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Review

Assessing environmental impacts of genetically modified plants on non-target organisms: The relevance of *in planta* studies

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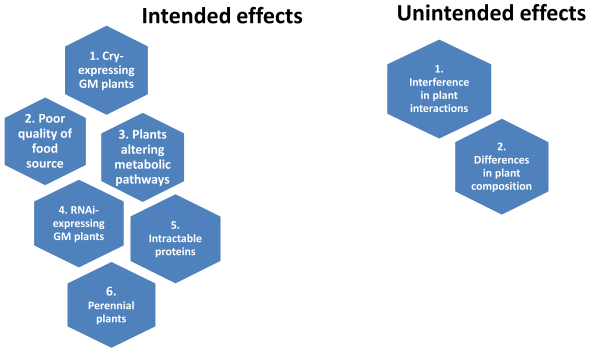
HIGHLIGHTS

- GM plants and their products are possible stressors for non-target organisms.
- In the EU it is compulsory to use GM plants in the experiments for risk assessment.
- Existing literature supports the use of such studies in environmental risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

In legal frameworks worldwide, genetically modified plants (GMPs) are subjected to pre-market environmental risk assessment (ERA) with the aim of identifying potential effects on the environment. In the European Union, the EFSA Guidance Document introduces the rationale that GMPs, as well as their newly produced metabolites, represent the potential stressor to be evaluated during ERA. As a consequence, during several phases of ERA for cultivation purposes, it is considered necessary to use whole plants or plant parts in experimental protocols. The importance of *in planta* studies as a strategy to address impacts of GMPs on non-target organisms is demonstrated, to evaluate both effects due to the intended modification in plant phenotype (e.g. expression of Cry proteins) and effects due to unintended modifications in plant phenotype resulting from the transformation process (e.g. due to somaclonal variations or pleiotropic effects). *In planta* tests are also necessary for GMPs in which newly expressed metabolites cannot easily be studied in vitro. This paper reviews the scientific literature supporting the choice of *in planta* studies as a fundamental tool in ERA of GMPs in cultivation dossiers; the

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Risk management

evidence indicates they can realistically mimic the ecological relationships occurring in their receiving environments and provide important insights into the biology and sustainable management of GMPs.

Contents

1. Introduction

The evaluation of possible environmental impacts of genetically modified plants (GMPs) and derived food/feed products is an important part of the authorization process, under which such biotechnological innovations are regulated for cultivation by authorities worldwide (e.g. the European Food Safety Authority (EFSA), US EPA, Health Canada, FSANZ). Among other areas of environmental concern (e.g. persistence and invasiveness, horizontal gene transfer, interactions with biogeochemical cycles, etc.), studies on non-target organisms (NTOs) (Arpaia, 2010) are conducted on a regular basis by developers of commercial GM crops in order to assess potential effects of GMPs on biodiversity in and around cultivated areas.

In agro-ecosystems, hundreds of species are sustained in food webs above and below ground, based on cultivated plants as the main primary producers (Mészáros et al., 1984a, 1984b), although most of these species are not economically relevant crop pests, or keystone organisms in supporting Ecosystem Services (Mace et al., 2012). Numerous species at higher trophic levels can come into contact with plants and their metabolites either directly or indirectly, e.g. through feeding on herbivorous hosts/prey (Andow et al., 2006). The concerns arising from the exposure of NTOs to GMPs is that intentional or unintentional changes in expression of some GMP metabolites or changes in plant composition and structure (e.g. lignin, cuticle, hairiness, etc.), may affect ecological interactions and thus ultimately harm sensitive NTOs when sufficiently exposed.

In some areas where GMPs were first cultivated (e.g. USA, Argentina), regulatory bodies adopted a testing system largely based on the eco-toxicological approach, traditionally used for testing noxious chemicals (e.g. pesticides). According to this 'tiered' system, testing starts with small-scale and short-term laboratory tests conducted on surrogate species in which the potentially noxious chemical is mixed with artificial diet in doses much larger than the expected environmental concentration. Only if negative effects on the studied organisms appear at this stage, is scaling up of the experimental setup required to greenhouse semi-field or field experiments using growing plants (Romeis et al., 2008).

In the European Union (EU), the assessment of effects on NTOs in Environmental Risk Assessment (ERA) is discussed in EFSA (2010a, 2010b). These two key Guidance Documents (GDs), adopted by all EU Member States (MS), present guidelines for applicants wishing to introduce GMPs in the European Union for cultivation purposes. The two documents refer to different existing ERA approaches, but also introduce a series of initiatives aimed at further rooting the ERA in sound ecology. These GDs include the requirement to consider the whole GM plant, in addition to the introduced traits (e.g. *Bt* proteins), as the possible environmental stressor. The rationale is that processes involved in genetic modification, as with most other plant breeding techniques, not only introduce intended novel traits but can also cause additional unintended changes to the plant's phenotype which affect its interactions with the receiving environment.

Therefore EFSA (2010a, 2010b) requires the generation of experimental data using viable GM plants or plant parts in the ERA process for cultivation purposes. This provides the basis for a comparative analysis, in which impacts of the GMP are assessed relative to an appropriate (usually genetically similar) non-GM plant comparator and differences are assessed for their potential hazards and risks. *In planta* tests study ecologically representative organisms (e.g. insects, microorganisms) by exposing them to the test plant(s); exposure can be to plant parts or organs (e.g. leaves, stems, seeds, pollen, flowers) or to whole plants in glasshouse, semi-field or field experiments. For NTO risk assessment in cultivation dossiers, *in planta* generated data are deemed necessary in two situations:

1. To evaluate effects on NTOs *due to the intended modification* in plant phenotype (e.g. expression of *Bt* Cry proteins active against certain insect pests);
2. To evaluate possible effects on NTOs *due to unintended modifications* in plant phenotype (e.g. pleiotropic effects, interference with other metabolic pathways, etc.). EFSA (2010b) considers such unintended

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effects to be event-specific. These may either be identified during the 'problem formulation' phase due to results from compositional or molecular studies of the GMPs, resulting in an explicit testable hypothesis, or from a general hypothesis that pleiotropic or other effects which may affect NTOs might have occurred during the genetic transformation process (e.g. due to the random insertion site of the transgene(s)).

In this review the evidence and theory supporting the relevance of *in planta* tests for ERA in the two areas highlighted above are discussed, with reference to existing scientific literature.

2. The use of *in planta* studies to assess effects of intended modifications

The reasons for requiring specific studies tailored for GMPs are manifold and performing *in planta* studies may lead to the rejection of more than one risk hypothesis at the same time (Andow et al., 2006) improving efficiency.

The following examples (Sections 2.1–2.6) illustrate the most common cases in which using *in planta* tests can inform the ERA.

2.1. Insect resistant plants expressing Cry proteins

One of the most widely adopted approaches in producing insect-resistant GMPs is the use of genes that produce insecticidal proteins and thereby confer pest resistance (James, 2015). The genes used for expressing the insecticidal proteins in GMPs often originate, or are derived from genes occurring in spore-forming bacteria summarised under the species name *Bacillus thuringiensis* Berl. (*Bt*). These bacteria have the capacity to produce insecticidal crystal proteins (Cry proteins, delta-endotoxins). Different *Bt* strains can produce variants of Cry proteins, which are active against specific groups of insects (van Frankenhuyzen, 2009).

While the crystal proteins in spores of *B. thuringiensis* are protoxins, requiring proteolytic cleavage before they become active inside the gut of insects, GM plants typically synthesize the activated toxin version inside their tissues. The protoxin and the activated toxin differ in their environmental persistence and abiotic interactions, e.g. adsorption to soil constituents (Venkateswerlu and Stotzky, 1992), see Table 1. To have sufficient amounts of proteins for eco-toxicological tests, the activated protein is often produced by genetically engineered bacteria (e.g. *Escherichia coli* K12 strains). The Cry proteins produced by *E. coli* and by the GM plant should be identical. However, protein synthesis by prokaryotic cells (bacteria) and eukaryotic cells (in GMPs) are not always the same, considering the possibility of post translational modifications by the latter (glycosylation), or modifications of the primary protein structure due to secondary mutations during the cloning procedure (Freese and Schubert, 2004). There are both differences and similarities between the use and mode of actions of *Bt*-based microbial pesticides and *Bt* expressing plants (see Table 1). Moreover, testing purified *Bt* proteins in artificial diets may not reflect the ecological reality in which many primary and secondary plant metabolites can act additively or synergistically and may interact with the newly expressed proteins, both in the plant and in the herbivorous host/prey upon which natural enemies feed (see Section 3.1).

Biological activity of the bacterium *B. thuringiensis* against insect pests can be influenced by the host plants, therefore these interactions need to be taken in duly considerations. The effect of chemicals from the surface of different plant species in combination with the use of Cry1Ac was evaluated by Paramasiva et al. (2016). The authors detected significant differences in the toxic activity on *Helicoverpa armigera* larvae. Larval weight and development, pupal weight and adult emergence were affected when plant extracts from different species were added to artificial diet containing a commercial formulation of Bt or Cry1Ac.

Therefore, existing knowledge about the toxicology of *Bt*-proteins expressed in microbial pesticides is insufficient to draw robust conclusions on potential hazards of GMPs, without testing the activated toxins expressed by the plants themselves (NAS, 2000).

2.2. Effects due to poor quality of food source

GM plants can have indirect effects on both target and non-target organisms at different trophic levels through food webs. As examples, the ERA would require *in planta* studies in the following exposure scenarios (Andow et al., 2006):

i) a NTO at third (or higher) trophic level feeds on hosts/preys which are herbivores that feed on GMPs and accumulate their products,
ii) a NTO at third (or higher) trophic level feeds on hosts/preys which are herbivores that feed on GMPs and are sub-lethally affected by their products and may therefore have a lower nutritional quality as host/prey;
iii) a GMP constitutes a poor quality diet for herbivores compared with the non-GM isogenic line, which in turn constitute poor quality host/prey for the NTO at higher trophic levels; and
iv) a GMP constitutes a poor quality diet (in comparison with the non-GM isogenic line) for NTOs at higher trophic levels that ingest plant material as a complement to their carnivorous diet (zoophytophagy).

None of the above mentioned risk hypotheses can be firmly rejected if only in vitro tests with purified proteins are conducted (see Section 4, below).

In addition to the experience gained with the first generation of GMPs, future applications may trigger different questions when dealing with biosafety and environmental risks. The level of plausibility of these risks will have to be based upon problem formulation and what is known about specific agronomic systems. The possible support to ERA gained through *in planta* studies is indicated in the following paragraphs.

2.3. Traits altering metabolic pathways

The ERA framework for GMPs must be robust enough to allow the efficient evaluation of qualitatively different events, including GMPs expressing new traits which are expected to be submitted for approval in the future. Some GM events currently entering commercialization do not produce novel proteins but purposefully alter specific plant metabolic pathways (e.g. starch production, oil composition, semiochemicals, etc.). As above, even when direct toxic effects can be excluded, possible secondary or indirect effects (e.g. Ricroch et al., 2011) require specific consideration during ERA.

2.4. Plants altered in RNAi post-transcriptional gene silencing

Recently, GMPs have been produced that achieve insect resistance using RNAi post-transcriptional gene silencing (Hamilton and Baulcombe, 1999; Hannon, 2002). While double strand (ds) RNA produced in crop plants as a means of pest control has been claimed to have a high degree of specificity (Bachman et al., 2016; Petrick et al., 2013; Whyard et al., 2009), other evidence suggests that it may have other unexpected effects (Lundgren and Duan, 2013). Therefore, dsRNA-expressing GMPs present new challenges for ERA. For instance, Terenius et al. (2011) were not able to confirm specificity of RNAi following examination of data from numerous studies with RNAi in insects. They proposed that variation in sensitivity of the target gene to RNAi and in the sensitivity of the tissue targeted may influence effects of dsRNA. Since mRNAs are transcribed when needed by the organism, it is important to recognize that the environment plays a significant role in gene expression and therefore, in which genes will be exposed to the inhibitory small siRNAs over time and space (Smith and

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Table 1
Similarities and differences between microbial Bt-based insecticides and Bt-expressing genetically modified plants, which are relevant for environmental risk assessment.

Issue for NTOs/ERA	*Bt* insecticides	*Bt* plants
Structure of primary constituents and gene fragments (if present)	Typically *Bt* formulations contain specialised crystal proteins or spores of the *Bt* bacteria with the crystal protein, as well as formulation additives such as wetting agents, sticking agents, UV protectants and undisclosed 'inert ingredients'. Diluted in water, e.g. 150–300 times, before application. Cry proteins are present as protoxins, whose activation requires several steps of processing after ingestion: solubilisation of crystal proteins, proteolytic activation of protoxins, binding to specific midgut receptors, irreversible insertion of the toxin into midgut membrane, passage of endotoxin and helper factors through target midget pores, leading to osmotic lysis, damage to midget membranes, starvation and septicaemia (Whalon and Wingerd, 2003).	Typically modified from naturally occurring *Bt* toxins; modified protein expressed in truncated gene constructs, expressing activated toxin form, which typically do not require proteolytic cleavage.
Mode of action	Possibly 2–3 different mechanisms involved, some poorly understood, especially factors determining pore formation in target midgut membranes (Graf, 2011; Vachon et al., 2012).	Broadly the same as in *Bt* insecticides, but with some unknown mechanisms. More potential for interactions of expressed *Bt* proteins with other plant metabolites (see below).
Interactions with plant metabolites (e.g. plant defence chemistry)	Interactions of *Bt* toxin(s) with endogenous plant metabolites is less likely, due to limited persistence on plant surfaces. However, applied insecticides which contain mixed toxins could interact with each other to increase or decrease efficacy and activity spectrum of the insecticide. Potential interactions with plant surface waxes, polar compounds which affect insect settling and feeding behaviour (indirectly affecting uptake).	Interactions with plant secondary metabolites such as phenolic compounds, trypsin inhibitors, e.g. *Bt-cotton* (Dong and Li, 2007). In stacked *Bt* events expressing multiple toxins, potential for additive interactions between different *Bt* toxin classes (Sharma et al., 2010) and endogenous plant metabolites inside the plant.
Specificity across taxa (orders)	Claimed to be genera specific, but some recent evidence of cross-taxa activity at high concentrations, if ecologically relevant: http://www.glfc.cfs.nrcan.gc.ca/bacillus/	Same potential for cross-taxa activity, depending on concentration and sensitivity of exposed non-target taxa.
Spatial exposure	Depends on penetration and coverage of *Bt* applications to the growing plant, persistence and degradation of *Bt* toxins. Usually spray coverage restricted to exposed foliage surfaces above ground and to soil surface, but not to internal leaf tissues or roots.	Variable, depending on gene promoter used, (e.g. whole plant expression including roots, or tissue-specific expression - only roots). Affected by plant age (Kranthi et al., 2005) environmental stressors (complex genotype × environment interactions possible).
Temporal exposure	Typically short persistence on exposed plant surfaces, due to UV (sunlight) inactivation (activity limited to 24 h–1 week). *Bt* insecticides may need repeated applications. Plant morphology also changes during crop growth, so different plant tissues become exposed to herbivores over time (e.g. new leaves, opening flowers).	Typically expressed in internal tissues rather than external surfaces. Expression can vary over time as the plant matures. (Dong and Li, 2007). Typically *Bt* expression in GM crops persists for weeks/months rather than days. Variable duration, depending on plant tissue, plant age, environmental stressors (Dong and Li, 2007).
Concentration in/on plant	Variable, typically declining rapidly due to UV exposure on plant surfaces (Whalon and Wingerd, 2003).	Variable duration, depending on tissue, plant age, leaf age, interactions with environmental stressors (Prager et al., 2014). Protein levels change through inhibited synthesis, degradation or translocation. Gene silencing via methylation of the promoter can occur during plant development. Efficacy not always directly correlated with tissue concentration, due to unknown factors affecting stability and efficacy (Dong and Li, 2007).
Post harvest exposure	Negligible	Decomposing roots, stubbles and leaves remaining on fields, with Cry proteins persisting until the following growing season (Baumgarte and Tebbe, 2005; Miethling-Graff et al., 2010). Viable root stubbles may continue to produce Cry proteins.

Kruglyak, 2008). The same issue of environmentally regulated expression of potential off-target genes (i.e., genes involved in different metabolic pathways which contain sequence similarity to RNAi) is relevant for testing effects on NTOs (US EPA, 2014). While in vitro assays can be conducted on a range of NTOs (Bachman et al., 2016), the experimental conditions imposed during *in planta* testing better reflect gene expression (in plants, target and NTOs) under typical field conditions and therefore increase the chance of detecting possible effects on NTOs.

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2.5. Plants expressing 'intractable proteins'

Other new GM events express 'intractable proteins' (Bushey et al., 2014). These proteins, including membrane proteins, signalling proteins, transcription factors, *N*-glycosylated proteins and resistance proteins, have properties that make them extremely difficult to isolate, purify, concentrate and quantify. While there is not much experience on this currently, it seems reasonable to predict that demonstrating their biological activity or comparing them with other proteins with in vitro assays will be practically impossible. In addition, *in planta* studies with NTOs in confined conditions are imperative when working with certain GMPs, such as GM plants designed to produce pharmaceuticals, for which risk assessors need to predict their possible ecological implications with limited practical experience under field conditions (Arpaia, 2010).

2.6. Perennial GM plants

Genetic modification and other novel plant breeding techniques are now applied to a wide range of plants including many perennials such as trees, shrubs and herbaceous species as well as biennial crops (Fernandez-Cornejo et al., 2014). Perennial species usually develop long term relationships with beneficial microbial species such as mycorrhiza and rhizobia in the soil and also with many arthropod species; therefore, *in planta* information on how these associations may be affected is an important part of the assessment of these GMPs. In addition, perennial plants develop longer term, more complex relationships with pest species, which include interactions with other associated biota, including beneficials, e.g. predators, parasitoids and antagonists of pests or diseases (Aguilera et al., 2013). Changes to plant metabolism, composition, phenotype and plant volatiles can affect these multi-trophic relationships over multiple growing seasons and result in changes in the equilibrium between beneficial and antagonistic associations in perennial ecosystems.

3. Using *in planta* tests to assess effects of unintended modifications

Many studies in the scientific literature have identified unintended modifications in GMPs (e.g. Lazebnik et al., 2017; Poerschmann et al., 2005; Ricroch et al., 2011). There are several reasons at the molecular level that can explain cases of unintended effects in a plant genotype (e.g. the construct, the insertional event, RNA production, etc.). Other changes may arise due to pleiotropic effects of new plant metabolites or properties of newly expressed proteins. These unintended changes at the molecular/genetic level can be translated into physiological or metabolic changes to the transformed GMP, which can in turn change the plant's phenotype and hence its biotic and other environmental interactions. While such unintended changes in the genome may not necessarily cause phenotypic changes that raise safety concerns, their potential effects on human health and the environment necessitate adequate examination. To resolve scientific uncertainties in this area, the consensus international approach to food-feed safety evaluation (reflected in the EU by EU Implementing Regulation No 503/2013) involves comprehensive comparative analyses of the chemical composition of GMPs and their conventional counterparts, for a large number of compounds in order to detect possible unintended changes in plant composition. Such tests for food safety do not involve explicit hypotheses, but are generic in nature.

Similarly, scientific uncertainties exist for ERA since unintended changes in the genome can change plant metabolisms and phenotypes, which may in turn impact interactions between plants and their biotic environment (Birch et al., 2002; Bruce, 2014; Bruce et al., 2015; Lazebnik et al., 2017). Some unintended changes are linked to characters which are normally not considered by plant breeders. For example, emission of plant volatiles is a trait not commonly considered during plant selection (unless associated with flavour traits) while this is a fundamental feature of many different defence mechanisms and pollinator interactions. Notwithstanding, a lack of explicit testable hypotheses is no more a reason not to study potential effects during ERA than it is for food safety.

3.1. Possible interference in plant interactions with other biota

The co-evolutionary 'arms-race' between herbivores and plants (Jander and Howe, 2008) is normally mediated by a series of plant chemicals in a delicate equilibrium which allow, or impair, alien organisms to feed on these plants. The full range of metabolites involved in plant-arthropod or plant-microorganism interactions can be split into two broad functional classes:

1. Chemicals stimulating colonisation or feeding stimuli (positive for the attacker) and
2. Chemicals involved in plant defence mechanisms (negative for the attacker).

Plants produce more than 35,000 metabolites with new ones being discovered regularly (Lunn, 2007). Carbohydrates, proteins and lipids are essential for plant growth, development and reproduction. The vast majority of plant metabolites, however, are so-called secondary metabolites that often play primary roles in ecological interactions with other organisms, particularly in plant defence. Several secondary plant substances have served as lead compounds for the development of new generations of synthetically derived insecticidal compounds, e.g. pyrethroids from *Pyrethrum* plants (Casida, 2010). Chemical plant defence mechanisms can act directly on plant-feeding insects by reducing feeding or oviposition (antixenosis) or by toxic or anti-nutritional effects (antibiosis); examples are listed in Table 2.

Moreover, plant nutrient variability can reduce insect herbivory performance and this phenomenon could even play a key role in suppressing herbivore populations (Wetzel et al., 2016).

Plant breeding has historically focussed more on genetic improvement of agronomic crop traits such as yield and quality than on pest resistance. Consequently, levels of certain classes of defensive secondary substances in crop plants have inadvertently become significantly reduced (typically by a factor of 5–10; Schoonhoven et al., 2005) which has often resulted in increased susceptibility to pests and pathogens. Genetic engineering of plant secondary metabolite biosynthetic pathways has the potential to provide enhanced resistance to insects (Birkett and Pickett, 2014; Bruce et al., 2015) and offer an alternative to *Bt*-based resistance against other important pests, such as aphids.

Plant chemical defences can also act indirectly by providing volatile chemical signals to parasitoids and predators, the natural enemies of plant-feeding insect pests (Schoonhoven et al., 2005) Plants respond to insect feeding by releasing herbivore-induced plant volatiles (HIPVs), (Dicke et al., 2009) which are detected by the olfactory system of parasitoids and predators of the plant feeders, guiding them to the location of their host/prey (Bruce and Pickett, 2011). Secondary plant substances acting in direct defence such as HIPV-emission, have been inadvertently affected by selecting crop species only for agronomically

Table 2
Examples of plant secondary metabolites involved in direct defence against insects.

Plant family	Crop species	Secondary plant metabolite classes
Solanaceae	Potato, tomato, eggplant	Steroidal glycoalkaloids, phenolic acids, flavonoid
Cucurbitaceae	Cucumber, gourds	Cucurbitacins
Gramineae	Maize, wheat, oats etc.	Benzoxazinoids, phenolic acids, flavonoids
Brassicaceae	Cabbages, oilseed rape	Glucosinolates, isothiocyanates; flavonoids
Fabaceae	Peas, beans, alfalfa etc.	Flavonoids; cyanogenic glycosides

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desirable traits. Among 11 maize cultivars and five wild maize progenitors, HIPV-blend composition showed qualitative differences and total HIPV-emission rates differed by factor 8 among cultivars (Gouinguené et al., 2001). Plant roots also produce HIPVs, of which the sesquiterpenoid β-caryophyllene plays a major role in attracting entomopathogenic nematodes that kill Western corn rootworm (*Diabrotica virgifera virgifera*) larvae, a key pest of maize (Hiltpold et al., 2010; Rasmann et al., 2005). Genetic engineering of HIPV-emission to enhance attraction of natural enemies of plant-feeding insects has been achieved in the model plant *Arabidopsis thaliana* Heynh. (Brassicaceae), maize (Kappers et al., 2005; Kos et al., 2013; Robert et al., 2013) and wheat (Bruce et al., 2015). Small changes in plant composition may also interfere more directly with the activity of organisms at higher trophic levels favouring or impairing herbivory (Hopkins et al., 2009). Therefore, predictions of consequences at higher trophic levels are not straightforward when only studies under simplified laboratory conditions are conducted. The results cannot be predicted from the assessment of the effects of purified proteins in vitro as constitutive and induced infochemicals from plants and natural enemies and competitors can affect herbivore behaviour and fitness (Dicke and Baldwin, 2010). Extrapolation of results obtained from the compositional and molecular analysis of the GM plant for food/feed safety purposes is insufficient, since the presence and the abundance of secondary metabolites usually differs between plant parts and behavioural effects depend on concentration (Hopkins et al., 2009). It is therefore difficult to extrapolate robust conclusions from GMP compositional analysis alone, particularly when the key components influencing the nutritional value for NTOs and secondary plant compounds relevant to plant defence mechanisms are not included in the composition analyses.

3.2. Differences in composition of GMPs

Chemically-mediated plant interactions may vary greatly among varieties of the same crop (Elzen et al., 1986). Comparison of novel plant germplasm (independently from the techniques adopted to obtain it) to its near isoline, often shows changes in the metabolism of the newly obtained GMP (Noteborn and de Wit, 2004). Examples include a celery with a high content of furanocoumarins (Beier, 1990), potatoes with increased levels of glycoalkaloids (Harvey et al., 1985), soybean which performed poorly under conditions of heat and drought stress (Gertz et al., 1999).

In GM crops, unintended changes in plant metabolism not linked to the metabolic pathway of the transgene have been reported by several authors (Birch et al., 2002; Poerschmann et al., 2005; Ricroch et al., 2011; Roessner et al., 2001).

For example, studies have addressed this in GM cotton events resistant to insects. Altered nitrogen metabolism was identified in some GM cotton lines by Chen et al. (2004, 2005). In particular, *Bt*-cotton cultivars showed a higher intensity ofChemicals stimulating colonisation or feeding nitrogen metabolism which Chen et al. (2004) and Ma et al. (2014) linked to the production of the new *Bt* proteins. They reported a reduction of gossypol and tannins in two *Bt*-cotton lines compared to their near isogenic counterparts.

There is a growing body of evidence suggesting that N deposition may substantially affect the interactions between plants and insect herbivores (Throop and Lerdau, 2004). The nitrogen and carbon metabolism are the major metabolic pathways in plants and normally an increase in one of the two triggers a decrease in the other (Baron et al., 2000; Lorio, 1986). It is then likely that an increased nitrogen metabolism might also weaken the synthesis of condensed tannins in the cotton plant, which are important component of natural resistance to herbivores in cotton (Ma et al., 2014).

Faria et al. (2007) found that Cry1Ab-expressing maize lines were significantly more susceptible to aphids than their near-isogenic counterparts. In studies with GM maize *Bt*11, Turlings et al. (2005) analysed volatiles collected from the GM event and its near isogenic control. The isogenic line released a larger amount of most volatiles, although there was no effect when the two maize genotypes were tested for their attractiveness to the parasitoid wasps *Cotesia marginiventris* and *Microplitis rufiventris*.

In eggplant, Arpaia et al. (2011) found a significant difference in the amount of volatiles produced by a Cry3Bb-expressing GM line and its near-isogenic control, although in this case the GM line produced significantly higher amounts of five of these compounds. In a greenhouse experiment, they observed the foraging behaviour of the bumblebee *Bombus terrestris* L. on the same eggplant lines. More bumblebees tended to visit GM eggplants compared to the near-isogenic control. Neither the number of flowers produced per plant, nor their size could explain the bumblebees' tendency to prefer GM eggplants. Six of the identified volatiles were also tested in electrophysiological bioassays on detached antennas from young bumblebees; a strong response was recorded in all six cases, suggesting the relevance of these chemicals for the bees' attraction to GM eggplant flowers.

4. *In planta* studies as predictors of effects on NTOs

4.1. Above-ground interactions

The scientific literature dealing with NTO effects has increased rapidly in recent years (Arpaia, 2010; Romeis et al., 2008; Wolfenbarger et al., 2008). Most data have been generated for *Bt* plants assessing the effects of Cry proteins on NTOs. Meta-analyses summarised the results obtained by many different authors and there is general agreement that direct effects on NTO species are mostly associated with the effects on related target organisms (Duan et al., 2009; Marvier et al., 2007). Indirect effects on higher trophic level species, particularly on parasitoids, have been highlighted (Lövei et al., 2009; Naranjo, 2009) and these appear more evident when tritrophic studies are considered separately from bitrophic studies. (Duan et al., 2009) also demonstrated that laboratory studies incorporating tri-trophic interactions of Cry1-expressing plants, herbivores and parasitoids provided also better explanation for the observed decreased field abundance of parasitoids than did direct exposure in vitro assays. Tritrophic studies including *in planta* tests were necessary to evaluate effects on aphid parasitoids in the study by Ramirez-Romero et al. (2007). Host-mediated effects of purified Cry1Ab protein on *C. marginiventris* were not evident when parasitoids were fed with larvae of *Spodoptera frugiperda*, which were grown on diet incorporating sublethal doses of purified Cry1Ab protein. However, several host-mediated effects on *C. marginiventris* were detected when Cry1Ab protein was delivered via *Bt*-maize tissue (*in planta*), affecting parasitoid developmental times, adult size, and fecundity.

Unintended effects were detected by Lazebnik et al. (2017) who reared the aphid *Myzus persicae* on several GM potato lines resistant to late blight. The authors found that aphid development and survival was affected by some GM events in the first generation, though effects disappeared during the second generation. However, comparing the non-transformed Désirée to the baseline variation among three other varieties showed that aphid intrinsic rate of increase and survival varied considerably more between different potato varieties than between Désirée and GM events.

While analysing effects of cultivating *Bt*-maize in Spain for three consecutive growing seasons, Pons et al. (2005) found that the effect on three herbivore groups differed. There were significantly higher aphid densities and leafhopper mature nymphs were more abundant on the *Bt*-maize. Similarly, Hagenbucher et al. (2013) reported that effective suppression of target herbivores by *Bt*-cotton inadvertently caused a decrease in the level of induced terpenoids, leaving plants more susceptible to feeding by the virus-transmitting cotton aphid *Aphis gossypii* Glov.

Clearly, studies purely conducted in vitro would be at risk of lacking predictive power for the formulation of testable hypotheses to be evaluated subsequently during ERA.

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4.2. Below ground interactions

Interaction between soil microorganisms and plants is to a great extent influenced by rhizodeposits, i.e., sloughed-off root cells and tissues, mucilage, volatiles, and soluble exudates (Dennis et al., 2010). These combinations of substrates and their variability during a growing season cannot be mimicked by laboratory assays excluding viable plants. Incubation of recombinant products directly in soil samples would in fact ignore the specific surface properties provided by roots for microbial colonisation and the implications of root respiration, which modifies the microbial habitat in the vicinity of the roots by depletion of oxygen and production of carbon dioxide. The latter possibility may be accompanied by a change in pH, having potential consequences for the mobility of micronutrients, bacterial diversity and adsorption affinities of proteins, including those expressed by GM plants. Thus, assessing effects of GM roots on soil microorganism requires studying viable plants rooted in soil.

In addition to rhizodeposits, NTOs in below-ground habitats are exposed to plant material from above ground, including pollen, deposited on the soil surface, and incorporated into soil, e.g. by the activity of earthworms (Schrader et al., 2008). Furthermore, after harvest, a significant amount of crop material remains in fields and its decomposition is an important ecosystem service mediated by micro- (bacteria, archaea, fungi, protozoa) and macro-organisms (e.g. nematodes, collembola, mites, earthworms, slugs and snails) (Birch et al., 2007). The EU ECOGEN studies (Griffiths et al., 2006) showed no in vitro effects on nematodes, micro-arthropods, earthworms and snails, while several mesocosm and field experiments (*in planta*) identified significant effects on these taxa. ECOGEN and some German studies concluded that a tiered approach including both in vitro and *in planta* tests at a range of realistic scales and multiple field sites are necessary to obtain ecologically relevant results on soil-inhabiting NTOs (Birch et al., 2007; Höss et al., 2008; Höss et al., 2011).

For assessing effects of GM plants on soil microorganisms, laboratory tests are limited to making very preliminary predictions on potentially adverse effects on ecologically relevant processes. This is because:

1. Only a minor fraction, generally assumed to be 0.1% of the total microbial community, is culturable on growth media in the laboratory (Rappé and Giovannoni, 2003).
2. In soil and rhizospheres microbial taxa ("species") typically exist in functional relationships to others within communities, where typically there are cross-feeding metabolites (Vos et al., 2013) and responses to signal molecules.

Thus, studies of microbial interactions with GM traits can only be performed in association with living root material and its microbial associations.

Furthermore, the rhizosphere is a soil microbial habitat characterized by an amount of carbon, limited by competition for nutrients but also by cooperation to access certain food sources. Bacteria or fungi cultivated in isolation on growth media, with sufficient food and without competition, promote habitat conditions which greatly differ from their natural environments. Under such artificial conditions, ecologically important genes may have different expression levels or be turned off and thus interactions through key enzymatic activities with stressors may be totally different.

3. Matrix effects, including sorption of chemical compounds and microbial cells, cannot be replicated without *in planta* studies at greenhouse or field scales (Grundmann, 2004; Neumann et al., 2013).
4. Studies of impacts on the efficiency of symbiotic plant-bacterial interactions such as legumes and rhizobia require viable roots and so there are no alternatives to *in planta* tests (Brockwell et al., 1995; Iaccarino, 2006; Mendes et al., 2013; Peoples et al., 1989). To evaluate symbiotic performance, the *in planta* tests for GM soybean cultivation in Europe would also require the addition of appropriate symbiotic bacterial partners (EFSA, 2014).

Unintended effects on the symbiotic interactions between mycorrhiza and GM plants have been reported (Castaldini et al., 2005; Cheeke et al., 2011; Turrini et al., 2005) and they may, under conditions of lower use of fertilizers, affect yield and plant health.

5. Implications for risk assessment

ERA is done on a case-by-case basis, so the information required varies with the GM plant and traits, the intended use, and the receiving environments. Compositional analyses are conducted routinely for GM food applications (EFSA, 2011) and are an important indicator of changes in a range of plant compounds. These provide useful information, but usually only of indirect relevance for ERA of NTO effects, in fact the chemical compounds involved in plant biotic interactions are not normally included as GMP compositional endpoints. Also, measurements are not usually derived from plant parts on which non-target organisms feed. For example, glycoalkaloids are important in regulating arthropod species assemblages on the leaves of potatoes but compositional analysis is normally conducted on potato tubers, not leaf tissue. In a greenhouse study Birch et al. (2002) found reduced levels of foliar glycoalkaloids in some transgenic potatoes that might adversely affect their defence against herbivory.

The data required for ERA should be no more than necessary to conclude and proportionate to the risk assessed. The use of *in planta* studies as suggested by EFSA ERA GD involves the requirement for plant exposure or plant-derived diet in the testing programme. An analysis was made of ERA dossier data within the public domain, from eight submissions to EFSA for cultivation made prior to publication of the European Food Safety Authority ERA guidance document (EFSA, 2010a, 2010b). In over half the cases examined no, or minimal further work would be required to meet this requirement for *in planta* exposure as set out in that new guidance (See Table S1, Supplementary Materials).

6. Strengths and weaknesses of in vitro and *in planta* tests

The strengths and weaknesses of both in vitro and *in planta* tests are summarised in Table 3. *In planta* tests are not intended to replace in vitro tests with purified proteins, especially when the potential hazards of a GMP require characterisation and the establishment of a dose-effect relationship. *In planta* tests can validate in vitro tests, which are subject to their own weaknesses and limitations (Andow and Hilbeck, 2004; Kimball and Levin, 1985) and can lead to misleading ERA evidence at an early stage in the process. *In planta* tests have added value, even when toxicological properties are well understood, as GMPs provide realistic in vivo exposure of test organisms to the stressor(s) being assessed.

In planta experimental designs may need to allow for large variability, for example in the induction and accumulation of defence signalling molecules to a higher level (Ricroch et al., 2011; Yan et al., 2015). Moreover, whole plant variability must be allowed for by standardised sampling protocols (e.g. plant age, growing conditions, fertilization, etc.) and may require larger replication to achieve adequate statistical power.

Some plant tissues are highly variable in their overall composition (Meissle et al., 2014), so design of *in planta* studies must allow for variability in composition of the starting material to obtain reliable baselines for bioassays. It is therefore paramount to use appropriate plant material, and experimental techniques should be uniform to allow differences due to unintended effects of genetic modification to be distinguished from other confounding factors.

In addition, NT insect populations can vary in sensitivity to expressed *Bt* proteins (Perry et al., 2012) and in response to different

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Table 3
Summary of main strengths and weaknesses of in vitro and *in planta* tests.

	Strengths	Weaknesses
In vitro tests	Testing environment and treatments are tightly controlled.	Purified proteins subject to anomalies (impurities, structural differences if produced from a different source than the GMP).
	Less experimental variability if well designed and good quality control.	Harder to test chronic exposure over multiple generations.
	Typically use acute high dose and short exposure, so easier to perform.	Indirect effects (e.g. through poor plant/prey quality) cannot be tested.
	Easier to assess dose responses of test species to individual chemical components.	Very limited possibilities to test trophic level interactions.
	Easier to use both positive and negative controls.	Only new metabolites are considered as stressors.
In planta tests	Genetic background effects of GMP taken into account.	Data more variable, so needs greater replication, more complex statistical analyses and experimental effort.
	Tests multiple trophic levels with GxE interactions concurrently.	Toxic effects can be hard to assign to a mechanistic cause (e.g. poor prey quality for predator or parasitoid).
	Greater ecological realism for testing environmental multiple stressors.	No dose-response can be estimated, so complementary in vitro tests are needed.
	More realistic testing of chronic exposure, sublethal effects over multiple generations.	
	Plants and their metabolites can be selected as stressors.	

genetic backgrounds of the same crop. This metabolic plasticity has been demonstrated in maize, (Degen et al., 2012; Degen et al., 2004; Tamiru et al., 2011) and GM potato (Plischke et al., 2012).

7. Conclusions

In their commentary, Devos et al. (2016) reported that some in the scientific community consider that GMO testing should be restricted to study effects (of GM traits) for which there was a distinct hypothesis for harm and that unintended changes to GM plants, as a result of the transformation process, did not constitute such hypotheses. However, since the international consensus is that such a naive approach is not appropriate for food safety evaluation, it is strange that it should be proposed for ERA.

In this review we have shown that genetic modification can indeed induce a range of effects on plant metabolism and overall phenotype, including unintended effects, though their biological relevances need to be evaluated case by case. Therefore, consideration of the GM plant itself and its crop management as potential environmental stressors is essential. This is particularly the case where realistic levels and times of exposure are required to test responses of different developmental stages of NTOs, which are relevant for assessing the safety and appropriate management of the new GM germplasm in relation to the biotic components of agro-ecosystems (e.g. non-target pests, natural enemies, pollinators etc.).

All the major regulatory authorities of the world (including USA FDA, Health Canada, FSANZ and OECD as well as the EU; see references in Perry (2015) concerned with risk assessment of GM plants require that unintended effects of the genetic modification process are assessed in the initial risk assessment of the GM 'event' in a single GM line prior to breeding into different genetic backgrounds (commercial varieties). The issue of evaluating unintended effects is not specific to GMPs, but is a component of the evaluation of new varieties produced by most conventional plant breeding systems. In the registration and approval of non-GM crop varieties in EU and OECD countries there are National List and Common Catalogue requirements for information on distinctness, uniformity and stability (DUS) as well as field performance characteristics which include biotic interactions as well as agronomic and quality traits. For GM crops there is a regulatory requirement for much of this information to be obtained in advance of variety registration, as part of the risk assessment process (EFSA, 2012).

In most cases this does not imply a significant increase in data requirements for applicants over previous requirements. The result is that applicants, as well as consumers, should gain more confidence that the ERA will be considered robust and appropriate, not least for future farming systems designed to improve sustainability and reduce pesticide inputs.

In addition, the benefits of this approach may also go beyond ERA, because *in planta* testing under ecologically realistic conditions at multiple scales will give insight into the GM crop's ability to withstand biotic and abiotic stressors, including multiple trophic levels, genotype × environment variability and insights into sustainable crop management/IPM options. Consequently this will provide more information to risk managers and decision makers and will help to assess the sustainability of these technologies for the future.

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