



**Secretariaat
Secrétariat**

O./ref.: WIV-ISP/BAC/2009_879

Title: Advice of the Belgian Biosafety Advisory Council concerning a research protocol on environmental risk proposed by the VIB in the context of a previous request for field trial with GM grey poplars.

Context

On the 1st of July 2008, the Federal Ministers Onkelinx (Minister for Social Affairs and Public Health) and Magnette (Minister for Climate and Energy) appealed to the Biosafety Advisory Council to give a scientific opinion concerning a research protocol on environmental risk ("Milieurisico-onderzoeksprotocol") provided by the VIB as a follow-up of a previous request for field trial with GM grey poplars (notification B/BE/07/V2 : "Field evaluation of poplars with an altered wood composition for the production of bio-ethanol").

The protocol has been drafted with the aim of providing answers to one of the arguments put forward by the Ministers in their negative decision regarding this field trial¹.

On 15 July 2008, the Biosafety Advisory Council gave a first and short advice concerning this protocol (ref. BAC_2008_790). However, owing the short delay in which this advice was asked for, the Biosafety Advisory Council was not able to organize a physical meeting, nor to ask advice to its experts in the preparation of its opinion.

Consequently, on 3rd October 2008, the Ministers addressed to the Biosafety Advisory Council a new request for a detailed analysis of the protocol. The current advice is an answer to this request. It complements the advice given in July 2008.

It is important to note that the Ministers' requests related to the above-mentioned protocol have been addressed to the BAC outside the official authorization procedure related to the evaluation of the notification B/BE/07/V2 (i.e. Chapter II of the Royal Decree of 21 February 2005).

Within the framework of the current evaluation of the environmental protocol, the Biosafety Advisory Council, under the supervision of two coordinators and with the assistance of its Secretariat, contacted

¹ This argument states (in the original Dutch language): "het dossier bevat geen specifiek evaluatieprotocol van de risico's die verband houden met de weerslag op de microfauna en de microflora van de bodem en de risico's die verband houden met de eventuele wijziging van de gevoeligheid van bewuste GGO populieren voor besmettingsagentia en klimaatstress".

experts to assess the dossier. One expert from the common list of experts drawn up by the Biosafety Advisory Council and the Division of Biosafety and Biotechnology (SBB), and one external expert answered positively to this request. These scientists were chosen for their relevant expertise in soil micro-organisms and insects respectively.

The main objective of the protocol is to collect (within the framework of the field trial proposed in the notification B/BE/07/V2) data about insects and soil micro-organisms that could be used later on for a more in depth risk analysis of the cultivation of the genetically modified grey poplars.

The experts were therefore asked to assess to what extent the protocol could contribute to gain a better knowledge as regards the potential impact on the environment associated with the growth of the GM grey poplars.

For the purpose of the scientific evaluation, the following documents have been considered:

- The full dossier of notification B/BE/07/V2, including additional information provided by the notifier during the official authorization procedure;
- The advice of the Biosafety Advisory Council concerning notification B/BE/07/V2 (doc. BAC_2008_733);
- A summary of the arguments put forward by the Ministers to motivate their negative decision for this field trial;
- The environmental protocol provided by the notifier to the Federal Ministers after the final decision on notification B/BE/07/V2.

A compilation of the contributions from the experts is provided in Annex I.

Summary of the scientific evaluation

a. Evaluation of the impact of GM poplars on insects

From the currently available scientific literature, one can conclude that only limited amount of information is available as regards the occurrence of insects on transgenic poplars. Moreover, data available relate mainly to insects which feed on leaves, and not on insects living on wood (whereas these insects represent an important group).

The environmental protocol proposed by the VIB foresees the study of one insect species only (the leaf beetle *Chrysomela populi*). It is important to note that the proposed field trial has not been designed in order to gather the maximum information concerning the topics dealt with in this evaluation.

The Biosafety Advisory Council is of the opinion that in its current settings, the proposed study will provide only limited information about the interaction between insects and transgene poplars given the fact that:

- (i) Poplars host a large and diverse community of insects;
- (ii) It is unclear from the protocol whether or not *Chrysomela populi* can be considered as an indicator species representative of other groups or species of insects.

The Biosafety Advisory Council is of the view that additional useful information could potentially be gathered by adapting the protocol according to the following suggestions:

- to include the study of other local species such as *Phratora laticollis* (Coleoptera, Chrysomelidae), given to the fact that *Chrysomela populi* is not very abundant in Belgium;

- to perform a larger study on the potential impact of transgenic poplars on a set of harmful and not harmful insects, looking both at insects feeding on leaves and on insects living on wood (such as root-, shoot- and stem-borers). Xylophagous species should be important to study because, on the one hand they constitute a group of pests particularly harmful to poplar and, on the other hand, the GM poplars will be modified in their lignin content, which is a particular trait of resistance towards wood-feeding species. Such a study could be run in two steps:

(i) A quantitative comparative inventory (at regular intervals during the growing season) of the insect fauna present should be run on the transgene poplars and on control lines. The Biosafety Advisory Council is however fully aware that due to the limited area of the trial, the limited numbers of trees involved and the particular harvest system of short-rotation coppice, which takes away the wood every few years, the study of xylophagous species will most likely provide only very limited information;

(ii) Because of this limitation in time and space, a detailed comparative study of the behaviour of some specific insect species (representatives of insects living on leaves or on wood), deliberately established on the transgene poplars and on control lines, should also be undertaken. This would have to be performed both under field and laboratory conditions, because of the difficulties in handling some insect species for experimental purposes in the field.

b. Evaluation of the impact of GM poplars on the soil microflora

Concerning the monitoring of microbial diversity (bacteria and fungi), the Biosafety Advisory Council considers that the sampling procedure and frequency is representative of the usual procedures used in soil microbiology. Pyrosequencing analysis is proposed by the VIB to study the effect of transgenic poplars on soil microbial biodiversity. The Council considers that this approach is currently one of the most suitable for drawing up a correct overview of soil microflora and can thus be considered as fully pertinent. It could however be useful to mention what kind of primers will be used to study the fungal diversity.

Conclusion

Based on the scientific assessment of the research protocol on environmental risk provided by the VIB, the Biosafety Advisory Council:

- Is of the opinion that the protocol offers the opportunity to gain scientific information concerning the impact of these transgenic trees on the biodiversity of soil bacteria and fungi;
- Recommends that in order to collect relevant scientific information concerning the impact on insects, the protocol could be completed with additional studies aiming at performing more in depth observations on the impact of the GM poplars on a set of harmful and so far harmless insects, including a local species such as *Phratra laticollis*.
- Notes that, due to the settings of the field trial, in particular its limited area, the limited numbers of trees involved and the short coppice system - which takes away the wood every few years - the protocol, even adapted following the above-mentioned suggestions, is expected to provide only limited information as regards potential long-term effects associated with possible future large scale trials or commercial plantation involving GM grey poplars;
- Emphasizes that the current advice relates strictly to the environmental protocol and that the advice of the Biosafety Advisory Council delivered as a result of the risk assessment of the notification B/BE/07/V2 (doc. BAC_2008_733²) remains valid;
- Reminds, as already mentioned in its final advice on notification B/BE/07/V2, that it is obvious that any other occasional establishment (e.g. a large scale trials or commercial plantation) of these GM poplars, either grown as a short-rotation coppice or as a timber production unit, should be the subject of a new advice, owing to the different conditions.



Prof. D. Reheul
President of the Biosafety Advisory Council

Annex I: Compilation of comments from experts who provided a scientific contribution in the framework of this assessment (ref. BAC_2009_878)

² available on http://www.bio-conseil.be/bac_advices.html



**Secretariaat
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**Compilation of comments of experts in charge of evaluating
the "milieurisico-onderzoeksprotocol" submitted by the VIB
to supplement notification B/BE/07/V2**

Mandate for the Group of Experts: mandate of the Biosafety Advisory Council (BAC) of 31 October 2008

Coordinators: Prof. Dr. ir. Dirk Reheul, Prof. Jean-Claude Grégoire

Experts: Michel Penninckx (ULB), Inge van Halder (INRA)

Domains of expertise of experts involved: Forestry entomology, soils microbial ecology, biodiversity

Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman

INTRODUCTION

Notification B/BE/07/V2 submitted by the VIB to the Belgian Competent Authority (CA) in November 2007 for a request of deliberate release in the environment of genetically modified higher plants for research and development according to Chapter II of the Royal Decree of 21 February 2005. This dossier did not receive authorization and as an answer to one of the arguments of the ministers, on 12 June 2008 the applicant submitted a "milieurisico-onderzoeksprotocol" proposing to collect during the field trial data about insects and soil micro-organisms that could later be used for a more in depth risk analysis of the cultivation of the genetically modified poplars. In November 2008 the Biosafety Council contacted 2 external experts, asking their opinion about the proposed protocol, in particular regarding its added-value at the biosafety viewpoint.

All the comments received from the experts are listed below and kept in the language used by the expert.

¹ revised version of document WIV-ISP/BAC_2009_871

List of comments received from the experts

Comment 1

Door bomen genetisch te wijzigen kunnen interacties met andere, niet-doelwitorganismen zoals insecten, veranderen. Transgene bomen kunnen een andere insectenfauna huisvesten dan wildtype bomen, en dit kan zowel veranderingen betreffen in het voorkomen van schadelijke insecten als van niet-schadelijke insecten. Het duidelijk meer voorkomen van schadelijke insecten op genetisch gewijzigde bomen zou inhouden dat deze bomen een lagere opbrengst hebben of dat deze insectenplagen bestreden moeten worden om economische schade te voorkomen. Daarnaast kan het ook zijn dat andere insecten, waaronder bedreigde soorten, minder of niet voorkomen op genetisch gewijzigde bomen en dat er dus effecten zijn op de biodiversiteit. Veranderingen in het voorkomen van herbivore insecten zullen bovendien effecten hebben op het voorkomen van predatoren en parasitoiden, waardoor mogelijk tot dan toe secundaire plagen primaire plagen kunnen worden.

Volgens het technisch dossier van de veldproef VIB (REG/07-2982) wordt het belangrijkste overzicht van mogelijk ecologische effecten van lignine gemodificeerde bomen gegeven in het review artikel van Halpin et al. (2007). In dit review artikel wordt één artikel geciteerd waarin gekeken is naar het voorkomen van insecten op genetisch gewijzigde populieren, namelijk het artikel van Pilate et al. (2002), en één artikel waarin vijf insectensoorten met bladeren van genetisch gewijzigde berken zijn gevoed (Timonen et al., 2005). Uit dit laatste onderzoek bleek dat twee insectensoorten een voorkeur toonden voor bepaalde transgene lijnen, maar dat deze voorkeur niet direct gekoppeld hoeft te zijn aan een lager lignine gehalte. In het artikel van Pilate et al. (2002) is alleen statistisch aangetoond dat het percentage populierenbomen met vraatschade niet verschilt tussen transgene en wildtype soorten. Volgens Pilate et al. toonden inventarisaties van insecten aan dat transgene en wildtype bomen een vergelijkbare insectenfauna herbergden, maar in het artikel worden geen cijfers gegeven om dit te onderbouwen en er worden geen statistische analyses vermeld.

Daarnaast wordt in het technisch dossier het artikel van Bordeur-Campbell et al. (2006) aangehaald. Dit artikel beschrijft een onderzoek naar de ontwikkeling van twee vlindersoorten op bladeren van lignine gemodificeerde *Populus tremuloides*. De overleving van één soort was significant lager op één transgene lijn, en de auteurs veronderstellen dat dit een indirect effect is van de genetische verandering en niet direct wordt veroorzaakt door een lager lignine gehalte.

De beschikbaarheid van literatuurgegevens over het voorkomen van insecten op transgene populieren is dus zeer beperkt en gedeeltelijk onvolledig. De schaarse beschikbare literatuur geeft aan dat er voor enkele insectensoorten verschillen zijn gevonden tussen transgene lijnen en controle lijnen.

In de geciteerde literatuur is bovendien alleen gekeken naar insecten die zich met bladeren voeden, en niet naar insecten die in het hout leven (wortelboorders, stam-, tak- en twijgboorders). Houtbewonende insecten vormen een belangrijke groep van insecten die op populier voorkomen, waaronder enkele soorten die schadelijk kunnen zijn.

In het Milieu-risico-onderzoeksprotocol wordt beschreven welke gegevens tijdens de veldproef zullen worden verzameld om de potentiële impact van genetisch gewijzigde bomen op het leefmilieu in te schatten. De verzamelde gegevens zouden gebruikt kunnen worden voor een latere, meer uitgebreide

risico-analyse. Wat betreft het effect op het voorkomen van insecten wordt in het onderzoeksprotocol aangegeven dat populierenhaantjes (*Chrysomela populi*)² zullen worden geteld.

Populieren herbergen een zeer diverse insectenfauna en het lijkt daarom weinig zinvol om maar één soort te inventariseren. In het onderzoeksprotocol wordt niet duidelijk gemaakt dat het voorkomen van het populierenhaantje gecorreleerd zou zijn met het voorkomen van andere plaag of niet-plaaginsecten, en dat dit insect dus model zou kunnen staan voor andere insectensoorten of insectengroepen. Het voorgestelde onderzoek kan daarom alleen iets zeggen over het voorkomen van het populierenhaantje en heeft derhalve een zeer geringe meerwaarde om in te schatten in hoeverre interacties met insecten op transgene lijnen verschillen van controle lijnen. Het zou veel zinniger zijn om een uitgebreider onderzoek op te zetten naar de effecten van transgene lijnen op het voorkomen van plaag- en niet plaaginsecten en daarbij zowel te kijken naar insecten die zich met bladeren voeden als die in het hout leven (wortelboorders, stam-, tak- en twijgboorders). Het is mogelijk dat de verlaagde en veranderde lignine-samenstelling op houtbewonende insecten een groter effect heeft dan op bladete insecten. In dit onderzoek zou gedurende verscheidene jaren meerdere keren in het groeiseizoen de insectenfauna aanwezig op de transgene lijnen en de controlelijn kwantitatief geïnventariseerd moeten worden om na te gaan of er verschillen bestaan in het voorkomen van insectensoorten op transgene lijnen en controlelijnen. Daarnaast zou meer gedetailleerd vervolgonderzoek uitgevoerd kunnen worden naar de overleving van enkele insectensoorten (bladeters, houtbewoners) op de transgene lijnen en de controle lijn.

Het zou jammer zijn om niet van de geplande veldproef gebruik te maken om deze gegevens te verzamelen en hiermee te wachten tot een latere meer uitgebreide risico-analyse kan worden uitgevoerd.

Comment 2

Objective of the expertise

The present evaluation concerns a particular aspect of the project, namely the pertinence of the analysis method proposed by the applicants for estimating the impact of GMO poplars on soil microbial diversity.

Background

Although development of transgenic plants, in particular poplars, has steadily increased, very few data concerning response of soil microorganisms to genetic modifications have been published. Using phospholipids fatty acids, neutral lipid fatty acids and extracellular enzymes assays analysis, Bradley et al (2007) came to the conclusion that *Populus tremuloides* altered in stem lignin may have effects on soil microbial communities in three different investigated USA soils (sandy loam, silt loam, and clay loam). It was however identified for now a long time ago that the procedures based on functional analysis and plate isolation reflects often very poorly total and/or active microflora in complex environments as soils (reviewed by Handelsman,2004).In the last twenty years, culture-independent bacterial survey in soils were developed (Pace et al, 1985; Torsvik et al., 1990). First approach used DNA isolated from soil in DNA: DNA hybridization procedure. Further attempts used the metagenomics approach. Metagenomics is a discipline in which genomic DNA analysis is applied to entire communities of microbes, bypassing the need to isolate and culture individual microbial species

² Additional comment from the coordinator : *Chrysomela populi* is not very abundant in Belgium. Local species should be preferred, such as *Phratona laticollis* (Coleoptera, Chrysomelidae)

(Chen and Pachter, 2005). Amplification and sequencing of the highly conserved 16S RNA gene from soil DNA was used to assess soil microbial diversity. Recent estimates of a number of nearly 10^7 microbial species per gram of soil have however highlighted that previous approaches using DNA sequencing technology were not accurate to verify this order of magnitude (Gans et al., 2005). Shotgun sequencing (Chen and Pachter, 2005) and pyrosequencing (Edwards et al., 2006) have been proposed as improved methods. In a recent contribution Roesch et al (2007) have shown that, although not absolute, the pyrosequencing method may enumerate and contrast soil microbial diversity more accurately than other procedures. In conclusion, pyrosequencing appears thus currently as one of the most suitable approach for drawing up a correct overview of soil microflora. The procedure was proposed by the VIB applicants to study the effect of transgenic poplars on soil microbial biodiversity.

Pertinence of the procedure for monitoring microbial diversity in the poplar project

The applicants will monitor soil microflora dynamic in the B/FR/07/06/01 field assay which is conducted in the framework of the European project ENERGYPOPLAR, where VIB is a partner. Ten transgenic poplar lines affected in different lignin biosynthesis enzymes will be considered. The applicants refer to the Roesch et al (2007) paper for the general monitoring protocol. Specifically, five samples will therefore be collected from the top of the soil surface around each genotype at three times spacing during growth season of the trees. Pyrosequencing analysis will be conducted on the samples for determining bacterial and fungal biodiversity. The present referee considers that the sampling procedure and frequency is representative of the usual procedures used in soil microbiology. The applicants mentioned that they will study not only bacterial diversity but also fungal diversity. Primers for determining fungi are not described. Hence it could be useful to mention what kind of primers will be used by the applicants to study this particular aspect.

As a general conclusion, pyrosequencing was initially developed for the detection of mutations and single nucleotide polymorphism in a variety of clinical applications (Nilsson and Johansson, 2004). The method was further successfully applied in Microbial Ecology. Technically speaking the procedure is rapid and easy, however requiring important data treatment. Until now, pyrosequencing was apparently never used in the particular aspect of GMO poplars impact on microbial diversity. Yet, provided the applicants have access to the technical and data treatment facilities needed for experiencing pyrosequencing, this referee consider that the approach is fully pertinent for this project.

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