) are referred to as living modified organisms (LMOs).

GMOs typically contain an insert or gene construct consisting of a promoter (that controls the expression of the gene), the gene (that expresses a particular trait) and a terminator (that functions as a stop signal to terminate the “reading” of the gene during protein production). Common promoter and terminator elements are used in combination with most transgenes to ensure adequate expression of the transgenes in the host organism. A gene construct can also contain

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a marker gene such as antibiotic resistance. The insertion of a transgene into the DNA of an organism is complex, and each transformation results in the integration of the insertion into a different region of the genome. GMOs that contain similar DNA elements for example the same promoter, transgene and terminator, but are obtained from different transformation experiments are considered to be event-specific GMOs (with different insertion points of the transgene into the DNA) and given different names.

Global status of GMOs

Currently, GM traits commercially available include herbicide tolerance (72 per cent of GMOs), insect resistance (19 per cent of GMOs) and a combination of insect resistance and herbicide tolerance (9 per cent of GMOs).¹ The most popular GM crops are soybean (60 per cent of GMO crops), maize (23 per cent of GMO crops), cotton (11 per cent of GMO crops) and canola (6 per cent of GMO crops) and together account for 29 per cent of global crop production in terms of area planted for these crops. The countries growing 99 per cent of GM crops are the USA (59 per cent), Argentina (20 per cent), Canada (6 per cent), Brazil (6 per cent), China (5 per cent), Paraguay (2 per cent), India (1 per cent) and South Africa (1 per cent).²

Common transgenes in GMOs

Insect resistance is mediated by different Cry genes (Cry1Ab, Cry1Ac, Cry1E, Cry2Ab, Cry3A, Cry3Bd1 and Cry9c) from different subspecies of *Bacillus thuringiensis*, a soil microbe (www.agbios.com). The genes code for the “Bt” toxin, a delta-endotoxin, toxic to different insect species, including crop pests. The endotoxin binds to receptors in the midgut of susceptible insects disrupting midgut ion flow, causing gut paralysis and subsequent death. Mammals do not have these receptors and are unaffected by this endotoxin.

There are two types of herbicide tolerance used in GM crops according to the active ingredient of the herbicide, glyphosate (commonly known as Roundup Ready) or phosphinothricin.³ Glyphosate prevents the synthesis of essential amino acids found in plants, specifically aromatic amino acids phenylalanine, tyrosine, and tryptophan, by acting as an amino acid analogue that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Herbicide tolerant GM crops contain a modified form of the EPSPS

enzyme that is tolerant to glyphosate, isolated from the soil microbe *Agrobacterium tumefaciens*. This gene is also used together with a gene encoding the enzyme glyphosate oxidoreductase (GOX) that inactivates glyphosate. The second type of herbicide tolerance involves the herbicide phosphinothricin. The active ingredient in phosphinothricin is glufosinate ammonium that inhibits the enzyme glutamine synthetase leading to the accumulation of phytotoxic levels of ammonia killing the plants. The gene conferring tolerance to phosphinothricin, was isolated from the soil microbe *Streptomyces viridochromogenes*, and encodes the enzyme phosphinothricin-N-acetyltransferase (PAT) that inactivates glufosinate. These genes allow for the use of specific herbicides to control post-emergence weeds.

GMO Regulations

A requirement for the release and development of GMOs is an effective regulatory system to ensure that GMOs pose no threat to human health or the environment. Many countries have also introduced mandatory GMO labelling (Table 1). Although GMO labelling does not have any bearing on the safety aspect of GMOs, it is used to give consumers an alternative choice allowing them to balance concerns of morality and perceived risk, with regard to genetic engineering.⁴

Mandatory labelling makes use of “threshold” labelling that specifies that foodstuffs must be labelled when containing GM at or above a predetermined level.⁵ The European Union has also introduced the requirement for traceability. This facilitates monitoring, the withdrawal of products in the event of an unforeseen risk to human health or the environment and the implementation of labelling requirements.⁶ In addition to country-specific or regional regulations, the “Cartagena Biosafety Protocol” puts into effect global rules that govern the “safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking into account risks to human health, and specifically focusing on trans-boundary movements” (Article 1) as well as “handling, transport, packaging and identification” of LMOs (Article 18).⁷ Thus global regulations make it imperative for governments and industry to develop reliable and accurate GMO/LMO detection systems for crops and foodstuffs to ensure compliance to international regulations and maintain international trade.⁸ Furthermore, events such as the release

Table 1: GMO Food Labelling Regulations and Thresholds for Different Countries

Country	Labelling	% Threshold	Scheme
Australia and New Zealand ^{1,2}	Mandatory	1.0%	GM
Brazil ^{2,3}	Mandatory	1.0%	GM
Canada ^{2,3,4}	Voluntary	5.0%	non-GM
China ^{2,5}	Mandatory	1.0%	GM
European Union ⁶	Mandatory	0.9%	GM
Indonesia ^{2,5}	Mandatory	5.0%	GM
Israel ⁷	Mandatory	0.9%	GM
Japan ^{2,3,7}	Mandatory	5.0%	GM
Philippines ^{2,5}	Voluntary	N/A	N/A
Russia ^{2,5}	Mandatory	0.9%	GM
Saudi Arabia ^{2,5,7}	Mandatory	1.0%	GM
South Korea ^{2,5,7}	Mandatory	3.0%	GM
Switzerland ⁵	Mandatory	0.9%	GM
Taiwan ^{2,5,7}	Mandatory	5.0%	GM
Thailand ^{2,7}	Mandatory	5.0%	GM
USA ^{7,8}	Voluntary	N/A5%	Non-GM/Organic

For further information:

- ¹ Food Standards Australia New Zealand: www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2000/labellinggenetically29.cfm
- ² Australian Government Agency: www.abareonlineshop.com/product.asp?prodid=12559
- ³ Agrifood Awareness Australia: Biotech Bulletin - [Global Labelling Laws](#) June 2004: www.afa.com.au
- ⁴ Health Canada: www.hc-sc.gc.ca/fn-an/gmf-agm/fs-if/faq_3_e.html#2
- ⁵ The Center for Food Safety: www.centerforfoodsafety.org/genetically15.cfm
- ⁶ Europa: europa.eu.int/scadplus/leg/en/lvb/l21170.htm
- ⁷ Global Knowledge Center on Crop Biotechnology: www.isaaa.org/kc/
- ⁸ The National Organic Program: www.ams.usda.gov/nop/NOP/NOPhome.html

of co-mingled Bt11 with Bt10 seed,⁹ the discovery of Starlink maize (deregulated for animal feed) in the human feed chain,¹⁰ the contamination of maize landraces in Mexico¹¹ and a report on the co-mingling of pharmaceutical GM crops with food crops,¹² has highlighted the need to develop GMO/LMO detection and traceability systems.¹³

GMO detection methods

Analytical methods to detect (qualitative or yes/no answer) and quantify (percentage content) GMOs fall into two main categories: protein analysis – to detect the specific protein expressed by the transgene in the GMO through the use of ELISA (enzyme-linked immunosorbent analysis) and lateral flow strip tests¹⁴ or DNA analysis – to detect the specific transgene in the GMO or specific elements associated with the transgene.¹⁵

General considerations for GMO detection

For both protein and DNA based GMO testing there are several general considerations. These include sampling, food matrix effects on protein/DNA extraction, reference materials, method validation, harmonization of standards and access to information databases. In brief, sampling is important as it determines how representative the result is of the lot from which it was taken. Sampling strategies must take into account the heterogeneity of the sample, the lot size, the sample size and particle size of the test portion being analyzed and the impact of this on the limits of detection (LOD) or quantification (LOQ).¹⁶ The method used to extract proteins or DNA from the sample must take into account the matrix of the sample (the constituent complexity of the sample) to ensure that “matrix effects” due to an “interferent” do not affect the outcome of the results.¹⁷ It is also important to use reference material (a food matrix containing a specific amount of a specific GMO) during GMO testing that can be used as an external standard, to validate a method or determine method sensitivity and specificity.¹⁸ Method validation is important to ensure that different testing methods give comparable results that are reliable and repeatable.¹⁹ It is also important to establish minimum performance criteria in terms of specificity and sensitivity, accuracy and reproducibility.²⁰ Most of these issues will have to be explored and developed through the Codex Alimentarius Commission of the UN. Access to information on commercial GMO releases, sequence and construct information and suggested detection methodologies are critical for the successful international implementation of GMO detection and will be discussed under “Capacity building”. Finally, all testing methods should include appropriate quality controls to determine that performance criteria are maintained.

Protein based testing

Protein identification requires the use of monoclonal antibodies raised against a specific protein encoded by a transgene. Protein methods can be used on raw and semi-processed samples, as long as the protein is not denatured or destroyed by processing.²¹ Protein testing is generally applied in two ways, through a lateral flow strip test (strip test) or ELISA.

When using the lateral flow test, the sample is homogenized to the appropriate particle size, buffer added for simplified protein extraction and the strip placed into the sample/buffer. After several

minutes, a positive result is indicated by a discoloured test line due to antibody-protein recognition.²² This is the simplest method to qualitatively detect a GMO.

Enzyme-Linked Immunosorbent Analysis (ELISA) requires a basic protein extraction followed by antibody detection in a micro-well plate. Positive reactions are determined by a colour reaction that can be read visually or by an optical reader for qualitative analysis. For quantitative analysis a standard curve is determined from reference material of known GM concentration by plotting the percentage GM against the OD (optical density) of the colour reaction. Positive unknown samples are quantified by a comparison of the sample OD, using an optical reader, to the standard curve of the reference material.²³

Antibody recognition identifies a protein product of a specific transgene.²⁴ Thus in order to determine that a product is non-GMO, different tests must be used for as many different GMOs as are available. Protein testing is often performed using only selected target proteins as an in-house initial screen. While protein testing is considered reasonably simple to apply, it is limited by the development and availability of protein antibodies for all types of available transgenes in the form of commercial kits and cannot identify event-specific GMOs.

DNA based testing

DNA identification makes use of Polymerase Chain Reaction (PCR) based methods.²⁵ PCR uses DNA polymerase and sequence specific primers (sequences that flank the region to be amplified) to selectively amplify target DNA sequences²⁶. PCR based methods can be used on raw and processed products as long as DNA can be extracted from the sample.²⁷

The basic process for Qualitative PCR testing is DNA extraction, PCR amplification of the target sequences and visualization of the amplified target DNA. DNA extraction is performed to obtain DNA of optimal quality and purity as previously reviewed.²⁸ After PCR the visualization of amplified DNA is performed using agarose gel electrophoresis. This allows the separation of amplified products based on fragment size and visualization in the gel matrix using dyes such as ethidium bromide or SYBR Green that binds selectively to the dsDNA.²⁹ Different methods can be used to confirm the PCR result including restriction enzyme cleavage of the PCR product, Southern blotting through hybridization with a DNA probe (specific for the target sequence), direct sequencing of the PCR product and nested PCR through

a two-step amplification approach that amplifies the target sequence followed by a second amplification of a smaller internal region of the product of the first amplification.³⁰

The selection of target sequence for PCR depends on the level of specificity required for GMO detection namely, GMO screening, transgene-specific, construct-specific and event-specific detection.³¹ GMO screening is used to determine whether a sample contains GMO through the detection of regulatory elements (promoter and terminator sequences) commonly associated with GMOs.³² For example, the 35S promoter and NOS terminator are found in more than 90 per cent of all commercial maize and soybean GMOs.³³ Transgene-specific identification identifies a specific gene, for example Cry1Ab, Cry9c (insect resistance) or EPSPS (herbicide tolerance). Construct-specific methods target the region between two DNA elements found within a particular transgene construct, such as the promoter and gene. The most specific method to identify a GMO is event-specific detection where the PCR target sequence is a junction between the host DNA and the inserted gene construct.³⁴

Semi-quantitative competitive PCR (QC-PCR) or double-competitive PCR (double QC-PCR) detection systems have also been described.³⁵ This method is based on the competitive PCR amplification of unknown amounts of a transgene in the presence of known amounts of an internal DNA standard using the same PCR primers. Although very effective, QC-PCR only determines GMO content relative to a predefined threshold and is not considered applicable for exact GMO quantification.³⁶

The most commonly used method to quantify GMOs is quantitative Real-time PCR.³⁷ Unlike conventional PCR where visualization of amplification takes place after the PCR is completed, during Real-time PCR the amplification of the target sequence is followed in “real time”. The PCR amplification is detected through the use of fluorescent dye or fluorescent probes. Double stranded DNA-binding dye (SYBR Green I) is used as a non-specific detection system for all amplified DNA fragments while specific detection makes use of fluorescent probes that recognize an internal segment of the PCR target sequence (hybridization (FRET) probes or hydrolysis (Taqman) probes).³⁸ For GMO quantification it is preferable to use specific detection to avoid problems of non-specific amplification. The use of probes has a further advantage in that it allows a one-step detection and verification of the target sequence.

During Realtime PCR, the incremental increase in fluorescence is measured above a predetermined cycle threshold (Ct value) where the amplification efficiency is constant. The Ct value is inversely proportional to the log of the initial amount of target molecules.³⁹ In order to compensate for different amounts of initial target template in the PCR, an endogenous reference gene target sequence is also amplified and acts as a measure of the total DNA present in the sample.⁴⁰ The reference gene is a readily amplifiable target sequence common to that specific species of GMO in single copy, and is used to normalize the amount of initial DNA present in the PCR. To quantify the GMO content of a sample, the amount of GM DNA is calculated relative to a GM DNA standard curve (using a serial dilution of known GM DNA copies) and expressed as a percentage ratio to the amount of reference DNA present. The amount of reference DNA is calculated relative to a reference DNA standard curve (using a serial dilution of known reference DNA copies).⁴¹

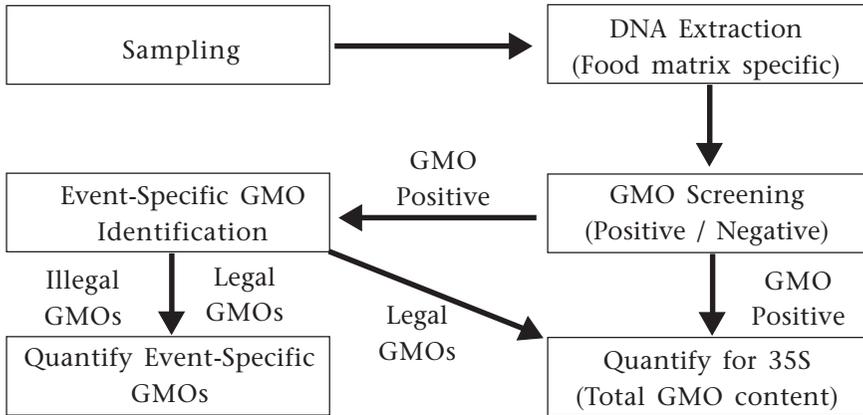
GMO quantification can either be based on total GMO content, using 35S quantification⁴² or event-specific GMO quantification.⁴³ Both approaches have pro's and con's. The 35S quantification is useful to determine overall GMO content in samples containing single or mixed genes, containing the 35S promoter, but does not discriminate between legal and illegal GMOs. Event-specific quantification only determines the content of a specific GMO and requires the development of event-specific methods.⁴⁴ Thus when multiple genes are present in a sample, these must be quantified individually and the percentages added together.

The most common operational procedure for GMO detection, identification and quantification begins with sampling followed by DNA extraction and GMO screening/detection (Figure 1). If a sample is positive, after screening, the total amount of GMO can be determined using 35S quantification or the individual GMOs identified, and if all legal, followed by 35S quantification or event-specific quantification depending on regulatory requirements.

Future technology for GMO testing

The technology for DNA sequence detection continues to develop and improve. Although this paper has been limited to the mainstream technology being used for GMO/LMO detection, it is important to take a look to what the future may hold. New developments include

Figure 1. A basic scheme for DNA based GMO detection and quantification



microarrays, capillary gel electrophoresis (CGE), biosensors and genosensors.⁴⁵ However, these technologies are currently in an early phase of development and it will be several years before they are practical and cost effective enough to replace current methodology.

Capacity Building

It seems like a mammoth task for developing countries to implement LMO testing when considering the different challenges and technical skill required. However, with 31 African, 28 Asian and Pacific, 16 Central and Eastern Europe and 22 Latin America and Caribbean countries already party to the protocol, this is exactly what will have to occur if the Biosafety Protocol is to succeed.

Unfortunately, there is a decided lag phase in methodology development on the one hand and consensus information dissemination on the other. Thus, while many academic publications deal with the different aspects of LMO detection, there is currently no international standard or consensus on available methods. In the absence of international standards, many countries are taking the initiative and developing their own LMO testing standards and methods which they will then try to justify and entrench in international discussions. Often companies in such countries develop proprietary methods based on national guidelines (where available) and internal research. Such information is unlikely to benefit the international

community, more specifically developing countries, due to a lack of coordination and information dissemination. Thus, countries without the necessary resources and infrastructure must either adopt a particular methodology or develop their own. Either way this is an overwhelming task for any developing country and means that these countries cannot participate as equal partners to develop international methodology guidelines and standards. Thus, most countries typically face a “cart before the horse” situation since the development of the Biosafety Protocol has preceded the development of the instruments to verify its implementation. The result is that the gap between accession/ratification and actual implementation will get bigger for developing countries as the discussions on methodology progress, unless this problem is specifically addressed. In other words, a pragmatic and practical approach to developing strategies for LMO detection including the practical implementation thereof will have to be taken.

The European Union has made great progress in establishing competent organizations to deal with the different issues regarding LMO detection since it introduced mandatory labelling. These include the ENGL (European Network of GMO Laboratories) under the coordination of the JRC (European Joint Research Centre) as well as other national organizations. Thus, while the EU can provide important leadership, it must be determined whether developed methods are applicable to developing countries.

Although the Codex Alimentarius Commission is responsible for harmonizing international guidelines for labelling and traceability, the time it takes to finalize these discussions continues to leave a vacuum in which developing countries must continue to function. Countries that produce large amounts of GMO food may actually welcome the protruded discussions on these issues as it delays the effective implementation of regulations that many feel are carefully disguised trade barriers⁴⁶ (usinfo.state.gov/ei/Archive/2004/Jan/30-39208.html).

The Biosafety Protocol takes into consideration the need for capacity building as stipulated in Article 22. Although probably not intending to do so, the requirement for the exporter to carry out the risk assessment of an LMO (if required) also benefits developing countries, assuming that the GMO was produced in a developed country which may not always be the case. However, Article 18 deals with “Handling, transport, packaging and identification” and places the

burden of proof on the country of production. Thus, if a country produces LMOs, even in small quantities, it must supply proof of the LMO status of an export. This requirement would certainly place a burden on developing countries in terms of exports.⁴⁷

The Biosafety Protocol also makes provision for the Biosafety Clearing-House (BCH) that acts as a database of relevant information on LMOs. With time it is hoped that the BCH will include simplified information on the LMOs present in each country and LMO detection systems and methodology. However, the BCH is unlikely to achieve its aim unless non-parties also contribute information, especially since non-parties are currently the largest producers of LMOs.

The process of overseeing the implementation of the Biosafety Protocol lies with COP-MOP (The Conference of the Parties to the Convention on Biological Diversity serving as the meeting of the Parties to the Protocol) which “is the governing body of the Cartagena Protocol on Biosafety”. “Its primary role is to keep under regular review the implementation of the Protocol and to make decisions necessary to promote its effective implementation”. At COP-MOP 2 (2005), a number of issues relating to LMO detection were discussed including the use of thresholds for the adventitious or unintentional presence of LMOs as well as a review of LMO sampling and detection techniques with a view to achieving harmonization.⁴⁸ The conclusion of the meeting was to encourage parties to exchange experiences in the “use and development of easy to use, rapid, reliable and cost-effective sampling and detection techniques for LMOs” and established the need to develop criteria for “harmonizing sampling and detection techniques at its fourth meeting taking into account the work of other competent regional and international organizations with a view to avoid duplication of efforts”.

Conclusion

Despite the obstacles that must be overcome, the importance of the Biosafety Protocol must be stressed, especially for developing countries. The Protocol uniquely commits developed countries to ensure that developing countries have the necessary regulations and requirements in place to deal with LMOs. For developing countries this is an exciting opportunity to transform their countries and develop the tools to deal with modern biotechnology.

The weakest link of the Biosafety Protocol will prove to be capacity building if it is not addressed adequately.

Endnotes

- ¹ James 2004.
- ² James 2004.
- ³ www.agbios.com
- ⁴ Ahmed 2002.
- ⁵ Anklam *et al.* 2002b.
- ⁶ Miraglia, *et al.* 2004.
- ⁷ www.biodiv.org
- ⁸ Ahmed 2002, Chaturverdi and Rao 2004.
- ⁹ www.syngenta.com/en/media/article.aspx?pr=040805&Lang=en
- ¹⁰ www.epa.gov/oscpmont/sap/2001/july/wetmilling.pdf
- ¹¹ www.cec.org/maize/
- ¹² www.foe.co.uk/resource/press_releases/20021115145406.html
- ¹³ Heinemann *et al.* 2004; Miraglia *et al.* 2004.
- ¹⁴ Stave 2002.
- ¹⁵ Meyer 1999.
- ¹⁶ Gilbert 1999, Lischer 2001a, 2001b, Ahmed 2002, Anklam *et al.* 2002a, 2002b, Bertheau *et al.* 2002, Trapmann *et al.* 2002.
- ¹⁷ Meyer 1999, Stave, 1999, Anklam *et al.* 2002b, Terry *et al.* 2002, Miraglia *et al.* 2004.
- ¹⁸ Meyer 1999, Bertheau *et al.* 2002, Trapmann *et al.* 2002, Miraglia *et al.* 2004.
- ¹⁹ Anklam *et al.* 2002a, 2002b, Bertheau *et al.* 2002, Miraglia *et al.* 2004.
- ²⁰ Schreiber 1999.
- ²¹ Gachet *et al.* 1999, Stave 1999, Anklam *et al.* 2002b, Van Duijn *et al.* 2002.
- ²² Ahmed 2002.
- ²³ Stave 1999, Ahmed 2002.
- ²⁴ Brett *et al.* 1999.
- ²⁵ Ahmed 2002, Anklam *et al.* 2002b.
- ²⁶ Gachet *et al.* 1999.
- ²⁷ Pauli *et al.* 2000.
- ²⁸ Gachet *et al.* 1999, Anklam *et al.* 2002b.
- ²⁹ Ahmed 2002.
- ³⁰ Zimmermann *et al.* 1998, Gachet *et al.* 1999, Ahmed 2002.
- ³¹ Miraglia *et al.* 2004.
- ³² Gachet *et al.* 1999; Lipp *et al.* 1999, Ahmed 2002, Anklam *et al.* 2002b, Forte *et al.* 2005.
- ³³ www.agbios.com
- ³⁴ Miraglia *et al.* 2004.
- ³⁵ Hubner *et al.* 1999, Wurz *et al.* 1999, Zimmermann *et al.* 2000.
- ³⁶ Anklam *et al.* 2002b, Wiseman 2002.
- ³⁷ Gachon *et al.* 2004.
- ³⁸ Ahmed 2002, Gachon *et al.* 2004.
- ³⁹ Anklam *et al.* 2002.
- ⁴⁰ Anklam *et al.* 2002b.
- ⁴¹ Wurz *et al.* 1999, Ahmed 2002, Anklam *et al.* 2002b.
- ⁴² Alary *et al.* 2002.
- ⁴³ Brodmann *et al.* 2002.
- ⁴⁴ Zimmermann *et al.* 2000, Brodmann *et al.* 2002.
- ⁴⁵ Feriotto *et al.* 2002, Garcia-Canas *et al.* 2002, Mariotti *et al.* 2002, Sheils *et al.* 2003, Meric *et al.* 2004.
- ⁴⁶ Oemke *et al.* 2001, Xue and Tisdell 2002.
- ⁴⁷ Xue and Tisdell 2002.
- ⁴⁸ UNEP/CBD/BS/COP-MOP/2/10

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