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## Public consultation on the draft Scientific Opinion on *in vivo* and *in vitro* random mutagenesis techniques in plants

European Food Safety Authority (EFSA)

### Abstract

The European Food Safety Authority (EFSA) carried out a public consultation to receive input from interested parties on *in vitro* random mutagenesis techniques. This draft scientific opinion was prepared by the GMO Panel, supported by the Working Group on Molecular Characterization. The draft opinion was endorsed by the EFSA GMO Panel for public consultation on the 5th May 2021. The written public consultation was open from 19 May 2021 until 30 June 2021. EFSA received comments from 16 different interested parties. EFSA and its GMO Panel wish to thank all stakeholders for their contributions to this work. The present report contains the comments received and details how they have been considered for finalisation of the opinion. The final opinion was adopted at the GMO Panel Plenary meeting on the 29th September 2021 and will be published in the EFSA Journal. © European Food Safety Authority, 2020

**Key words:** Random mutagenesis, *in vivo*, *in vitro*, chemical mutagenesis, physical mutagenesis, mutagen, mutation

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## 1. Introduction

### 1.1. Background and Terms of Reference as provided by the requestor

#### 1.1.1. Background

The judgment of the Court of Justice of the European Union (CJEU) in Case C-528/16<sup>1</sup> on mutagenesis held that Article 3(1) of Directive 2001/18 on the deliberate release of Genetically Modified Organisms (OGM)<sup>2</sup> must be interpreted as meaning that “only GMOs obtained by means of techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record” are excluded from the scope of that directive. The CJEU in its reasoning referred to the “application of conventional methods of random mutagenesis” without distinguishing further between *in vivo* and *in vitro* random mutagenesis and distinguished them from “new techniques/methods of mutagenesis which have appeared or have been mostly developed since Directive 2001/18 was adopted”.<sup>3</sup>

Following the ruling of the CJEU, the Conseil d’Etat of France issued on 7 February 2020 a judgment on organisms obtained by mutagenesis. In its judgment, the Conseil d’Etat describes conventional or random mutagenesis as a technique triggering random mutations in a DNA sequence through the action of chemical or physical mutagens. The French Conseil d’Etat distinguishes between *in vivo* and *in vitro* random mutagenesis techniques. *In vivo* random mutagenesis would consist in the application of chemical or physical mutagens to whole plants or parts of plants, which would then be subject to selection procedures in order to identify the interesting mutations. *In vitro* random mutagenesis would consist in subjecting plant cells to chemical or physical mutagenic agents. The modified cells would then be subject to techniques of *in vitro* cell culture in order to regenerate the whole plant.

EFSA, in its Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function<sup>4</sup>, examines conventional plant breeding techniques relevant for a comparison with Site Directed Nuclease-3 technique. Among these conventional techniques, EFSA describes mutation breeding by chemical and physical mutagenesis. While EFSA explains the various modes of action depending on the chemical mutagens or the type of radiation used, the Authority makes no distinction between the application of the techniques *in vitro* or *in vivo*.

Member States have never made a distinction between *in vitro* and *in vivo* either when implementing the seed legislation, the plant propagating material legislation or the GMO legislation.

It is therefore important to provide a robust scientific understanding of random mutagenesis techniques and a robust scientific analysis as to whether the distinction between *in vitro* and *in vivo* is scientifically justified.

#### 1.1.2. Terms of Reference

Against this background, the Commission asks EFSA, in accordance with Art 29 of Regulation (EC) No 178/2002:

- A. To provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*.
- B. To assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*.
- C. To assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*.

<sup>1</sup> Case C-528/16, *Confédération paysanne and Others*, Judgment of 25 July 2018, EU:C:2018:583.

<sup>2</sup> Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 106, 17.4.2001, p. 1), Article 4.

<sup>3</sup> Case C-528/16, *Confédération paysanne and Others*, Judgment of 25 July 2018, EU:C:2018:583, points 48 et 51.

<sup>4</sup> EFSA Panel on Genetically modified organisms (GMO); Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. EFSA Journal 2012;10(10):2943. [31 pp.] doi:10.2903/j.efsa.2012.2943. Available online: [www.efsa.europa.eu/efsajournal](http://www.efsa.europa.eu/efsajournal)

- D. To assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis techniques or on the contrary, if they are to be considered as a *continuum*.

## 1.2. Rationale for the public consultation and brief summary of the outcome

In line with EFSA's policy on openness and transparency, and in order for EFSA to receive comments on its work from the scientific community and stakeholders, EFSA engages in public consultations on key issues. Accordingly, the draft opinion was released for public consultation from 19 May 2021 until 30 June 2021 by means of an electronic comment submission tool together with explanatory text on the EFSA website (See Appendix A). Comments were received from 16 interested parties from 8 countries. Table 1 provides an overview on the interested parties that have submitted comments through the electronic submission. Two contributions from the OGM Danger and Groupe International d'Etudes Transdisciplinaires (GIET) from France were submitted as PDF by email within the deadline.

**Table 1:** Overview on stakeholder comments received

Stakeholder	Category <sup>(a)</sup>	Country
Alliance for Agriculture and Cooperation	Non-Governmental Organisation (NGO)	Romania
Anonymous	Industry, Small or Medium-Sized Enterprise (SME)	France
Anonymous	Other	France
Anonymous	Academia/Research Institute	Denmark
CropLife Europe	Industry, Multinational	Belgium
Euroseeds	Other	Belgium
Federal Office of Consumer Protection and Food Safety (BVL)	Public Authority in EU Member State	Germany
Groupe International d'Etudes Transdisciplinaires (GIET)	Non-Governmental Organisation (NGO)	France
Haut Conseil des Biotechnologies - Scientific Committee	Other	France
International Seed Federation (ISF)	International Organisation	Switzerland
Not Applicable (Submission on Personal Capacity)	-	Belgium
OGM dangers	Non-Governmental Organisation (NGO)	France
Plants for the Future ETP	International Organisation	Belgium
Plantum	Other	Netherlands
The Plant Variety Development Office	Other	Ireland
Wissenschaftlerkreis Grüne Gentechnik e.V. (WGG), Frankfurt	Non-Governmental Organisation (NGO)	Germany

<sup>(a)</sup> as specified by the commenter

## **2. Assessment of comments and use for finalisation of the opinion**

The comments received were duly evaluated by the EFSA GMO Panel WG on Molecular Characterization. Wherever appropriate these comments were taken into account for finalisation of the draft opinion.

Table 2 provides a detailed list with all comments received from interested parties together with EFSA responses and explanations how the comments were considered for finalisation of the draft opinion. Some comments, especially those suggesting editorial changes, have been directly addressed in the text of the opinion, if they were considered appropriate.

**Table 2:** Stakeholder comments and EFSA responses

Stakeholder	Section	Comment	Number	EFSA response
CropLife Europe	Abstract	General comments: CropLife Europe welcomes the EFSA's GMO Panel scientific opinion on <i>in vivo</i> and <i>in vitro</i> random mutagenesis techniques in plants, the comprehensive review of the underlying molecular processes and the range of resulting genetic modifications. The GMO Panel's conclusions that the molecular mechanisms underlying different mutagenesis approaches (spontaneous or induced) are the same and that mutagens act at the cellular level irrespective of whether the cell is part of a cultivated tissue <i>in vitro</i> or is any part of a plant <i>in vivo</i> are supported by indisputable scientific evidence and can support risk analysis and policy discussions. We agree with the final conclusion that the distinction between plants obtained by <i>in vivo</i> or <i>in vitro</i> approaches is not justified and that the same mutation can potentially be obtained by different methods. While we support the GMO Panel's review and conclusions, we note that there are areas in the text that can benefit from further clarification. We highlight these in our detailed comments below. Finally, while EFSA may have been constrained by the Terms of Reference (ToRs) to specifically address 'random' mutagenesis, the text would gain in clarity if a better context is provided for the distinction between 'random' in relation to mutagenesis in general.	1	The GMO Panel thanks for the comment. The GMO Panel developed the scientific opinion by adhering to the ToRs provided by the EC. Comparing random mutagenesis to mutagenesis in general was out of the scope of the mandate.
Euroseeds	Abstract	Euroseeds welcomes the opportunity to comment on this scientific opinion on <i>in vivo</i> and <i>in vitro</i> random mutagenesis techniques in plants.	2	The GMO Panel thanks for the comment.
International Seed Federation (ISF)	Abstract	The International Seed Federation (ISF) is a non-governmental, non-profit organization. ISF represents more than 7500 seed companies in 75 countries active in breeding, seed production and trading and is widely regarded as the voice of the global seed sector. ISF thanks for the opportunity of commenting on the draft EFSA report. Mutation breeding has a very important role in creating new genetic variation which is the source material for new plant characteristics. Mutation breeding is applied globally and has contributed to bringing to the market thousands of plant varieties with improved, agronomic, and nutritional characteristics and resistances to various biotic and abiotic stresses.	3	The GMO Panel thanks for the comment.
Not Applicable (Submission on Personal Capacity)	Abstract	line 18: change into : largely independent from the tissue line 19: change into: difference between application of the mutagen <i>in vivo</i> or <i>in vitro</i>	4	Text regarding both comments has been amended accordingly
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	Abstract	The European Commission's closed and biased questions lead to the expected response from EFSA, which does not correspond to either the letter or the spirit of the legal opinion of the French Conseil d'État. Asking to consider whether the techniques applied are the same <i>in vivo</i> and <i>in vitro</i> is like asking whether sunlight changes depending on whether it will illuminate Petri dishes or plants in the field. The restrictive definitions of so-called "genetic" mutations that can be transmitted to offspring do not consider the scientific results acquired over the last 50 years. As a result, this Abstract does not answer the questions raised by the Conseil d'État's opinion and is of no interest other than to use a reusable language that allows the European Commission to avoid asking the proper questions that make people angry. This abstract is thus not an abstract of a scientific document but a political one. As reminded by EFSA, the Commission defines :	5	The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved.

		<p>' In vivo random mutagenesis would consist in the application of chemical or physical mutagens to whole plants or parts of plants, which would then be subject to selection procedures in order to identify the interesting mutations. In vitro random mutagenesis would consist in subjecting plant cells to chemical or physical mutagenic agents. The modified cells would then be subject to techniques of in vitro cell culture in order to regenerate the whole plant.'</p> <p>As a strict consequence, at least one question, unraised in the whole EFSA's text, is whether regenerating a whole plant does add mutations. The reply is well-known for decades (somaclonal variation even used by some breeders) and is positive. But EFSA does not discuss this without the faintest justification.</p>		
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	2. Data and Methodologies	By staying within the circular questions of the European Commission, it is clear that EFSA, is unlikely to bring forward any new scientific evidence that could provide information on the biological effects of the application of random mutagenesis techniques <i>in vivo</i> and <i>in vitro</i> . Using only reviews and book chapters without checking methodological items and their inherent limits mainly contribute to this soothing effect of unfunded assertions and political orientation.	6	The scientific opinion was developed by adhering to the ToRs as provided by the European Commission.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	3.1. Problem formulation	<p>No grey literature is reported. No set of external experts was sought or consulted, no reference documents to test the completeness of the study were identified, and their presence verified.</p> <p>Moreover:</p> <ul style="list-style-type: none"> <li>• Biological random mutagenesis is absent (Anderson et al., 2016; Combiér et al., 2003; Filipecki and Malepszy, 2006; Wilson et al., 2006).</li> <li>• <i>In vitro</i> culture is by itself an uncontrolled random mutagenesis (inducing <i>somaclonal variation</i>) technique (and not very usable in varietal selection before the description of Tilling's technique and the development of several other selection tools) that should have been integrated since the conditions for its use in micropropagation aim to reduce induced random mutations and epimutations (Bednarek and Orłowska, 2020; Bobadilla Landey, 2013; McCallum et al., 2000; Neelakandan and Wang, 2012; Rout et al., 2006). There is a logical flaw here that is difficult to understand if not due to a preeminent political decision.</li> </ul>	7	The GMO panel considered that the grey literature would not add value, as EFSA only relies on peer reviewed publications. Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, Agrobacterium mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved.

OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	3.1 Extent of planning	EFSA cannot refer to a tight schedule: questions on the definition of GMOs existed since 2007 (COGEM). Moreover, questions to the CJEU are from 2017, while the opinion of the Conseil d'État is dated 2020. Limited time is thus clearly a false excuse to submit sloppy work while supporting a preexisting political opinion. Nothing prevented EFSA, which does not lack resources compared to national bodies, from calling on external experts, as in the case of SRLs, or an <i>ex-ante</i> public consultation on this report. To rely solely on the pseudo-consensus of reviews and book chapters hardly bodes well for the critical quality of the data collected and reported.	8	EFSA worked within the timeline as agreed with the European Commission.
CropLife Europe	3.3.1. Literature search	CropLife Europe agrees that mutagenesis has an extensive history and that a restriction to reviews and book chapters is therefore justified. We recommend taking into account the recent publication by Stacy D. Singer et. al (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826.	9	The suggested citation has been added in section 4.3.1.1.
Euroseeds	3.3.1. Literature search	Euroseeds agrees that random mutagenesis has an extensive history and that a restriction to reviews and book chapters is therefore justified. Euroseeds recommends that the very extensive and most recent review by Stacy D. Singer, John D. Laurie, Andriy Bilichak, Santosh Kumar & Jaswinder Singh (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826 which includes a very comprehensive overview and references to original literature should be taken into account.	10	The GMO Panel thanks for the comment. The suggested citation has been added in section 4.3.1.1.
Plants for the Future ETP	3.3.1. Literature search	There is an issue with the reference in lines 234 and 235	11	The text has been amended.
The Plant Variety Development Office	3.3.1. Literature search	We support the basis of the Literature search. The PVDO recommends the recent comprehensive review by Stacy D. Singer, John D. Laurie, Andriy Bilichak, Santosh Kumar & Jaswinder Singh (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826 should be considered.	12	The GMO Panel thanks for the comment. The suggested citation has been added in section 4.3.1.1.
Plantum	3.3.1. Literature search	The literature concerning random mutagenesis is vast. Given the time constraint we support the decision of EFSA to focus on reviews and book chapters.	13	The GMO Panel thanks for the comment.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	3.3.1. Literature search	Despite the quasi "flavour" of a SRL, the work carried out is not up to scratch because it does not call upon grey literature, committees of experts independent of the fields concerned, and does not test the relevance of the questions and the completeness of the type of bibliographic databases queried, nor of the responses from the databases queried, through reference documents. So, the style of this report is thus highly confusing for uninformed, lay people. EndNote, Zotero and BibTeX files should have been provided for proper transparency of the study for the public consultation. All 517 references should have been provided at least in the form of a listing to verify the completeness of the responses during the public consultation. Biological random mutagenesis ( <i>Agrobacterium in vitro</i> and <i>in vivo</i> , agroinfiltration, floral dip, virus...) and somaclonal variation due to <i>in vitro</i> cultures of isolated cells or tissues should have been included in the mutagenesis techniques studied. Indeed, physical and chemical mutagenic agents only increase the frequency, modify some types and locations of mutations and epimutations.	14	The GMO panel considered that the grey literature would not add value, as EFSA only relies on peer reviewed publications. Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, <i>Agrobacterium</i>



		Overall, because of the poor scoping (National Research Council, 2009; Pham et al., 2014; Speirs et al., 2015), the <i>a priori</i> choices and the questions of the European Commission, an important ethical question arises from this study <sup>5</sup> and the report. It would probably be appropriate to ask for their opinion the European Ombudsman and the ethics bodies independent from the European Commission's on such practices of the European Commission and EFSA.		mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	3.3.2. Screening of the literature search results	The screening conditions are not explicitly described as they should be in such a document mimicking systematic literature reviews. The criteria for rejecting articles are not specified, nor should the list of rejected articles with the reasoning have been provided in complete transparency. Although this work does not claim to be a real RSL and only two evaluators <sup>6</sup> were involved, a detailed description - even a statistical analysis comparing the evaluators - should have been reported.	15	A systematic literature search was performed in this mandate. The GMO Panel considers that the information reported in the section sufficiently describe the selection procedure performed on the outcome of the literature search.
CropLife Europe	4.1. Introduction	Line 263: Edit for clarity. 'Genomes are subjected to mutations' implies that this is not an intrinsic feature of life. Suggestion to change with 'Genomes undergo' mutations. Line 265-266: Edit for clarity. 'Naturally occurring spontaneous mutations' Please consider using consistently the expression 'spontaneous mutations' only; delete 'naturally occurring' here and elsewhere in the text. Line 267: the evolution which "can" result in new individuals. Line 276: add 'DNA changes or' before 'modifications' Line 276: Revise - Is the following statement factual' "called 'mutations' when the affected cells are able to pass them on to the daughter cell'. Are mutations only 'mutations' when inherited'	16	Regarding comment to line 263, 265-266, 267, the text has been amended accordingly. Regarding comment to line 276, the sentence has been removed.
Not Applicable (Submission on Personal Capacity)	4.1. Introduction	line 267: change to kingdoms	17	The text has been amended accordingly.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.1. Introduction	Spontaneous as well as induced mutations do not occur "essentially" at random. There are chromosomal regions that are more resistant or, on the contrary, more 'vulnerable' (recombination hotspots, for example), which vary according to the proximity of specific structures (centromeres, telomere, mutation hotspot...). In contrast, tissues such as meristematic cells, cell lines and cells at the origin of germ cells are, on the contrary, preserved from spontaneous and probably induced mutations (Halldorsson et al., 2019). Moreover, differences are noted between nuclei and organelles with mutation hotspots and exchanges between organelles, whereas horizontal transfers seem to occur preferentially in	18	As the text clearly explain and in line with the references cited in this comment, although the GC composition, the presence of repetitive sequences and TEs, the heterochromatic nature of a region, or its transcriptional status can influence the mutation rate (Weng et al., 2019), mutations essentially happen at random in the genome . However,

<sup>5</sup> <https://www.covidence.org/blog/the-difference-between-a-systematic-review-and-a-literature-review/>

<sup>6</sup> As more than two raters could have easily taken part in the study inclusion assessment, a Randolph's Kappa coefficient would have been appreciated.

		<p>hotspots, e.g. bacterial in the absence of such research on eukaryotes (Gao et al., 2020; Oliveira et al., 2017).</p> <p>It is deplorable that the European Commission and EFSA have decided to ignore 50 years of scientific results on the organisation and regulation of genomes and epigenomes and stick to the molecular biology of nucleotide sequences of the 1970s. This is when one remembers that the nuclear genome (human in particular, as it is obviously the most studied) has gone through various forms of interpretation: with very many genes, then with junk DNA, and finally with the current interpretation of extensive parts involved in the regulation of "genes", an entity that is still so poorly defined that it continues to evolve (Allen et al., 2017; Galli et al., 2020). Another example is that radiation-induced DSB repair systems influence the 3D structure of genomes, gene regulation, with responses varying between cell types (Sanders et al., 2020).</p>		<p>mutations can result in a wide range of phenotypic effects. Mutations which have a strong negative effect in the phenotype are not retained and are removed by purifying selection, therefore they will not be maintained in subsequent generations.</p>
CropLife Europe	4.1.1. Spontaneous and induced mutations in the context of plant breeding	<p>Line 279-280: Edit for clarity. The sentence ".causes of mutations, which could be divided into spontaneous or induced events" is confusing as it is not clear if it refers to the causes of mutations or the events (outcomes) that are classified as spontaneous or induced. Suggestion to modify the sentence to 'Mutations are often categorized as spontaneous or induced depending on whether they occurred with or without human intervention.' Line 283: Suggestion to modify the sentence or split into two to clarify that polymerases and ROS are not part of the same mechanisms. Line 285: it is not the presence but the movement of these elements that could be a cause of mutations. Line 291: 'natural' genetic variability to be changed with 'spontaneous' genetic variability Line 292: 'favourable' agronomic characteristics to be changed with 'desirable' Line 294-296: Edit for clarity. The sentence " variability induced by spontaneous mutations is limited, and it may be difficult in most cases to obtain a desired phenotype by screening natural populations" could be misleading. The variability induced by spontaneous mutations is not limited, but the frequency of these mutations is very low, and would require the screening of unrealistic number of individuals to identify desired changes. Please consider the following edit: Replace " variability induced by spontaneous mutations is limited, and" with " frequency of spontaneous mutations is very low, and" Line 305: Suggestion to add a new paragraph describing the use of site directed nucleases as tools for induced mutagenesis. Such information can be obtained from EFSA's own scientific opinion on SDN1/2 and ODM.</p>	19	<p>Regarding comment to line 279-280, the sentence has been removed.</p> <p>Regarding comment to line 283, the text has been improved.</p> <p>Regarding comment to line 285, the text has been improved.</p> <p>Regarding comment to line 291, the GMO Panel considers that the text is correct since 'natural genetic variability' is a well know concept.</p> <p>Regarding comment to line 292, the text has been amended accordingly.</p> <p>Regarding comment to line 294-296, the sentence has been rephrased to improve its clarity.</p> <p>Regarding comment to line 305, a new sentence has been added to indicate that some techniques for targeted mutagenesis in plants have been addressed in the EFSA recent opinion on SDN-1, SDN-2 and ODM (EFSA GMO Panel, 2020).</p>
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.1.1. Spontaneous and induced mutations in the context of plant breeding	<p>It should be noted that EFSA does not define (even in its glossary) essential wordings such as spontaneous, neutral, natural and finally induced mutations. What should scientific, technical or legal limits be introduced? Is it possible to distinguish what is unclearly defined (natural mutation versus non-natural mutations)? However, according to some results, only 5% of the genome undergoes' neutral evolution, although it is unknown what neutral or natural mutation evolution precisely means (Harris, 2018; Pouyet et al., 2018). This lack of clear definitions allows the European Commission and EFSA to include what it wishes when it wishes, i.e. to perniciously manipulate the discourses.</p>	20	<p>The scientific opinion explains the differences between induced and spontaneous mutation in section 4.1.1 with several considerations about their frequency. Physical and chemical mutagenesis aim at increasing the frequency of mutations. Epimutations</p>

<sup>7</sup> <https://phys.org/news/2018-10-faulty-yardstick-genomics-cope.html#jCp> and <https://elifesciences.org/articles/41491>

	<p>How should we qualify, for example, all the mutations and epimutations linked to the subjection of growing plants in fields or greenhouses to thermal and/or hydric stress conditions? Are the growing conditions considered to induce artifactual or spontaneous mutations when plants grow in artificial and controlled (greenhouses or air-conditioned rooms) or natural (e.g. Northern European plants transplanted in North Africa for experiments) environments?</p> <p>It is helpful to remind the readers that the domestication syndrome drives the search for genetic diversity due to human selection, which has led to significant losses of genetic and epigenetic diversity (Flint-Garcia, 2013; Shi and Lai, 2015; Van Tassel et al., 2020). Moreover, that many other varietal selection schemes are available.</p> <p>Not to point out the difficulty of estimating spontaneous and induced mutation rates (because until now, averages have been taken from tissue sets with different replication, age, function status...). Sequencing, sequences' assembly, annotation, and comparison techniques are error-prone with inadequate quality assessment and incomplete software. Sequences databases are full with errors (Steinegger and Salzberg, 2020). These mutations estimations vary between species, evolution and diversification. It shows a lack of methodological rigour and critical thinking in the reviews and chapters collected and reviewed by EFSA (Dulieu, 2005; Hua and Bromham, 2017; Katju and Bergthorsson, 2019; Kondrashov and Kondrashov, 2010). This is also – due to the EFSA restrictive definition of "genetic mutation" - without counting all the epimutations – <i>sensu lato</i> - of the exon skipping, exon shuffling, intron retention, or moonlighting protein type... (Ariel and Crespi, 2017; Clark et al., 2019; de Souza et al., 2013; Halldorsson et al., 2019; Huberts and van der Klei, 2010; Jeffery, 2014; Kiegle et al., 2018; Pievani and Serrelli, 2011; Sharpe and Cooper, 2017; Singh and Bhalla, 2020).</p> <p>Among the genome, there are hot spots (natural mutations more likely) and also safe harbors (much less natural mutations). Even if scientists do not know the reasons for this non-uniformity, one must acknowledge it. Irradiation makes uniform mutations (mainly on outer parts of the chromosomes). So the statistics of the two is necessarily different, even if the reason for the difference is unknown.</p> <p>EFSA's text states</p> <p>"In other words, mutation breeding consists of increasing the genetic variability of plant species of agronomic interest by inducing mutations at a higher frequency compared to spontaneous processes."</p> <p>So there are induced/artificial mutations and spontaneous mutations. They are not at the same frequency. But then, the only scientific question is to know whether the difference is one, two, three or five orders of magnitude. Only such a dimensionnalizing could help reply to the question. Since irradiation uses between six and seven orders of magnitude more than the strongest natural irradiation and that the breeders claim they need at least that, it is awkward to claim the two vary continuously.</p> <p>Finally, in the absence of systematic sequencing of isolated cells (single-cell sequencing) and of any dynamic vision of the evolution of the genomes and epigenomes, we can only argue that average approaches group together both spontaneous and induced mutations and epimutations of unrelated cell types.</p> <p>Therefore, the results presented should be considered with extreme caution as to the interpretations drawn from them.</p>	<p>were not included in the ToRs, therefore they were not addressed.</p>
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Euroseeds	4.1.2. Historical view on random mutagenesis in mutation breeding	Euroseeds would like to point out that the number of varieties provided voluntarily to the IAEA database is not reflecting the total number of varieties resulting from direct or indirect (via crossing and selection) mutagenesis. Even though -as EFSA states- most of the mutagenesis work in the EU was done by Germany and Sweden (line 338), the actual number of varieties from those countries as listed in the database is less than 200 (out of over 3000 database entries in total). Also, for France as the largest seed producer in the EU only 39 entries (latest from 1990) are listed and e.g. none of the herbicide tolerant oilseed rape varieties mentioned in the French Draft Decree (2020/280/F `Decree amending the list of techniques for obtaining genetically modified organisms traditionally used without any noted drawbacks with regard to public health or the environment`; 2020/281/F `Order laying down the list of varieties mentioned in Article 2 of Decree [xx]`; 2020/282/F `Order amending the Official Catalogue of Species and Varieties of Cultivated Crops in France (rape seeds and other crucifer seeds) are among them. This is also recognized in a publication in Frontiers (Bartsch D, Ehlers U, Hartung F, Kahrman J, Leggewie G, Sprink T and Wilhelm R (2020) Questions Regarding the Implementation of EU Mutagenesis Ruling in France. Front. Plant Sci. 11:584485. doi: 10.3389/fpls.2020.584485) `Conventional mutagenesis is applied mostly as physical mutagenesis by the help of irradiation. Seventy percent of the mutant varieties at the FAO/IAEA database were obtained via irradiation, the first one (tobacco, Chlorina F1) as early as 1928. Meanwhile more than 3,300 varieties are registered in this database. As these are voluntary registrations, even more mutagenized varieties and crossings thereof might be traded at present.'	21	The GMO Panel takes note of the comment.
The Plant Variety Development Office	4.1.2. Historical view on random mutagenesis in mutation breeding	The PVDO would like to note that the number of varieties provided voluntarily to the IAEA database is not reflecting the total number of varieties resulting from direct or indirect (via crossing and selection) mutagenesis. Even though -as EFSA states- most of the mutagenesis work in the EU was done by Germany and Sweden (line 338), the actual number of varieties from those countries as listed in the database is less than 200 (out of over 3000 database entries in total). Also, for France as the largest seed producer in the EU only 39 entries (latest from 1990) are listed and e.g. none of the herbicide tolerant oilseed rape varieties mentioned in the French Draft Decree are among them. This is also recognized in a publication in Frontiers ( <a href="https://www.frontiersin.org/articles/10.3389/fpls.2020.584485/full?utm_source=F-NTF&amp;utm_medium=EMLX&amp;utm_campaign=PRD_FEOPS_20170000_ARTICLE">https://www.frontiersin.org/articles/10.3389/fpls.2020.584485/full?utm_source=F-NTF&amp;utm_medium=EMLX&amp;utm_campaign=PRD_FEOPS_20170000_ARTICLE</a> ) `Conventional mutagenesis is applied mostly as physical mutagenesis by the help of irradiation. Seventy percent of the mutant varieties at the FAO/IAEA database were obtained via irradiation, the first one (tobacco, Chlorina F1) as early as 1928. Meanwhile more than 3,300 varieties are registered in this database. As these are voluntary registrations, even more mutagenized varieties and crossings thereof might be traded at present.'	22	The GMO Panel takes note of the comment.
Plantum	4.1.2. Historical view on random mutagenesis in mutation breeding	As IAEA is a voluntary database, the actual number of varieties listed in the database as resulting from random mutagenesis might differ from reality. It should also be noted that mutagenesis is not only used directly for creating new varieties, it is also used as a tool during the research-stage to uncover e.g. gene function. This information can be used during selection and crossing in a later stage of development.	23	The GMO Panel takes note of the comment.
Anonymous	4.1.2. Historical view on random	(line 338-341) and (line 392-396) Numerous scientific publications report the application of <i>in vitro</i> mutagenesis as early as the late 1970s with the development of <i>in vitro</i> culture methods themselves (see references 1 in the document attached). They show that, not only, the <i>in vitro</i>	24	The GMO Panel thanks for the comment.

	mutagenesis in mutation breeding	random mutagenesis was developed and used before the adoption of Directive 2001/18/EC, but that it was also a plant breeding technique that was already well known before that date and therefore taken into account by the legislator. In over 50 years, applications of <i>in vitro</i> random mutagenesis have led to the development of many varieties. The first varieties in rapeseed obtained using this technique for their tolerance to herbicides were marketed in Canada as early as 1995. However, the process for obtaining these mutations was described as early as 1988 (see references 2 in the document attached). The Mutant Variety Database, a joint FAO/IAEA programme, lists (as of 20 June 2020) 3332 varieties worldwide obtained by random mutagenesis. It includes applications to species as diverse as wheat, sweet potato, rice, maize, pea, potato, tomato, plum, cherry, etc. The characteristics obtained in these plants concern protein content, nutritional quality, disease resistance, cold tolerance, yield, etc... As this inventory of the FAO/IAEA database is voluntary, it is not exhaustive. It is therefore certain that other applications have been developed by international public and private research.		
Not Applicable (Submission on Personal Capacity)	4.1.2. Historical view on random mutagenesis in mutation breeding	line 364, change into: For example, at the beginning of the 1950s line 367: change into: and became widely used to generate mutations line 371: remove the from the Aegilops	25	The text has been amended accordingly.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.1.2. Historical view on random mutagenesis in mutation breeding	<p>EFSA's historical shortcut is biased. The mutants obtained by physical mutagens and recorded by the IAEA were obtained at the beginning of the first quarter of the 20th Century but only by <i>in vivo</i> mutagenesis. <i>In vitro</i> techniques did not develop until long after the laboratory developments of the 1970s, with very few practical spin-offs, particularly in the food sector, until the 2000s and the publication of Tilling's method, the use of marker-assisted selection and the setting up of heavy and expensive mutant screening platforms (McCallum et al., 2000).</p> <p>The conflation of laboratory research with practical applications that may provide a proven safety record is misleading to political and lay people.</p> <p>That purpose is very well summarised by (Thorpe, 2012):</p> <p><i>"During the 1990s, continued expansion in the application of in vitro technologies to an increasing number of plant species was observed. Tissue culture techniques are being used with all types of plants, including cereals and grasses (154), legumes (155), vegetable crops (156), potato (157), other root and tuber crops (158), oilseeds (159), temperate (160) and tropical (161) fruits, plantation crops (162), forest trees (163), and, of course, ornamentals (164). As can be seen from these articles, the application of in vitro cell technology went well beyond micropropagation, and embraced all the in vitro approaches that were relevant or possible for the particular species, and the problem(s) being addressed. However, only limited success has been achieved in exploiting somaclonal variation (165) or in the regeneration of useful plantlets from mutant cells (166); also, the early promise of protoplast technology has remained largely unfulfilled (167)."</i></p> <p>as well as by (Vasil, 1999):</p> <p><i>"The isolation (Cocking 1960) and fusion (Power et al. 1970) of plant protoplasts, and regeneration of plants from them (Takebe et al. 1971), generated much optimism for crop</i></p>	26	The chapter on historical view is meant to provide a general view on the history on mutation breeding. Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, Agrobacterium mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The opinion addressed the ToRs which asked whether there are differences between genetic mutations obtained by the application of random mutagenesis <i>in vivo</i> and <i>in vitro</i> in plants.

	<p><i>improvement by the production of somatic hybrids. In spite of much effort, however, no commercially useful novel hybrids of any major crop species have been obtained by protoplast fusion.</i></p> <p>The lack of simple screens for the majority of mutated traits of interest (apart from a few herbicide or toxin resistances or characterised by an easily identifiable phenotype such as a loss or deficiency of chloroplasts) and the instability of genomes after <i>in vitro</i> cultures (EFSA requires 5 years of genome stability studies after any mutational set) (Comai and Tan, 2019; Fossi et al., 2019; Henry et al., 2018; M Lee and Phillips, 1988) explain this gap of almost 70 years between the use of some mutants obtained <i>in vivo</i> and those recently obtained through <i>in vitro</i> techniques.</p> <p>Paragraph 4.1.2 should therefore be revised entirely by incorporating the accurate and precise history of mutants used in food (the first cultivars obtained <i>in vivo</i> concerned ornamental plants with more accessible selection criteria), differentiating between developments in the laboratory, such as the discovery of mutagenic agents <i>in vitro</i>, and their effects, which mainly concern the frequencies of the practical developments necessary to produce mutants used in agricultural production for food. The amalgams practiced do not reflect historical reality and are therefore misleading.</p> <p>Surprisingly, EFSA forgot to incorporate random biological mutagenesis into its study. The same applies to <i>in vitro</i> cultures of isolated cells or tissues. The mutations and epimutations induced in the latter case are minimised as much as possible by changing the culture conditions for micropropagation. Physical and chemical mutagens (<i>in vivo</i> and <i>in vitro</i>) only increase the frequency and type of mutations available.</p> <p>In the 3 cases of cell cultures (with and without added mutagenesis agents), only a few cases were selected before 2000.</p> <p>While it is indeed helpful to recall the questions posed by MacKey and Konzak in the 1950s, it would be beneficial to recall other questions. Thus, certain elements suggest that the mechanisms for inducing DNA damage and repair may differ between <i>in vivo</i> and <i>in vitro</i> (Brash and Hart, 1978; Krishna et al., 1987). As for the phenomena of <i>ex vivo</i> mutations (<i>in vivo</i> mutations that can be fixed <i>in vitro</i>) that can be distinguished, questions remain as to the mechanisms involved (and which may differ between <i>in vivo</i> and <i>in vitro</i>) (Hedde et al., 2000).</p> <p>More prosaically, it is fundamental to recall the enormous technical difficulties in trying to differentiate the mechanisms involved at the tissue and cellular levels <i>in vivo</i> and <i>in vitro</i> both concerning genome and epigenome damage and their repair/resilience and to cell death such as apoptosis that may be induced by adjacent cells <i>in vivo</i> (Azqueta et al., 2014; Bajpayee et al., 2019; Figueroa-González and Pérez-Plasencia, 2017; Ganapathy et al., 2015; Hoeijmakers et al., 1990; Lehmann, 2011; Meyn et al., 1986; Surova and Zhivotovsky, 2013).</p> <p>Inducing hypotheses of similarity and continuity between <i>in vivo</i> and <i>in vitro</i> when the techniques do not allow situations to be distinguished at the cellular level dynamically is highly misleading. State of the art, as much as the modes favouring research orientations and funding, are the basis of knowledge gaps. It is dishonest to provide results without recontextualising them, particularly by indicating that the methods leave something to be desired in terms of the possible interpretations of the results. These truths observed in the</p>		
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		human and animal domains, particularly for studies in cancer, therapy and genotoxicity, are even more prevalent for plants. In conclusion, giving a survey result without providing uncertainties is of the same order of misleading assertion as the work presented in this study without critical evaluation of the results and methods. This misinformation in the report is mainly related to how EFSA works, based on reviews and book chapters, thus avoiding any critical approach to the published results and protocols used.		
CropLife Europe	Summary	Line 36-37: Edit for clarity. The mutagenesis by itself does not accelerate the selection process, suggest to replace 'natural process to accelerate the selection of varieties with important agronomic traits' with 'spontaneous processes to increase the genetic variability in breeding programs'.	27	Text has been amended to improve clarity.
Plants for the Future ETP	Summary	Plants for the Future ETP (Plant ETP) welcomes this very detailed and exhaustive overview of random mutagenesis techniques, including the myriad of mutagens that can be used, as well as the mutations they induce. Plant ETP supports the findings and conclusions of the EFSA draft scientific opinion on <i>in vitro</i> random mutagenesis techniques. More specifically, Plant ETP supports that mutations derived from random mutagenesis are the same in nature, whether they occur as a result of <i>in vivo</i> or <i>in vitro</i> application, and that they therefore should not be considered as separate techniques, but rather a continuum. One point that could be emphasised further, is the fact that whether a mutagen is applied <i>in vivo</i> or <i>in vitro</i> is most often determined by the specific plant species, rather than the type of mutagen used or the resulting mutation(s). For many sexually propagated plant species, seeds are the preferred plant material for mutagen application (i.e. <i>in vivo</i> ), while treatment of plant tissue or cells and regeneration of entire plants (i.e. <i>in vitro</i> ) is most common for vegetatively propagated plants species.	28	The GMO Panel thanks for the comment. The GMO Panel considers that the aspects related to the mutagen and the type of plant material used are sufficiently described in section 4.2.
Anonymous	Summary	In its response to the European Commission regarding the difference between mutagenesis performed on plants <i>in vivo</i> or <i>in vitro</i> , the EFSA Panel on Genetically Modified Organisms provides a thorough and comprehensive account of the history and scope of mutagenesis techniques in plant breeding during the last century. Furthermore, a comprehensive review is provided on the range of mutagens used in mutation breeding and on the mechanisms by which the mutagens exert their effects in the plants. This forms the basis for the conclusion by the panel that there is no scientific basis for a distinction between <i>in vivo</i> and <i>in vitro</i> mutagenesis, since the final mutations are similar and the mechanisms involved, based on the available information, are the same. We support this conclusion.	29	The GMO Panel thanks for the comment.
Not Applicable (Submission on Personal Capacity)	Summary	line 60: remove "to" after "applied"	30	The text has been amended accordingly
Wissenschaftlerkreis Grüne Gentechnik e.V. (WGG), Frankfurt	Summary	The EFSA report "In vivo and in vitro random mutagenesis techniques in plants" gives a very comprehensive and complete overview of random mutagenesis methods. It does not only describe in detail the methods used, but also discusses very critically their effects on the changes /modifications at the DNA level. In most cases, it is intensively investigated whether different or the same mechanisms lead to changes on the DNA level (mutations) when using <i>in vivo</i> and/or <i>in vitro</i> methods for their generation. Based on the available data and publications, EFSA concludes that there are no different mechanisms leading to mutations when using <i>in vitro</i> and <i>in vivo</i> methods and that a distinction between <i>in vivo</i> and <i>in vitro</i> mutagenesis is	31	The GMO Panel thanks for the comment.

		not justified. This statement is scientifically correct and the Wissenschaftskreis Grüne Gentechnik e.V. supports it.		
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	Summary	<p>This Summary does not bring anything new compared to the previous Abstract and is understandable by the Abstract's comments.</p> <p>The recalled protocol suggests to the layman that an exhaustive study has been carried out, worthy of the level of a "Systematic Review of the Literature" (SRL), whereas, as we shall see below, this is not the case<sup>8</sup>. It is becoming increasingly common to present biased reviews mimicking SLRs in order to indirectly assert, via a false 'state of the art', an argument of authority used to push through political options (Allen and Baker, 2017; Baker, 2016; Ioannidis, 2016; West and Bergstrom, 2021).</p> <p>The fact that EFSA has confined itself only to reviews and book chapters means that EFSA and the Commission wish to avoid finding disturbing results in the literature and not go into the issues in any depth, thus creating a 'smoothing out' of the mainstream, even though the issues do not address the letter nor the spirit of the legal opinion of the Conseil d'État.</p> <p>Assuming that random mutagenesis in plants does not evolve rapidly is a distortion of the Conseil d'État's opinion issues. Consequently, it is a significant denial of the accumulated knowledge of the biological effects of mutagenesis, of whatever origin, over the last 50 years. Furthermore, nothing is said about the kinds of mutations, their frequencies, their locations (e.g. vs centromere or telomere), and more generally about reactions to stress and their inheritability, such as over 13 generations the cells' hypomethylation issued from cell cultures (Berdasco et al., 2008; Bertheau, 2021; Quadrona and Colot, 2016). Finally, by limiting the study to applied techniques and not induced responses, the fundamental <i>in vivo</i> and <i>in vitro</i> difference in stress response introduced by intercellular and intertissular communication is not addressed but discarded as disturbing a political opinion (Belting and Wittrup, 2008; Gilroy et al., 2018; Lee and Frank, 2018; Lim et al., 2016; Peters et al., 2021; Thieme et al., 2015). Compared to the Abstract, however, the remainder of the European Commission's closed questions (ToRs) underlines, in particular ToR4, the Commission's biased view of the biological issues and implications raised by the legal opinion of the Conseil d'État in a way that suits it. The European Commission's closed and biased questions are a typical example of circular questioning in which the questions provide (contain) the answers.</p> <p>This is not the Summary of a scientific paper but a political-economic oriented paper with some brief historical background, typical of the <sup>9</sup>increased politicization of science and the oriented "scientization" of politics (Barker and Peters, 1993; Bolsen and Druckman, 2015; Demortain, 2004, 2017; Everson and Vos, 2009; Marris, 2004).</p>	32	The Terms of Reference 2 and 3 provided by the EC asked EFSA to assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied <i>in vivo</i> or <i>in vitro</i> , and to assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied <i>in vivo</i> or <i>in vitro</i> , respectively. Therefore, potential differences between the application <i>in vivo</i> and <i>in vitro</i> of random mutagenesis have been extensively assessed in the opinion regarding the type and the mechanisms leading to genetic mutations.
CropLife Europe	4.2.1.1. Mutation breeding: summary of the main steps	<p>Line 401: Consider deleting the word 'random'. It is not clear why physical and chemical mutagenesis techniques are also described as 'random'. Line 404: Add "and selection" after 'is the testing' Lines 404-405: Suggestion to delete 'and the release for commercialization' as it does not seem appropriate to be included for the three-step process of mutation breeding. Line 418: Replace 'interesting' with 'desired' Line 419: It is more appropriate to use 'may' instead of 'will'</p>	33	Regarding comment to line 401, 404, 404-405, 418, the text has been amended accordingly.
Euroseeds	4.2.1.1. Mutation	Euroseeds would like to point out that the description of step 3 ('step 3 is the testing for the desired characteristics and the release for commercialization') would benefit from adding that	34	The text has been amended to improve clarity.

<sup>8</sup> <https://www.milbank.org/quarterly/articles/mass-production-redundant-misleading-conflicted-systematic-reviews-meta-analyses/>

<sup>9</sup> <https://www.nature.com/articles/d41586-020-03067-w> and <https://blogs.scientificamerican.com/guest-blog/science-has-always-been-inseparable-from-politics/>



	breeding: summary of the main steps	after the application of mutagenesis it requires certain rounds of backcrossing to discard unwanted mutations and to achieve homogeneity for the desired mutation/characteristic. Only then, the plant material will be subjected to further field testing and variety development as well as official testing. The currently used text in the draft EFSA opinion provides the impression that plants resulting from mutagenesis treatment can directly be released to the market without further breeding and official testing.		
The Plant Variety Development Office	4.2.1.1. Mutation breeding: summary of the main steps	It is important to highlight that the explanation of step 3 ('step 3 is the testing for the desired characteristics and the release for commercialization') would benefit from adding that after the application of mutagenesis it requires some rounds of backcrossing to discard unwanted mutations and to achieve homogeneity for the desired mutation/characteristic. Only then, the plant material will be subjected to further field testing and variety development as well as official testing. The description in the draft report gives an understanding that plants resulting from mutagenesis are market ready without further breeding and official testing.	35	The GMO Panel thanks for the comment. The text of the opinion referring to 'Step 3' has been amended to address this comment.
Plantum	4.2.1.1. Mutation breeding: summary of the main steps	Plantum feels that the description of steps taken in the application of random mutagenesis techniques is incomplete. The last step ('step 3 is the testing for the desired characteristics and the release for commercialization') seems to suggest that application of random mutagenesis is immediately followed by commercialization. There are, however, many steps between these two, such as backcrossing to eliminate unwanted mutations and testing for homogeneity. This aspect might be included in the EFSA opinion to provide a more realistic sense of the process.	36	The text has been amended to improve clarity.
Anonymous	4.2.1.1. Mutation breeding: summary of the main steps	(line 400-404) UFS would like to point out that the description of step 3 ('step 3 is the testing for the desired characteristics and the release for commercialization') would benefit from adding that after the application of mutagenesis it requires certain rounds of backcrossing to discard unwanted mutations and to achieve homogeneity for the desired mutation/characteristic. Only then, the plant material will be subjected to further field testing and variety development as well as official testing. The currently used text in the draft EFSA opinion provides the impression that plants resulting from mutagenesis treatment can directly be released to the market without further breeding and official testing.	37	The GMO Panel thanks for the comment. The text of the opinion referring to 'Step 3' has been amended to address this comment.
Not Applicable (Submission on Personal Capacity)	4.2.1.1. Mutation breeding: summary of the main steps	line 433: change into to screen large populations in a relatively line 442: remove the slash at the beginning of the line	38	The text has been amended accordingly.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.1.1. Mutation breeding: summary of the main steps	<i>In vitro</i> cultures that induce mutations (somaclonal variation) at rates higher than natural, spontaneous mutations resulting from, for example, cell replication should be included (Bhatia et al., 2004; Bhatia and Dahiya, 2015; Maliga, 1984; Tapingkae et al., 2012). The same applies to random biological mutagenesis. The three steps succinctly recalled by EFSA are highly misleading. They omit the very many difficulties encountered in EFSA's Newspeak of "mutation breeding", a new neologism introduced to lose the layman like the subsequent renaming of NBTs or of NGTs, a classic rhetorical technique.	39	Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, Agrobacterium mediated transformation) is taken into account in the Commission Implementing

				Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved.
CropLife Europe	4.2.1.2. General considerations on <i>in vivo</i> and <i>in vitro</i> random mutagenesis techniques in plants	Line 422: We made a general comment that random mutagenesis can be put in the context of mutagenesis in general. We believe that in many parts of the review, EFSA has addressed precisely mutagenesis in general, and we recommend that this is clearly acknowledged in the text for the benefit of the readers. We believe that it would be also helpful to the reader that the GMO Panel explains what is the basis of calling some mutagenesis techniques 'random' and to further explain when the expression 'random mutagenesis techniques' was first introduced and why.	40	The text of the opinion has been revised to address this comment.
Anonymous	4.2.1.2. General considerations on <i>in vivo</i> and <i>in vitro</i> random mutagenesis techniques in plants	Mutagenesis is the process whereby mutations, or modifications, are generated in the DNA sequences that make up the genetic material of a cell or individual. It may be spontaneous, caused by natural agents (exogenous or endogenous), or induced, caused by mutagens (chemical, physical or biotechnological), which are used in plant breeding to increase genetic variability in plants in order to select for new traits and, subsequently, varieties of agronomic interest. On the physical mutagens, X and gamma rays, fast neutrons and ions are the most widely used. On the chemical mutagens, the three main chemical mutagens are ethyl methanesulfonate (EMS), methyl nitrosourea (MNU) and sodium azide (SA). Use of T-DNA insertion and transposon systems has also been reported. Lastly, it should be noted that the CRISPR/Cas system, known for its ability to induce targeted mutations, can also cause random mutations in localized regions when a special protocol is used, allowing allelic variability to be created for a given gene. Physical and chemical random mutagenesis techniques can be applied: - <i>In vivo</i> - To plant cells <i>in vitro</i> - To other plant materials <i>in vitro</i> Random mutagenesis can also result from the applications of <i>in vitro</i> culture, without agents intended to induce mutations.	41	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.2.1.2. General considerations on <i>in vivo</i> and <i>in vitro</i> random mutagenesis techniques in plants	Mutagenesis is the process whereby mutations, or modifications, are generated in the DNA sequences that make up the genetic material of a cell or individual. It may be spontaneous, caused by natural agents (exogenous or endogenous), or induced, caused by mutagens (chemical, physical or biotechnological), which are used in plant breeding to increase genetic variability in plants in order to select for new traits and, subsequently, varieties of agronomic interest. On the physical mutagens, X and gamma rays, fast neutrons and ions are the most widely used. On the chemical mutagens, the three main chemical mutagens are ethyl methanesulfonate (EMS), methyl nitrosourea (MNU) and sodium azide (SA). Use of T-DNA insertion and transposon systems has also been reported. Lastly, it should be noted that the CRISPR/Cas system, known for its ability to induce targeted mutations, can also cause random	42	The GMO Panel takes note of the comment.

		mutations in localized regions when a special protocol is used, allowing allelic variability to be created for a given gene. Physical and chemical random mutagenesis techniques can be applied: - In vivo - To plant cells in vitro - To other plant materials in vitro Random mutagenesis can also result from the applications of in vitro culture, without agents intended to induce mutations.		
Not Applicable (Submission on Personal Capacity)	4.2.1.2. General considerations on in vivo and in vitro random mutagenesis techniques in plants	line 464: pollen is always plural, pollens does not exist line 468: change into However, the use of a DH system	43	Regarding comment to line 464, the text has been amended accordingly. Regarding comment to line 468, the text has been revised.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.1.2. General considerations on in vivo and in vitro random mutagenesis techniques in plants	<p>The fact that random mutagenesis techniques can be applied to many living forms organised in tissues or organisms or in isolation cannot predict the ability to revert to plants with stable and transmissible genomes (Aguilera and García-Muse, 2013; Ariel et al., 2015; Arnholdt-Schmitt, 2004; Benson, 2000; Ckurshumova and Berleth, 2015; De Saeger et al., 2020; Ramon et al., 2014; Walters et al., 2013). The circular reasoning induced by the European Commission's questions in no way takes into account the letter or the spirit of the French Conseil d'Etat's opinion on the biological consequences of mutagenesis techniques. Consequences which are not addressed at any point in the EFSA report, an agency nevertheless primarily concerned with the risk assessment and management of the techniques applied.</p> <p>EFSA could have recalled that <i>in vivo</i>, there are diffusion gradients for both treatments and nutrients, and it is one of the causes of instability, chimerism, etc. Thus, there are obvious differentiation mutagenesis conditions between <i>in vivo</i> and <i>in vitro</i>. These differences are also found in intercellular and intertissular signalling through plasmodesmata and conducting vessels such as the phloem via signal traffic with effects that are generally difficult to understand in our state of knowledge (do Amaral and Souza, 2017; Shinozaki et al., 2018); the genetic code of plants is not always representative of proteins derived from mRNAs (Vélez-Bermúdez and Schmidt, 2014). Overall, the molecular data (from the three compartments: nuclei with their chromosomes and episomes, mitochondria and chloroplasts) are all in favour of a break-in behaviour between <i>in vivo</i> and <i>in vitro</i> environments due to changes in contact pressures, electricity, information flows/cell and tissue continuities, via plasmodesmata and between tissues through proteins, DNA, RNA of various types, hormones flowing between tissues and regulating gene expressions<sup>10</sup>, chromosomal rearrangements, mutations (indels...) and epimutations (<i>sensu lato</i>), mobilisation of transposable elements... and at the organismal level and intercellular relationships, plasmodesmata with RNAs/ RNAs and cell-autonomous proteins (Bloemendal and Kück, 2013; Lee, 2015; Lee and Frank, 2018; Lim et al., 2016; Lucas et al., 2009).</p> <p>Given the importance of these signals in animals (e.g. between the embryo and pregnant mother) and of imprinting phenomena, it can be easily inferred that the importance in plants</p>	44	The section of the scientific opinion focuses on the techniques of random mutagenesis which are applied <i>in vivo</i> and <i>in vitro</i> in plants. The section does not focus on the differences in the cellular "behaviour" or "reactions" when the plant cell is either <i>in vitro</i> or <i>in vivo</i> . The GMO Panel was asked to assess whether i) the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied in vivo or in vitro and ii) to assess whether the molecular mechanism underlying formation of new mutations induced by random mutagenesis techniques is different if the techniques are applied in vivo or in vitro. As described in the scientific opinion, the GMO Panel considers that the same mechanisms and the same genetic mutations can be found both <i>in vivo</i> and <i>in vitro</i> when applying random mutagenesis techniques. The literature cited in this comment does not provide additional evidence that would change the conclusions of the opinion.

<sup>10</sup> Moreover, the definition of gene is still changing: no consensus (3D... far from the conception of the 70s)

		<p>of these signals differing between <i>in vivo</i> and <i>in vitro</i> is also essential. Therefore, reactions of plant genomes and epigenomes to these signals differ <i>in vivo</i> and <i>in vitro</i>.</p> <p>Finally, it should be remembered that microbiota (absent in classic <i>in vitro</i> cultures) plays a role in <i>in vivo</i> in regulating various genes in both animals and plants, and it is understandable that the reactions of organisms to mutations and epimutations differ between <i>in vivo</i> and <i>in vitro</i>.</p> <p>The evidence of an <i>in vivo</i> - <i>in vitro</i> discontinuity seems to prevail over any possible <i>in vivo</i> - <i>in vitro</i> continuity (of techniques and particular effects) assumed by EFSA and the European Commission.</p> <p>There are currently no published data to suggest that mutations are truly random. However, there do appear to be genomic islands of speciation (i.e. with particular mutation rates) with hybridisation barriers (Campbell et al., 2018) while mutation hotspots seem to be obliterated by 'hidden' genes, at least in animals, which are better studied than plants (Hargreaves et al., 2017; Hron et al., 2015; Lovell et al., 2014; Marx, 2016).</p> <p>More generally, it should be remembered that GWAS studies<sup>11</sup> have not given as good qualitative results as hoped<sup>12</sup> and, more generally, that genotype-phenotype correlations are still tricky and inaccurate, as demonstrated, for example, by the 1,001 Genomes Consortium project when compared to the initial assertions about single "model organisms" (Egea-Cortines and Doonan, 2018; Kawakatsu, 2016; Koch, 2016; Ledford, 2016; Lobos et al., 2017; Sanders et al., 2020; The 1001 Genomes Consortium, 2016; Yaish, 2017).</p> <p>Examples include (Rasheed et al., 2017):</p> <p><i>Our understanding of epigenetic variation and its phenotypic effects are very limited in crop plants. For example, it was demonstrated that identical isogenic populations in Brassica napus had distinct agronomic characteristics for energy use efficiency despite their identical DNA sequences (Hauben et al., 2009).</i></p> <p>Trying to make people believe that knowing more or less a sequence of a few nucleotides is enough to determine the risks induced by any mutagenesis (random or directed, desired or unexpected) is a misleading message to politicians and fellow citizens. Only time and observation can give a small idea of the risks involved. A perspective that militates in favour of specific and general GMOs' surveillance (2001/18 Directive) and the constant application of the precautionary principle and the associated research.</p> <p>Environmental stresses, such as <i>in vitro</i> cell cultures, can even lead to hypermutations or even chromothripsis, which have not always been studied in plants - or only very late - compared to studies on humans and animals (Roberts and Gordenin, 2014). However, signatures of DNA damage and repair by different mutagens and other stresses exist (Bertheau, 2021; Doitsidou et al., 2016; Flibotte et al., 2010; Lehrbach et al., 2017; Smith and Yun, 2017; Volkova et al., 2020; Zheng et al., 2017).</p> <p>The effect of stress on isolated cells or multicellular clusters (i.e. <i>in vitro</i> vs natural environments), on their subsequent development into an organism and on genetic reprogramming, was recognised as early as 1999 by Barker and is currently well documented in animals (Ventura-Juncá et al., 2015). <i>In vitro</i> culture methods with injections are similar, if only by the sizes of the molecules to be inserted involved, to the injections of the NBT nucleic</p>		
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<sup>11</sup> Genome Wide Association Studies

<sup>12</sup> [https://www.lemonde.fr/journalectronique/donnees/libre/20180912/index.html?article\\_id=1327067](https://www.lemonde.fr/journalectronique/donnees/libre/20180912/index.html?article_id=1327067)

		<p>acid, protein and/or ribonucleoprotein complex 'packages'. They induce profound disorders in the genomes and epigenomes, transcriptome perturbations, etc. of the targeted cells and organisms, all of which are transmissible, as in the "fat calf syndrome" (Ventura-Juncá et al., 2015). Finally, the simple analysis of cells for <i>in vitro</i> fertilisation and pre-implantation diagnosis already induce mutations and epimutations unknown in natural fertilisation (Cyranoski, 2017; Kucab et al., 2019; Peters et al., 2015; Song et al., 2015).</p> <p>Even EFSA states "Somaclonal variation can be coupled with random mutagenesis techniques when applied <i>in vitro</i> to further increase the mutation frequency (Suprasanna et al., 2014)". If it "further" increases, the question is whether it is of the same order of magnitude or not. There is no discussion of the magnitude. At least it is not the same.</p> <p>Further, EFSA states</p> <p>"The application of random mutagenesis techniques to plant material <i>in vitro</i> offers some advantages compared to the application <i>in vivo</i>, such as the uniformity of the treatment and the possibility to apply a selective agent more easily, to screen large population in relatively small space and to handle disease-free plant material (Suprasanna et al., 2012)."</p> <p>Again the two are acknowledged to be different (they give different products), but "a continuum". No scientific discussion of the magnitudes is provided.</p>		
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.1.3. Physical mutagenesis techniques applied in mutation breeding	<p>This paragraph succinctly but clearly recalls that there are differences in the effects of physical mutagens <i>in vivo</i> <i>in vitro</i>, if only in the choice of cells, stages of mutagenesis, differential penetration rates, mutation signatures.... This confirms what has been published elsewhere (Bertheau, 2021).</p> <p>Still missing is a paragraph on random biological mutagens and the effect of cell cultures <i>per se</i>.</p>	45	<p>Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, <i>Agrobacterium</i> mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved.</p>
CropLife Europe	4.2.1.4. Chemical mutagenesis techniques	<p>Line 537-538: Please consider adding explanation on what are the most common DNA changes triggered by the application of different chemical classes.</p>	46	<p>The explanation of the most common DNA changes triggered by different mutagens is already addressed in the</p>

	applied in mutation breeding			opinion (section 4.4 and related sub-chapters).
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.1.4. Chemical mutagenesis techniques applied in mutation breeding	<p>This paragraph again succinctly reminds us that there are differences in the effects of chemical mutagens between <i>in vivo</i> and <i>in vitro</i>, if only in the choice of cells, stages of mutagenesis, differential penetration rate, mutation signatures.... This confirms what has been published elsewhere (Bertheau, 2021).</p> <p>Still missing is a paragraph on random biological mutagens and the effect of cell cultures <i>per se</i>. Let us remind that regeneration of a whole plant requires cell culture and so somaclonal variation applied to isolated cells that would die out in nature. This is one more difference.</p> <p>This subsection states "The plant material should preferably be in the active growing stage." but it does not stress that no reason for this is known. This incidentally proves that this science makes things that it is not able to explain. And after it claims the risks are under control?</p> <p>This subsection states also "the possibility to apply a selective agent more easily, to screen large population in relatively small space and to handle disease-free plant material (Suprasanna et al., 2012)." This proves that tissues are not the same as isolated cells. At least for physical reasons of contact between the mutagen agent and the target cells protected by the other cells constituting the tissue.</p>	47	<p>Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, Agrobacterium mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. Genetic transformation is not one of the techniques used for <i>in vivo</i> or <i>in vitro</i> mutagenesis for developing commercial varieties and it is therefore not included in the scope of this mandate. It should be noted that the possible mutations introduced following plant transformation with established plant transformation techniques (for example, Agrobacterium mediated transformation) is taken into account in the Commission Implementing Regulation (EU) No 503/2013 and all EFSA guidances for the risk assessment of genetically modified organisms. The scientific opinion discusses the increase of spontaneous mutations associated with the culture and regeneration of plants in <i>in vitro</i> conditions, which is known as somaclonal variation, in sections 4.1.1 and 4.2.1.2. Although somaclonal variation was already presented in the text, section 4.1.1 has been improved.</p>

				The statement referring to the 'active growing stage' which is the preferred plant material in mutagenesis does not imply any safety considerations, but rather reports data which is found in protocols routinely used in mutagenesis. The statement referring to 'the possibility to apply a selective agent more easily' using <i>in vitro</i> settings refers to some advantages in the technical aspects when applying protocols <i>in vitro</i> compared to protocols applied <i>in vivo</i> .
CropLife Europe	4.2.1.5. Examples of <i>in vivo</i> and <i>in vitro</i> random mutagenesis applications in plants	Line 580-581: suggestion to delete the following part as the statement is speculative: 'but also because the treatment is simpler compared to chemical mutagenesis' Line 583: suggestion to replace 'subjected' with 'developed within' Line 660: suggestion to delete 'random'	48	Regarding comment to line 580-581, the text has been amended accordingly. Regarding comment to line 583, the sentence has been rephrased to improve clarity. Regarding comment to line 660, the GMO Panel considers that the word 'random' is necessary to distinguish between 'in vitro random mutagenesis' and 'in vitro targeted mutagenesis'.
Anonymous	4.2.1.5. Examples of <i>in vivo</i> and <i>in vitro</i> random mutagenesis applications in plants	- Genes for dwarfism - Genes involved in fatty acid composition of oilseeds - Genes involved in growth traits of ornamental plants - Genes involved in growth traits of fruit trees - Imidazolinone resistance genes See the file attached for development of these examples.	49	The GMO Panel is aware of these examples. The examples reported in the scientific opinion were retrieved from the results of the literature search.
Haut Conseil des Biotechnologies - Scientific Committee	4.2.1.5. Examples of <i>in vivo</i> and <i>in vitro</i> random mutagenesis applications in plants	- Genes for dwarfism This trait had a substantial economic impact for wheat and rice. Dwarf oilseed rape varieties produced through EMS seed mutagenesis have been registered with the Official French Catalogue of Species and Varieties of Cultivated Crops since 1999. Since this growth trait cannot be selected <i>in vitro</i> , <i>in vitro</i> mutagenesis was not specifically considered. - Genes involved in fatty acid composition of oilseeds To meet demands from nutritionists, oilseed breeders have had to modify the fatty acid composition of some oils. Low erucic acid varieties of oilseed rape have thus been created from a spontaneous erucic acid-free mutant, while the varieties grown in France up to 1972 were producing an oil containing roughly 50% of this fatty acid (a trait controlled by two genes). However, because of the lack of diversity in the species, in order to produce oilseed rape oils better suited to frying, two cycles of seed mutagenesis were used in succession to obtain first a low level of linolenic acid and then a high oleic acid content (over 75% as opposed to 60%). Mutant screening was performed by analysing the fatty acid composition of several thousand M2 offspring. As in the case of dwarfism, since this seed growth trait cannot be selected <i>in vitro</i> , <i>in vitro</i> mutagenesis was not specifically considered, apart from the work of Albrecht et al. on erucic acid (1995) and Möllers	50	The GMO Panel takes note of the comment.



		et al. on oleic acid (2000), who have studied the fatty acid composition of one of the two cotyledons of an embryo taken <i>in vitro</i> , prior to regeneration of the plant. Also : - Genes involved in growth traits of ornamental plants - Genes involved in growth traits of fruit trees - Imidazolinone resistance genes To read more details about these 3 last examples, please see the document attached.		
Anonymous	4.2.1.5. Examples of <i>in vivo</i> and <i>in vitro</i> random mutagenesis applications in plants	The panel states in section 4.2.1.5 that most of historic mutants have been generated by physical muta-genesis. That is probably right when looking several decades back. However, with the advance of TILLING populations in recent years, we wonder whether that still holds, since most TILLING populations are now made with chemical mutagens in order to obtain a high mutation density in the populations and, hence, to facilitate an efficient screening for specific mutations.	51	The text of the opinion has been amended to improve clarity.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.1.5. Examples of <i>in vivo</i> and <i>in vitro</i> random mutagenesis applications in plants	<p>This paragraph is again misleading by deliberately mixing <i>in vivo</i> and <i>in vitro</i>, ornamental and food mutants and by not differentiating the history of laboratory developments from actual large-scale food applications.</p> <p>Otherwise, to point out that a series of mutations can also be created by adding physical and chemical mutagens is a truism on the level of adding a chemical mutagen to <i>in vitro</i> cultures undergoing somaclonal variation.</p> <p>The accumulation of examples of mutants obtained following the action of mutagenic agents has never constituted a decisive argument proving continuity of biological reactions between <i>in vivo</i> and <i>in vitro</i>. This kind of argument of authority is misleading lay people.</p> <p>Otherwise, the techniques and products used (ENU, sodium azide, gamma rays, etc.) remain the same depending on whether they are applied <i>in vivo</i> or <i>in vitro</i>, a fallacy for which it was not necessary to fill many pages of circular reasoning.</p> <p>The fundamental question is elsewhere, as the authors of this report are well aware. We note here that the oldest articles cited are from 2003. Clearly, this research applied on isolated cells <i>in vitro</i> is recent. The reason is simple: whole plant regeneration is a technique that is not yet mastered on all crop plants. We talk about "recalcitrant" plants. It should be noted that in the same species, some varieties are recalcitrant and not others. It happens that the elite varieties are often recalcitrant and not the wild varieties. This explains why industrialists modify wild varieties, and then have to make crosses with elite varieties. But this fact explains that many agronomic traits were indeed present in Herbicide Tolerant Varieties (HTV), but other traits had been lost. The paper (Darmency "Pleiotropic effects of herbicide-resistance genes on crop yield: a review", 2013 <a href="https://doi.org/10.1002/ps.3522">https://doi.org/10.1002/ps.3522</a>) shows that "Pleiotropic effects on yield are reported in half of the case" and "Breeders' efforts to produce better varieties could compensate for the yield loss, which eliminates any possibility of formulating generic conclusions on pleiotropic effects that can be applied to all resistant crops."</p> <p>Finally, the conclusion that this has already been used is therefore true but confusing since it cannot be said that these "genetic modification techniques [...] have been traditionally used for various applications and have a long history of proven safety." (c. 17 of Directive 2001/18, which is indeed wise).</p>	52	The section of the scientific opinion providing examples of the application of mutagenesis techniques <i>in vivo</i> and <i>in vitro</i> reports several cases where the same technique has been used both <i>in vitro</i> and <i>in vivo</i> to the same or different plant species. The section does not aim to differentiate between food and non-food applications since this is irrelevant considering the ToRs of the mandate (in particular for ToR2 and ToR3). Moreover, the mandate does not request to provide considerations on the safety aspects of these techniques and the obtained products.
CropLife Europe	4.2.2. Are all these techniques	Line 682: suggestion to delete 'random'	53	The GMO Panel considers that the word 'random' is necessary to distinguish



	applicable both in vivo and in vitro?			between ' <i>in vitro</i> random mutagenesis' and ' <i>in vitro</i> targeted mutagenesis'.
Anonymous	4.2.2. Are all these techniques applicable both in vivo and in vitro?	Mutagenesis techniques can be applied in different contexts and to different materials. They were originally used in vivo, but with the development of in vitro methods, the inherent benefits of changing to the latter have been exploited in many processes, including mutagenesis. The following sections will shed light on the specific conditions in which it is worth applying in vitro mutagenesis techniques to certain plant materials of certain species. Here, mutagenesis techniques can be applied to a wide range of in vitro plant materials varying in differentiation and structure, covering not only single cells but also whole plants, as well as calli, tissues and organs. See the file attached for development about: - In vitro culture : Diversity of methods and applications - Added value of in vitro mutagenesis - Phenotypes induced by in vivo vs in vitro mutagenesis	54	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.2.2. Are all these techniques applicable both in vivo and in vitro?	Mutagenesis techniques can be applied in different contexts and to different materials. They were originally used in vivo, but with the development of in vitro methods, the inherent benefits of changing to the latter have been exploited in many processes, including mutagenesis. There are specific conditions in which it is worth applying in vitro mutagenesis techniques to certain plant materials of certain species. Here, mutagenesis techniques can be applied to a wide range of in vitro plant materials varying in differentiation and structure, covering not only single cells but also whole plants, as well as calli, tissues and organs. For more details about in "vitro culture : diversity of methods and applications" ; "added value of in vitro mutagenesis", "phenotypes induced by in vivo vs in vitro mutagenesis", please read the document attached.	55	The GMO Panel takes note of the comment.
Not Applicable (Submission on Personal Capacity)	4.2.2. Are all these techniques applicable both in vivo and in vitro?	line 682: illustrates	56	Text has been amended accordingly.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.2.2. Are all these techniques applicable both in vivo and in vitro?	This is yet another circular reasoning whose answer was included in the Commission's question, whereas the spirit of the Conseil d'État's opinion is clearly elsewhere: are there differences in biological reactions, a continuity, between in vivo and in vitro? Indeed, the reagents remain the same whether they are applied in vivo or in vitro. And there is no known alchemical transmutation despite the egreore of some... Yet, the reactions and consequences are not necessarily the same because of the different environments, interactions and induced regulations reflected at the levels of genomes and epigenomes. Assuming the environment is irrelevant in these discussions amounts to assuming an organism can be reduced to a set of cells.	57	Please refer to the responses provided to the comments above.
CropLife Europe	4.3.1. What are the underlying molecular mechanisms which generate the mutations?	Line 692-699: Consider editing the introductory text of this section to provide the context for what follows next. Suggested text: 'All living organisms, including plants, have to deal with natural mutagens that may cause changes in their genomes by triggering DNA damage or alteration. When an alteration is detected by the cells repair machinery, the eukaryotic cells slow down or stop cell cycle at an available checkpoint to repair the damaged DNA. Repairing DNA damage or alterations is an important process to preserve the stability and transmission of genetic information to the next generations. This efficiency of the repair process varies, and	58	Regarding comment for lines 692-699, the text has been amended accordingly.

		can result in the full repair to the original DNA sequence (conservative repair), or be more error prone and (non-conservative) leading to repair with integration of DNA sequence changes, i. e. mutations. In this paragraph we will first review the mechanisms by which the mutagens generate lesions (breaks) in the DNA and then the.'		
Anonymous	4.3.1. What are the underlying molecular mechanisms which generate the mutations?	Prior to any molecular descriptions of induced genetic variability, it should be emphasised that mutation is taken to mean any transmissible change in a genomic DNA sequence, whether or not it results in a change of phenotype. A mutation is the outcome of introduction of a difference between a parent sequence and a daughter sequence. Once introduced, mutations are subject to selection. If a mutation persists throughout a population, the differences observed are called polymorphisms. The frequency and number of differences between individuals across the whole genome reflect the genetic variability in a given species. Mutations are not errors, as long as they are consubstantial with living beings. Mutations can occur at any time in the life of a cell, through the processes explained below. Mutations appearing in germ cells (which give rise to gametes) are transmitted to the progeny. Mutations occurring in non-germ cells are known as somatic mutations. In some cases, germinal mutations have been shown to be less frequent than somatic mutations; specific mechanisms for control of virus and transposon expression have been selected in germ cells in the course of evolution (Parrilla-Doblas et al., 2019). In plants, cellular totipotency means that somatic mutations may be transmitted to plants regenerated from somatic tissue, which will in turn be able to transmit them to their progeny.	59	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.3.1. What are the underlying molecular mechanisms which generate the mutations?	Prior to any molecular descriptions of induced genetic variability, it should be emphasised that mutation is taken to mean any transmissible change in a genomic DNA sequence, whether or not it results in a change of phenotype. A mutation is the outcome of introduction of a difference between a parent sequence and a daughter sequence. Once introduced, mutations are subject to selection. If a mutation persists throughout a population, the differences observed are called polymorphisms. The frequency and number of differences between individuals across the whole genome reflect the genetic variability in a given species. Mutations are not errors, as long as they are consubstantial with living beings. Mutations can occur at any time in the life of a cell. Mutations appearing in germ cells (which give rise to gametes) are transmitted to the progeny. Mutations occurring in non-germ cells are known as somatic mutations. In some cases, germinal mutations have been shown to be less frequent than somatic mutations; specific mechanisms for control of virus and transposon expression have been selected in germ cells in the course of evolution. In plants, cellular totipotency means that somatic mutations may be transmitted to plants regenerated from somatic tissue, which will in turn be able to transmit them to their progeny.	60	The GMO Panel takes note of the comment.
Not Applicable (Submission on Personal Capacity)	4.3.1. What are the underlying molecular mechanisms which generate the mutations?	694: stop the cell cycle 696: I would add here: However it is also important that the DNA repair is finished rapidly, even when imperfectly, in order to retain cell functionality. 699: end point is missing	61	The text of Section 4.3.1 has been improved.
CropLife Europe	4.3.1.1. Mechanisms	General: To provide better understanding to readers about mutations, a new paragraph, or section is recommended that explains the chemical and physical structure of DNA and where "changes" in the DNA can be triggered. Line 717: Delete 'caused' as they do not cause	62	The GMO Panel considers the text to be sufficiently clear. The proposed section will not be introduced to avoid

	leading to DNA damage	"phenotypes" but DNA changes. Line 744: Add 'because of protocols that have been developed' at the end of the sentence		increasing the complexity of the document. Regarding comment to line 717, the text has been improved. Regarding comment to line 744, the GMO Panel considers the text to be sufficiently clear.
Anonymous	4.3.1.1. Mechanisms leading to DNA damage	A mutation may be initiated by different mechanisms: (1) by modification of a base, of the bond between a base and its sugar or of the bond between two nucleotides (base + sugar), (2) by insertion of a sequence or (3) by rearrangement of a sequence within or between chromosomes. These modifications vary in type depending on the mutagens responsible. So-called exogenous agents come from the environment. Ionising radiation (X rays, gamma rays, etc.) tends to introduce breaks between nucleotides on one or two strands. Ultraviolet rays introduce thymine dimers. Some viruses, when integrated in cell genomes, introduce new sequences (Takahashi et al., 2019). So-called endogenous agents are generated by the cell's biological activity. The main source of mutations consists in nucleotide insertion errors during replication, which result in mismatches. Although DNA copy enzymes have high specificity, a noncomplementary nucleotide may be inserted. These changes are responsible for single nucleotide variations, which are point mutations that give rise to single nucleotide polymorphisms (SNPs). Furthermore, recombination events frequently occur during meiosis and more rarely during mitosis. They may result in deletions, insertions, translocations, inversions, etc. Lastly, genomes contain several families of repeated elements found in high copy numbers, and many of them are mobile (transposons, retrotransposons). If the latter move as a result of various factors, whether biotic or abiotic, this may lead to mutations through insertion.	63	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.3.1.1. Mechanisms leading to DNA damage	A mutation may be initiated by different mechanisms: (1) by modification of a base, of the bond between a base and its sugar or of the bond between two nucleotides (base + sugar), (2) by insertion of a sequence or (3) by rearrangement of a sequence within or between chromosomes. These modifications vary in type depending on the mutagens responsible. So-called exogenous agents come from the environment. Ionising radiation (X rays, gamma rays, etc.) tends to introduce breaks between nucleotides on one or two strands. Ultraviolet rays introduce thymine dimers. Some viruses, when integrated in cell genomes, introduce new sequences (Takahashi et al., 2019). So-called endogenous agents are generated by the cell's biological activity. The main source of mutations consists in nucleotide insertion errors during replication, which result in mismatches. Although DNA copy enzymes have high specificity, a noncomplementary nucleotide may be inserted. These changes are responsible for single nucleotide variations, which are point mutations that give rise to single nucleotide polymorphisms (SNPs). Furthermore, recombination events frequently occur during meiosis and more rarely during mitosis. They may result in deletions, insertions, translocations, inversions, etc. Lastly, genomes contain several families of repeated elements found in high copy numbers, and many of them are mobile (transposons, retrotransposons). If the latter move as a result of various factors, whether biotic or abiotic, this may lead to mutations through insertion.	64	The GMO Panel takes note of the comment.

OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.3.1.1. Mechanisms leading to DNA damage	<p>As already mentioned above, this type of question cannot be validly answered without reliable and precise techniques allowing dynamic studies at the cellular and tissular level of mutagenesis application reactions.</p> <p>Are repair mechanisms dependent on the whole organism or only on single cells? Clearly the latter is wrong and the former is right as we can see with apoptosis, P53 and son on. Even though scientists (who do not know everything) do not know the mechanism, one must acknowledge they exists since a whole organism induces very very few mutations inside its parts (see above).</p> <p>The answers reported by EFSA are generalities on the consensus of the general mechanisms of DNA damage, erroneously considered a simple concatenation of nucleotides, thus a tiny part of the elements transmitted to the descendants, a scientific aberration given the current knowledge in molecular biology. Generalities do not contribute anything because they group the responses of hundreds to tens of thousands of cells of various types (somatic, meristematic...), replicative states, ages...</p> <p>Tools, such as the sequencing of genomes, epigenome and epitranscriptome of single-cells or instantaneous atomic microscopy pictures, are being developed, but funding will still have to be found for issues on <i>in vivo</i> and <i>in vitro</i> differences, wich are of little interest to research funding agencies.</p> <p>The fundamental question raised incidentally by the Conseil d'État as to whether cells react differently <i>in vivo</i> and <i>in vitro</i> thus remains unanswered, although there is some evidence to suggest that the mechanisms of DNA damage induction and repair may differ between both environments (Brash and Hart, 1978; Krishna et al., 1987).</p>	65	The opinion extensively describes the mechanisms leading to DNA damage, occurring in living cells upon the action of a mutagen, either <i>in vivo</i> or <i>in vitro</i> . As suggested by the conspicuous body of literature available, the outcome of mutations obtained <i>in vivo</i> and <i>in vitro</i> is the same.
CropLife Europe	4.3.1.2. Mechanisms leading to repair	<p>Line 773-779: Consider editing the introductory paragraph as some statements are speculative or misleading. Suggested text: 'The detection of DNA damage triggers cellular repair mechanisms than can lead to the restoration of the original sequence. However, the fidelity of the repair is not always complete (100%) and can lead to the introduction of DNA sequence change. If the repaired DNA sequence is transmitted to the next generation, it will be fixed as a mutation. It is important to notice that the DNA damage and consecutive cellular repair mechanisms described below are identical, whether the damage is caused by an induced or by a spontaneous event.' Line 780: What does 'localized alterations' mean? Line 784: Suggestion to add 'DNA sequence' damage; word 'tackled' to be replaced by a more appropriate verb Line 798: Add 'pathway' after '(NER)' Line 800: Replace 'is capable of' with 'typically removes' Line 807-808: Suggestion to delete 'which gives an idea of the complexity and intricacy of these mechanisms' as this is not informative. Line 810: Word "alterations" is used throughout the text in different ways. In some cases to describe DNA breaks/damage, in others to indicate mutations. "Lesions" "mutations", and other words are used interchangeably. We suggest a consistent use of the terminology. Line 812: add 'DNA' before 'replication fork' Line 813: add 'DNA' before 'breaks'</p>	66	<p>Regarding comment to line 773-779, the text has been amended accordingly.</p> <p>Regarding comment to line 780, the text has been improved.</p> <p>Regarding comments to lines 798, 800, 807-808, the text has been amended accordingly.</p> <p>Regarding comment to line 784, the term 'damage' has been changed to 'DNA damage', the term 'takled' has been changed to 'dealt with'.</p> <p>Regarding the comment to line 810, the GMO Panel thanks for the comment and revised the entire text to make sure the term alteration is used consistently.</p> <p>Regarding comment to line 812 and 813, the text has been amended accordingly.</p>
Anonymous	4.3.1.2. Mechanisms leading to repair	<p>The modifications described above can be detected by specialised cell proteins that activate repair systems. A particular type of repair tends to be activated for each type of exogenous or endogenous modification. Thus DNA modifications caused by ionising radiation are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). If, during NHEJ, sequences are lost or added or the bonded strands do not come from the same chromosome,</p>	67	The GMO Panel takes note of the comment.

		<p>a mutation appears. Homologous recombination may also produce mutations; the mechanisms involved are more complex. DNA modifications caused by reactive oxygen species (ROS), which may be generated by the cell metabolism, are repaired by specific systems. There are two types of system: nucleotide excision repair (NER) and base excision repair (BER). Each involves a substantial number of genes. After these systems have been activated, mutations can occur if the 'repaired' base or nucleotide fails to match the nucleotide on the anti-sense strand: during replication, the mutation will be fixed when this new nucleotide is copied. Thymine dimers are repaired by BER. Alterations caused by integrative viruses have no short-term repair system and are retained and subject to natural selection. For changes introduced by endogenous pathways, replication errors activate the mismatch repair system (MMR). This system can fail to function properly. In this case, a new pair of nucleotides replaces the original pair, thus introducing a mutation. For recombination events, in general, there is no system that repairs such modifications. The functional consequences of these events determine whether they persist. If a cell is highly disrupted, it is removed by cell death. If the damage is less extensive, it will persist. The same applies to movement of endogenous sequences (transposons, retrotransposons).</p>		
Haut Conseil des Biotechnologies - Scientific Committee	4.3.1.2. Mechanisms leading to repair	<p>The modifications described above can be detected by specialised cell proteins that activate repair systems. A particular type of repair tends to be activated for each type of exogenous or endogenous modification. Thus DNA modifications caused by ionising radiation are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). If, during NHEJ, sequences are lost or added or the bonded strands do not come from the same chromosome, a mutation appears. Homologous recombination may also produce mutations; the mechanisms involved are more complex. DNA modifications caused by reactive oxygen species (ROS), which may be generated by the cell metabolism, are repaired by specific systems. There are two types of system: nucleotide excision repair (NER) and base excision repair (BER). Each involves a substantial number of genes. After these systems have been activated, mutations can occur if the 'repaired' base or nucleotide fails to match the nucleotide on the anti-sense strand: during replication, the mutation will be fixed when this new nucleotide is copied. Thymine dimers are repaired by BER. Alterations caused by integrative viruses have no short-term repair system and are retained and subject to natural selection. For changes introduced by endogenous pathways, replication errors activate the mismatch repair system (MMR). This system can fail to function properly. In this case, a new pair of nucleotides replaces the original pair, thus introducing a mutation. For recombination events, in general, there is no system that repairs such modifications. The functional consequences of these events determine whether they persist. If a cell is highly disrupted, it is removed by cell death. If the damage is less extensive, it will persist. The same applies to movement of endogenous sequences (transposons, retrotransposons). For more details, please read the document attached.</p>	68	The GMO Panel takes note of the comment.
Not Applicable (Submission on Personal Capacity)	4.3.1.2. Mechanisms leading to repair	<p>713-714: from this sentence it seems that DSBs are always the result of corresponding SSBs that tend to be in their vicinity. Is this correct or can DSBs also originate from a double break directly affected by the ionising radiation? 714-716: is this not rather a result of the DNA repair processes, rather than an example of the effect of ionising radiations? The change in the context to the concept of deletions and inversions should perhaps be clearer made, e.g. by introducing "as a result/consequence" instead of "for example" 490: change into: kinds of damages 494: change into: is filled with the help of polymerases and ligases 843: has ATR</p>	69	<p>Regarding comment to lines 490 and 494, the text has been amended accordingly.          Regarding comments to lines 713-714, the text has been amended.          Regarding comment to lines 714-716, the text has been amended as</p>

		<p>been defined before? 851-852: should it not rather be strand invasion of these ...overhangs by a homologous sequence? 849-866: this summary of the DNA repair processes is rather short and incomplete, e.g. it does not mention that resection of the broken DNA ends may happen at various extents, leading to different repair pathways, including single-strand annealing. It also fails to mention that in classical NHEJ, both nucleases and polymerases may act alternatively to remove or add nucleotides, which at the end are ligated. Line 895 suggests that KU70/80 prevents loss of DNA, however, both small and large end resection may occur in NHEJ, leading to small or large deletions. Further reading can be found in the recently released JRC study on NGTs.</p>		<p>suggested. Regarding comment to line 843, text has been improved. Regarding comments to lines 849-866, 851-852 and 895, text has been revised.</p>
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.3.1.2. Mechanisms leading to repair	<p>As already mentioned above, this type of question cannot be validly answered without reliable and precise techniques allowing dynamic studies at the cellular and tissular level of mutagenesis application reactions.</p> <p>The answers reported by EFSA are generalities on the consensus of the general mechanisms of DNA damage, erroneously considered a simple concatenation of nucleotides, thus a tiny part of the parts transmitted to the descendants, an aberration given the current knowledge in molecular biology. Generalities do not contribute anything because they group together the responses of hundreds to tens of thousands of cells of various types (somatic, meristematic...), replicative states, ages...</p> <p>Tools, such as the sequencing of genomes, epigenome and epitranscriptome of single-cells or instantaneous atomic microscopy pictures, are being developed, but funding will still have to be found for issues about differences between <i>in vivo</i> and <i>in vitro</i> environments that are of little interest to research funding agencies.</p> <p>The fundamental question raised incidentally by the Conseil d'Etat as to whether cells react differently <i>in vivo</i> and <i>in vitro</i> thus remains unanswered, although there is some evidence to suggest that the mechanisms of DNA damage induction and repair may differ between <i>in vivo</i> and <i>in vitro</i> (Brash and Hart, 1978; Krishna et al., 1987).</p>	70	<p>The opinion extensively describes the molecular mechanisms leading to DNA repair, occurring in living cells in response to a DNA damage caused by a mutagen, either <i>in vivo</i> or <i>in vitro</i>. As suggested by the conspicuous body of literature available, the outcome of mutations obtained <i>in vivo</i> and <i>in vitro</i> is the same.</p>
Euroseeds	4.3.2. Is there any difference between these molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> ?	<p>Euroseeds agrees with EFSA's conclusions from a thorough literature analysis in section 4.3.1.1, that the molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> and the repair mechanisms that are triggered by the mutagens are acting at the cellular level and therefore are the same irrespective if the cell is part of a cultivated tissue <i>in vitro</i> or an organ of a plant <i>in vivo</i>.</p>	71	<p>The GMO Panel thanks for the comment.</p>
The Plant Variety Development Office	4.3.2. Is there any difference between these molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> ?	<p>Considering the EFSA's comprehensive analysis, the PVDO agrees with the conclusions in section 4.3.1.1, that the molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> and the repair mechanisms that are triggered by the mutagens are acting at the cellular level and therefore are the same irrespective if the cell is part of a cultivated tissue <i>in vitro</i> or an organ of a plant <i>in vivo</i>.</p>	72	<p>The GMO Panel thanks for the comment.</p>
Plantum	4.3.2. Is there any difference between these	<p>Since random mutagenesis affects mechanisms on a cellular level, the location of that cell (be it <i>in vivo</i> or <i>in vitro</i>) is irrelevant. Plantum fully supports this conclusion from EFSA.</p>	73	<p>The GMO Panel thanks for the comment.</p>



	molecular mechanisms whether they happen in vivo or in vitro?			
Anonymous	4.3.2. Is there any difference between these molecular mechanisms whether they happen in vivo or in vitro?	UFS agrees with EFSA's conclusions regarding the lack of a possible distinction between <i>in vitro</i> and <i>in vivo</i> random mutagenesis and notes that this opinion is also shared by the french High Council for Biotechnology (HCB), which was seized of this issue in the context of the decisions of the French Council of State. The distinction between <i>in vitro</i> and <i>in vivo</i> mutagenesis is therefore not supported either by legal data such as the decision of the Court of Justice of 25 July 2018, and EU legislation, or by scientific data. Mutagenesis can occur spontaneously in nature, or following exposure to mutagenic agents: this process consists of treating plants <i>in vivo</i> or <i>in vitro</i> with chemical substances or physical processes. <i>In vitro</i> mutagenesis (applied to cells grown in tubes) is a simple evolution of <i>in vivo</i> mutagenesis (applied to parts of plants or whole plants). In the context of the decisions of the french Council of State, the Scientific Committee of the HCB was asked the question "From a biological point of view, how does <i>in vitro</i> random mutagenesis as defined by the decree differ from other plant breeding techniques?". In its opinion of 29 June 2020, it concluded that there were no biochemical differences between the various mutations, whether obtained spontaneously or by random mutagenesis <i>in vitro</i> or <i>in vivo</i> , on isolated cells, parts of plants or whole plants. This shows that the same plant characteristics can be obtained by either method. The choice of technique used depends on the suitability of the plant species. <a href="http://www.hautconseildesbiotechnologies.fr/fr/avis/avis-hcb-sur-projet-decret-modifiant-l'article-d531-2-code-lenvironnement">http://www.hautconseildesbiotechnologies.fr/fr/avis/avis-hcb-sur-projet-decret-modifiant-l'article-d531-2-code-lenvironnement</a>	74	The GMO Panel thanks for the comment.
Anonymous	4.3.2. Is there any difference between these molecular mechanisms whether they happen in vivo or in vitro?	Whether used <i>in vitro</i> or <i>in vivo</i> , induced mutagenesis increases the frequency of DNA lesions in comparison with the frequency of lesions induced in natural conditions, thus increasing the rate of mutation as compared with that occurring spontaneously. Cell or tissue culture - especially over a long period, in an undifferentiated state and in the special conditions of <i>in vitro</i> culture - can lead to an accumulation of spontaneous mutations and entail epigenetic adaptation mechanisms. But the lesion repair mechanisms that give rise to the mutations found are the same whether the cells are grown <i>in vitro</i> or <i>in vivo</i> . For a given mutagen, types of modification are the same, but the frequency of each type can vary according to the conditions.	75	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.3.2. Is there any difference between these molecular mechanisms whether they happen in vivo or in vitro?	Whether used <i>in vitro</i> or <i>in vivo</i> , induced mutagenesis increases the frequency of DNA lesions in comparison with the frequency of lesions induced in natural conditions, thus increasing the rate of mutation as compared with that occurring spontaneously. Cell or tissue culture - especially over a long period, in an undifferentiated state and in the special conditions of <i>in vitro</i> culture - can lead to an accumulation of spontaneous mutations and entail epigenetic adaptation mechanisms. But the lesion repair mechanisms that give rise to the mutations found are the same whether the cells are grown <i>in vitro</i> or <i>in vivo</i> . For a given mutagen, types of modification are the same, but the frequency of each type can vary according to the conditions.	76	The GMO Panel takes note of the comment.
Not Applicable (Submission on Personal Capacity)	4.3.2. Is there any difference between these	888: change into: type of mutations 891: change low into small 892: before 2), add : and the position of the amino acid in relation to the active site(s) of the protein 892: change into:	77	Regarding comments to lines 888 and 891, the text has been amended accordingly.

	molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> ?	consequence on the final protein,... 900-902: change into: and cause rearrangements within the DNA (remove endpoint)		Regarding comment to lines 900-902, the text has been improved. Regarding both comments to line 892, the GMO Panel considers the original text sufficiently clear.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.3.2. Is there any difference between these molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i> ?	<p>EFSA's answer is not based on specific facts but on the well-known general mechanisms of DNA damage and repair (not to mention the lack of knowledge for epigenomes...). The presumption made by EFSA that the same causes make the same consequences, whatever the environment ensures that there is no difference between <i>in vivo</i> and <i>in vitro</i> reactions. It is a rather original error of inference.</p> <p>EFSA's reasoning is of the type that since the basic atoms are the same in both cases, there is no difference between <i>in vivo</i> and <i>in vitro</i> situations. Except that the interactions between cells constitute a major difference, the role of which has not yet been fully studied.</p> <p>This difference in cellular reactions between <i>in vivo</i> and <i>in vitro</i> can also be observed at the level of organoids, which appear to be increasingly necessary for studying the effects of drugs, including genotoxic drugs, and for screening them, thereby indicating fundamental differences in cellular behaviour between <i>in vitro</i> and <i>in vivo</i> (National Research Council, 2007; Thompson, 1986). The differences between <i>in vitro</i> and <i>in vivo</i> tests are that multicellular models are no longer an option, even for cancer studies (Akdemir et al., 2020; Benfenati et al., 2010; Edmondson et al., 2014; Tennant, 1991). These results obtained for animals are most likely to be extrapolated to plants.</p> <p>In the case of <i>in vivo</i> / <i>in vitro</i> equivalence, it should then be sufficient to leave an isolated pluripotent/meristematic cell, or even an animal IPS, in a three-dimensional environment to obtain organs, or even a complete organism, which is not observed, among other things, because of certain stochasticity of 'rebel' cells (Mojtahedi et al., 2016; Richard et al., 2016). Only changes in the environment, including electrical and mechanical stresses, allow for the creation/regeneration of organs/organisms. Environmental constraints are necessary to such an extent that work is being done on their reconstitution, if only for organoids. On the other hand, whole plants can quickly regenerate missing parts (broken, grazed, etc.) (Asahina et al., 2011; Pulianmackal et al., 2014; Reid and Ross, 2011). However, the totipotency of an isolated plant cell comes from the lack of intra-tissue contacts rather than from the cell lineage (Caboche, 2010). Therefore, there are differences in plant cells' behaviour when faced with mutations and epimutations induced either <i>in vivo</i> or <i>in vitro</i>.</p> <p>It is currently not possible to measure somatic variation precisely <i>in vivo</i> (only approximation by extrapolation of <i>in vitro</i> results) and even less so according to selection pressure or allele rarity (Dou et al., 2018) implies that the genetic mosaicism (and therefore gene expression) observed <i>in vivo</i> has no equivalent for cells in phase culture (Oota, 2020). Without an appropriate analytical measurement tool (Orr et al., 2020) and despite technical improvements in sequencing isolated cells (but requiring 'ultra-deep' sequencing), it, therefore, seems presumptuous, to say the least, with very different results between papers (Brody et al., 2018; Milholland et al., 2017) to ensure that all things are equal with regard to possible continuity <i>in vivo</i> and <i>in vitro</i>.</p> <p>Exocytosis is only known <i>in vivo</i>, without any <i>in vitro</i> model (Žárský et al., 2009).</p>	78	The opinion does not assume that an organism has no internal regulation. The sentence refers to the fact that the described mechanisms take place in living cells and that the outcome of the mutagenesis process is the same, either <i>in vitro</i> or <i>in vivo</i> .



		<p>Irradiation of cells <i>in vivo</i> can induce unintended and delayed changes when cultured with unirradiated cells (possibly inducing transgenerational effects) (Morgan, 2003). An argument for a discontinuity between <i>in vivo</i> and <i>in vitro</i> cells' facing mutations.</p> <p>Finally, as already noted, some evidence suggests that the mechanisms of DNA damage induction and repair may differ between <i>in vivo</i> and <i>in vitro</i> (Brash and Hart, 1978; Krishna et al., 1987).</p> <p>Tools such as the sequencing of genomes, epigenome and epitranscriptome of single-cells or dynamic atomic microscopy are being developed and should be able to address these issues shortly. Nevertheless, funding will still have to be found for questions that are of little interest to research funding agencies.</p> <p>Therefore, EFSA cannot answer in the current state of knowledge that there is no difference since there is no factual evidence to support its claim. This is a classic error of inferential logic usually committed when one wants to impose a conclusion. On the contrary, the differences in cells' behaviour between <i>in vivo</i> and <i>in vitro</i> suggest that differences in responses to mutations and epimutation should be discernible with the appropriate tools. One do not see what one refuses to look for.</p> <p>All the data currently available on genetic and epigenetic mechanisms in isolated cells and organised tissues demonstrate more of a discontinuity between the effects of induced mutations <i>in vivo</i> and <i>in vitro</i> than any continuity. The difference between continuity and discontinuity is non-scientific. It should not be possible for EFSA to reply. Yet, one may notice EFSA ventures out of its field. Similarly there is continuity of wavelength between green (573 to 490 nm) and blue (490 to 466 nm) color. Yet everyone sees the difference between a green grass and a blue sky.</p> <p>Efsa states:      "There is no difference between these molecular mechanisms whether they happen <i>in vivo</i> or <i>in vitro</i>. As mentioned in section 4.3.1.1, both physical and chemical mutagens cause alteration at the DNA level. These processes and the repair mechanisms that are triggered by the mutagens are acting at the cellular level and therefore are the same irrespective if the cell is part of a cultivated tissue <i>in vitro</i> or an organ of a plant <i>in vivo</i> ."</p> <p>The beginning is right when it denotes <i>mutation processes</i>. But when it claims in addition that "repair mechanisms [...] are acting at the cellular level", it gets out of the road by assuming an organism is a set of cells with no internal regulation. The whole text states right things on mutations and neglects repair mechanisms and even regeneration of a whole plant.</p>		
CropLife Europe	4.4.1. What type of alterations at the DNA level are induced by random mutagenesis?	The text so far does not address the possible categories of DNA changes . Including such text would be helpful to readers. It can provide context to the description of different mutagens and the type of possible changes that can be triggered by these. We also suggest highlighting, in a clearer way, that mutagens are a "trigger" of DNA change, but the interplay with DNA repair mechanism provides the final outcome. This should be highlighted more clearly, and reflected in the executive summary.	79	The GMO Panel considers the aspects raised in the comment sufficiently addressed in the opinion.
Anonymous	4.4.1. What type of alterations at the DNA level	Mutations caused by random mutagenesis result from application of physical or chemical mutagens. Of the physical mutagens, X and gamma rays, fast neutrons and ions are the most widely used (in some 90% of cases for rice). X and gamma rays are high-energy photons. Gamma rays tend to cause small deletions and insertions by double strand breaks in DNA.	80	The GMO Panel takes note of the comment.

	are induced by random mutagenesis?	They can also generate larger deletions, inversions, breaks and chromosome rearrangements. X rays cause ROS formation and therefore result in mutations through base modification, thus mostly point mutations. Fast neutrons tend to cause breaks in DNA and result in substitutions (point mutations), duplications, insertions and deletions. Ion beam radiation (IBR) produces high energy carbon ions and protons that cause mainly large deletions but also point variations. For example, the frequencies recorded for physical mutagens vary according to the energy used: approximately 10 mutations per Mb for X and gamma rays, 30-80 per rice genome for fast neutrons, and little data for IBR. The three main chemical mutagens are ethyl methanesulfonate (EMS), methyl nitrosourea (MNU) and sodium azide (SA). These molecules give rise to nucleotide base modifications, which will be associated with introduction of point mutations during a replication cycle. The number of mutations that can be obtained depends on dosage and time of exposure. Similarly, for most mutagens, the mutations found vary according to sequence context and DNA methylation. Thus, by altering the chromatin context, cell culture can change the frequency of some of the profiles obtained. Generally speaking, protocols are adapted to combine mutagenic effectiveness with less toxicity to be able to select more mutants. These mutagens can also give rise to chromosome rearrangements, at lower rates. For example, the frequency of mutations found in rice is, depending on the agent: 2 to 10 per Mb for EMS (Mb:106pb), 1 per 135 kb for MNU (kb:103bp) and 1.4 to 2.9 per Mb for SA.		
Haut Conseil des Biotechnologies - Scientific Committee	4.4.1. What type of alterations at the DNA level are induced by random mutagenesis?	Mutations caused by random mutagenesis result from application of physical or chemical mutagens. Of the physical mutagens, X and gamma rays, fast neutrons and ions are the most widely used (in some 90% of cases for rice). X and gamma rays are high-energy photons. Gamma rays tend to cause small deletions and insertions by double strand breaks in DNA. They can also generate larger deletions, inversions, breaks and chromosome rearrangements. X rays cause ROS formation and therefore result in mutations through base modification, thus mostly point mutations. Fast neutrons tend to cause breaks in DNA and result in substitutions (point mutations), duplications, insertions and deletions. Ion beam radiation (IBR) produces high energy carbon ions and protons that cause mainly large deletions but also point variations. For example, the frequencies recorded for physical mutagens vary according to the energy used: approximately 10 mutations per Mb for X and gamma rays, 30-80 per rice genome for fast neutrons, and little data for IBR. The three main chemical mutagens are ethyl methanesulfonate (EMS), methyl nitrosourea (MNU) and sodium azide (SA). These molecules give rise to nucleotide base modifications, which will be associated with introduction of point mutations during a replication cycle. The number of mutations that can be obtained depends on dosage and time of exposure. Similarly, for most mutagens, the mutations found vary according to sequence context and DNA methylation. Thus, by altering the chromatin context, cell culture can change the frequency of some of the profiles obtained. Generally speaking, protocols are adapted to combine mutagenic effectiveness with less toxicity to be able to select more mutants. These mutagens can also give rise to chromosome rearrangements, at lower rates. For more details, please read the file attached.	81	The GMO Panel takes note of the comment.
OGM dangers/ Groupe International d'Etudes	4.4.1. What type of alterations at the DNA level are induced by	The different effects of "genetic mutations" as defined by EFSA (effect on nucleotide sequences) are insufficient to describe the whole range of cellular and tissue reactions between these two cellular organisations. EFSA lacks a holistic view of these environmental situations of cells.	82	In ToR2 EFSA was requested to address the types of genetic modifications. Therefore, this section of the opinion focuses on the alteration at the DNA level.

Transdisciplinaires (GIET)	random mutagenesis?			
CropLife Europe	4.4.1.1. Types of mutations	Line 901: The authors use different descriptions of mutations. Here mutations are called "chromosomal" . There is a need to more rigorously align the use of terms and to include these in the glossary. Line 901: Suggestion to replace 'is not properly' with a more appropriate term because there is no proper or improper way to repair a break. Line 905: It is misleading to contrast deletions with other types of DNA sequence changes and to imply that only deletions are linked to los of function.	83	Regarding comment to line 901and 905, the text has been improved
The Plant Variety Development Office	4.4.1.1. Types of mutations	The PVDO agrees, again taking in consideration the depth of research and analysis undertaken (section 4.3, 4.4.1 and 4.2.1.5), that mutations are the final results of molecular mechanisms that cause alterations to the DNA and that the repair mechanisms are the same in vivo and in vitro and thus the type of mutations obtained by in vivo and in vitro mutagenesis are the same including deletions, insertions of single or multiple base pairs as well as single and multiple base pair exchanges and chromosome re-arrangements. As mentioned in the context of the chapter 'literature search', we strongly recommend to include the review from Stacy D. Singer, John D. Laurie, Andriy Bilichak, Santosh Kumar & Jaswinder Singh (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826 which represents the most recent overview and specifically elaborates on the description of types of mutations that can occur by random mutagenesis (page 78 B.).	84	The GMO Panel thanks for the comment. The suggested citation has been added in section 4.3.1.1.
Anonymous	4.4.1.1. Types of mutations	These different mutagens can be employed in combination: gamma rays and EMS, for example. Use of T-DNA insertion and transposon systems has also been reported. Lastly, it should be noted that the CRISPR/Cas system, known for its ability to induce targeted mutations, can also cause random mutations in localised regions when a special protocol is used, allowing allelic variability to be created for a given gene (Li et al., 2020). Since different mutagens have different molecular targets, the mutation profiles are different, with some overlap nevertheless, enabling different phenotypes to be obtained (Belfield et al., 2012; Shirasawa et al., 2016; Viana et al., 2019). While specific features have been found (ionising mutagens are associated with more chromosome breaks, for example), the changes recorded could also have occurred in field conditions. It has been noted that since environmental conditions (biotic or abiotic stress) alter chromatin organisation, the rates at which particular genes may be modified can vary.	85	The GMO Panel takes note of the comment.
Haut Conseil des Biotechnologies - Scientific Committee	4.4.1.1. Types of mutations	These different mutagens can be employed in combination: gamma rays and EMS, for example. Use of T-DNA insertion and transposon systems has also been reported. Lastly, it should be noted that the CRISPR/Cas system, known for its ability to induce targeted mutations, can also cause random mutations in localised regions when a special protocol is used, allowing allelic variability to be created for a given gene (Li et al., 2020). Since different mutagens have different molecular targets, the mutation profiles are different, with some overlap nevertheless, enabling different phenotypes to be obtained (Belfield et al., 2012; Shirasawa et al., 2016; Viana et al., 2019). While specific features have been found (ionising mutagens are associated with more chromosome breaks, for example), the changes recorded could also have occurred in field conditions. It has been noted that since environmental conditions (biotic or abiotic stress) alter chromatin organisation, the rates at which particular genes may be modified can vary.	86	The GMO Panel takes note of the comment.

OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.4.1.1. Types of mutations	<p>A simple description of a final result observed at the level of a few nucleotides is</p> <ul style="list-style-type: none"> <li>(i) in no way representative of what happened before and</li> <li>(ii) above all of the effects other than at the level of the sequence of nucleotides considered by EFSA: modification of the epigenome, of the epitranscriptome, aberrant proteins... with alternative splicing, 3D modifications of the genome, exchanges between organelles and with the nucleus...</li> </ul> <p>There are no publications apart from those we have cited above because this is not a subject funded by research agencies or the European Commission. The absence of publications does not mean that there are no differences, a regrettable lack of critical analytical thinking of this report.</p>	87	In ToR2 EFSA was requested to address the types of genetic modifications, meaning the alterations at the DNA level. The opinion does not address the possible consequences of mutations on gene expression.
Euroseeds	4.4.2. Is there any difference between the mutations whether they are obtained in vivo or in vitro?	Euroseeds agrees with EFSA's detailed and comprehensive analyses of the literature in section 4.3, 4.4.1 and 4.2.1.5 showing that mutations are the final results of molecular mechanisms that cause alterations to the DNA and that the repair mechanisms are the same in vivo and in vitro and thus the type of mutations obtained by in vivo and in vitro mutagenesis are the same including deletions, insertions of single or multiple base pairs as well as single and multiple base pair exchanges and chromosome re-arrangements. As mentioned in the context of the chapter 'literature search', we strongly recommend to include the review from Stacy D. Singer, John D. Laurie, Andriy Bilichak, Santosh Kumar & Jaswinder Singh (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826 which represents the most recent overview and specifically elaborates on the description of types of mutations that can occur by random mutagenesis (page 78 B.).	88	The GMO Panel thanks for the comment. The suggested citation has been added in section 4.3.1.1.
Anonymous	4.4.2. Is there any difference between the mutations whether they are obtained in vivo or in vitro?	UFS agrees with EFSA's detailed and comprehensive analyses of the literature in section 4.3, 4.4.1 and 4.2.1.5 showing that mutations are the final results of molecular mechanisms that cause alterations to the DNA and that the repair mechanisms are the same in vivo and in vitro and thus the type of mutations obtained by in vivo and in vitro mutagenesis are the same including deletions, insertions of single or multiple base pairs as well as single and multiple base pair exchanges and chromosome re-arrangements. The Scientific Committee of the french High Council for Biotechnology (HCB) also mentions in its opinion of 29 June 2020 that biochemically, applied in vivo or in vitro, mutagenesis induces the same type of mutations, but at a lower frequency. The DNA repair mechanisms activated by alterations induced by a mutagenic agent and/or the culture conditions are identical, whether the cells are grown in vitro or in vivo. As a result, the mutations observed are biochemically identical. However, their type, their frequency, and thus the frequency at which each gene may present a mutation, depend on the agent used, its dosage, the genotype and the culture conditions.	89	The GMO Panel thanks for the comment.
Anonymous	4.4.2. Is there any difference between the mutations whether they are obtained in vivo or in vitro?	In biochemical terms, induced mutagenesis, whether applied in vivo or in vitro, increases the frequency of DNA lesions in comparison with the frequency of lesions induced in natural conditions, thus increasing the rate of mutation as compared with that occurring spontaneously. Cell or tissue culture ` especially over a long period, in an undifferentiated state and in the special environmental conditions of in vitro culture (culture media, oxygenation, climatic environment of growth chambers, etc.) can lead to an accumulation of spontaneous mutations, at a lower rate, and entail epigenetic adaptation mechanisms. But the lesion repair mechanisms that give rise to the mutations found are the same whether the cells are grown in vitro or in vivo. For a given mutagen, types of modification are the same, but the frequency of	90	The GMO Panel thanks for the comment.

		each type can vary according to the conditions. Thus, the HCB Scientific Committee has found no biochemical differences between mutations, whether obtained spontaneously or by <i>in vitro</i> or <i>in vivo</i> random mutagenesis, in single cells or multicellular entities. The HCB did not really work on molecular comparisons, but compared induced phenotypes, obtained <i>in vivo</i> or <i>in vitro</i> . Given that the molecular mechanisms involved are the same for <i>in vitro</i> and <i>in vivo</i> mutagenesis, the same types of mutation may be expected to occur. The phenotypes that can be selected by <i>in vitro</i> mutagenesis of plant cells by comparison with spontaneous mutagenesis and other mutagenesis techniques are therefore identical. By contrast, the rates at which a gene may be modified vary according to technique (mutagen, exposure time, culturing). Depending on the desired phenotypes, it will be easier to identify variation in phenotype expression by <i>in vitro</i> culture rather than <i>in vivo</i> mutagenesis, especially if the variation can be screened during the <i>in vitro</i> culture phase by applying selection pressure to the large number of entities being treated.		
Haut Conseil des Biotechnologies - Scientific Committee	4.4.2. Is there any difference between the mutations whether they are obtained <i>in vivo</i> or <i>in vitro</i> ?	In biochemical terms, induced mutagenesis, whether applied <i>in vivo</i> or <i>in vitro</i> , increases the frequency of DNA lesions in comparison with the frequency of lesions induced in natural conditions, thus increasing the rate of mutation as compared with that occurring spontaneously. Cell or tissue culture ` especially over a long period, in an undifferentiated state and in the special environmental conditions of <i>in vitro</i> culture (culture media, oxygenation, climatic environment of growth chambers, etc.) can lead to an accumulation of spontaneous mutations, at a lower rate, and entail epigenetic adaptation mechanisms. But the lesion repair mechanisms that give rise to the mutations found are the same whether the cells are grown <i>in vitro</i> or <i>in vivo</i> . For a given mutagen, types of modification are the same, but the frequency of each type can vary according to the conditions. Thus, the HCB Scientific Committee has found no biochemical differences between mutations, whether obtained spontaneously or by <i>in vitro</i> or <i>in vivo</i> random mutagenesis, in single cells or multicellular entities. The HCB did not really work on molecular comparisons, but compared induced phenotypes, obtained <i>in vivo</i> or <i>in vitro</i> . For more details, please read the file attached.	91	The GMO Panel takes note of the comment.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.4.2. Is there any difference between the mutations whether they are obtained <i>in vivo</i> or <i>in vitro</i> ?	First, <i>in vitro</i> mutagenesis, as reminded by EFSA, and Commission, requires the step of regeneration of a whole plant. This requires isolated cell culture which, in itself makes somaclonal variation. So it is different. Again, there is no way for EFSA to say whether or not there are intrinsic differences because we do not know the mechanisms dynamically involved, due to the lack of appropriate techniques. We can only note final partial similarities and differences because at no time has EFSA looked at other modifications that can be transmitted to descendants and horizontally. On the contrary, the questions raised by various authors (cf. above) and the differences in the behaviour of the cells according to their background ( <i>in vivo</i> or <i>in vitro</i> environments) allow to suspect that globally there are differences in these two environments even though we cannot point out the deep mechanisms. In addition, there are some different signatures (epigenetic, ...) to the two environments despite nucleotide sequences are common to the two situations. But it is known for a long time that genetic does not reduce to the mere nucleotide sequence. EFSA's lack of a holistic approach does not allow it to answer the question posed but to note the lack of data.	92	In ToR2 EFSA was requested to address the types of genetic modifications, meaning the alterations at the DNA level. The opinion does not address the possible consequences of mutations on gene expression. Somaclonal variation has been described in section 4.1.1.
Anonymous	4.5.1. Are <i>in vitro</i> and <i>in vivo</i> random	In conclusion, the HCB Scientific Committee has found no biochemical differences between mutations, whether obtained spontaneously or by <i>in vitro</i> or <i>in vivo</i> random mutagenesis, in single cells or multicellular entities. Nor are there any differences between the phenotypes	93	The GMO Panel takes note of the comment.

	mutagenesis techniques considered to be different or not?	resulting from these techniques. It is only the ease of selection and the likelihood of producing these mutations that vary.		
Haut Conseil des Biotechnologies - Scientific Committee	4.5.1. Are <i>in vitro</i> and <i>in vivo</i> random mutagenesis techniques considered to be different or not?	In conclusion, the HCB Scientific Committee has found no biochemical differences between mutations, whether obtained spontaneously or by <i>in vitro</i> or <i>in vivo</i> random mutagenesis, in single cells or multicellular entities. Nor are there any differences between the phenotypes resulting from these techniques. It is only the ease of selection and the likelihood of producing these mutations that vary.	94	The GMO Panel takes note of the comment.
Not Applicable (Submission on Personal Capacity)	4.5.1. Are <i>in vitro</i> and <i>in vivo</i> random mutagenesis techniques considered to be different or not?	920-921: "if they are to be considered as a continuum": this is not specifically addressed in the text, and perhaps it should be clarified that the answer is a no, otherwise it may still be thought that there is a continuum of mutagenesis techniques, with <i>in vitro</i> techniques on one end of the continuum and <i>in vivo</i> methods on the other hand. This is clearly not the case. 925, shorten sentence by ending first sentence after conditions, and starting new sentence with "Within" 934: text is correct, but should it not be mentioned somewhere that <i>in vitro</i> techniques would require a tissue-culture step, which may induce additional mutations?	95	Regarding comment to text line 920-921, the GMO Panel considers the text sufficiently clear. Regarding comment to text line 925, the text has been changed accordingly. Regarding comment to text line 934, the GMO considers the text sufficiently clear, as somaclonal variation has been discussed elsewhere in the text.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	4.5.1. Are <i>in vitro</i> and <i>in vivo</i> random mutagenesis techniques considered to be different or not?	This is arguably the worst ToR ever, as it completely distorts the letter and spirit of the Conseil d'État's opinion. The difference between continuity and discontinuity is non-scientific. It should not be possible for EFSA to reply. It should be the mission of Commission. Yet, one may notice EFSA ventures out of its field. Similarly there is continuity of wavelength between green (573 to 490 nm) and blue (490 to 466 nm) color and yet everyone sees the difference between a green grass and a blue sky. But there is a scientific convention that green and blue must be regulated as colors even if there is continuity of wavelength. Similarly a documentary traceability is enforced in all European Union, even if there might be cheaters (rape's oil from GMO for instance). So the debate is not scientific and EFSA is being manipulated to give a scientific foundation to a political decision of the Commission which refuses to take its own responsibility. We do fear all this is going to undermine the trust in science and even in our institutions in the medium range time. EFSA's answer is contained in this circular question from the European Commission (so the answer is contained in the question) about techniques and not about differences in biological responses between <i>in vivo</i> and <i>in vitro</i> to similar mutagenesis techniques. Of course, EFSA answers positively: a neutron flux remains a neutron flux whether <i>in vivo</i> or <i>in vitro</i> applied to cells, at least in our Einsteinian universe... what another nice truism. CQFD. In conclusion: 23 pages to arrive at a false conclusion already contained in the premises to a twisted question.	96	Physical and chemical mutagenesis can be achieved following two distinct methodologies, <i>in vivo</i> or <i>in vitro</i> , that have the advantages and disadvantages explained in the opinion, and that allow to achieve the same type of mutations. The continuity refers to the fact that these two techniques lead to the same type of mutants and the final products are not distinguishable.



Federal Office of Consumer Protection and Food Safety (BVL)	5. Conclusions	The Federal Office of Consumer Protection and Food Safety as the German Competent Authority for Directive 2001/18/EC agrees with the scientific conclusions of the EFSA GMO Panel.	97	The GMO Panel thanks for the comment.
CropLife Europe	5. Conclusions	Lines 955-958: Suggestion to rephrase the last sentence for clarity as follows: 'The DNA sequence changes (mutations) are the result of a process that involves several consecutive steps - from disruption or damage to the DNA sequence to its repair by cellular mechanisms. At a molecular level, these processes are identical, irrespective of the way the mutagenizing agents are delivered to the cell, whether in vivo or invitro.'	98	Regarding comment to lines 955-958, the text has been improved
Euroseeds	5. Conclusions	Euroseeds welcomes the very comprehensive report and agrees with its conclusions that the distinction between plants obtained by in vivo or in vitro approaches is not justified, and that the same mutation and the derived trait can be potentially obtained using both in vivo and in vitro random mutagenesis and the resulting mutants would be indistinguishable. All this is based on a detailed and thorough analysis of the available scientific evidence as referenced in the report.	99	The GMO Panel thanks for the comment.
The Plant Variety Development Office	5. Conclusions	The PVDO welcomes the report and supports its conclusions,, which are all based on the available scientific evidence - the distinction between plants obtained by in vivo or in vitro approaches is not justified, and that the same mutation and the derived trait can be potentially obtained using both in vivo and in vitro random mutagenesis and the resulting mutants can not be distinguished.	100	The GMO Panel thanks for the comment.
Plantum	5. Conclusions	EFSA has drafted a clear report on random mutagenesis. Plantum welcomes this clarity and supports the conclusion that there should be no distinction between plants obtained by in vivo or in vitro random mutagenesis. Since the resulting plant (or trait) from the use of random mutagenesis is indistinguishable, it would not be logical to make such a distinction. Plantum welcomes the conclusions of EFSA, which are based on extensive literature analysis and scientific evidence.	101	The GMO Panel thanks for the comment.
Anonymous	5. Conclusions	UFS welcomes the report's conclusions that the distinction between plants obtained by in vivo or in vitro approaches is not justified: the same mutation and the derived trait can be potentially obtained using both in vivo and in vitro random mutagenesis. Furthermore, the resulting mutants would be indistinguishable. We rely on the scientific body (HCB, french High Council of Biotechnology) which concluded in the same way as the EFSA.	102	The GMO Panel thanks for the comment.
Anonymous	5. Conclusions	The opinion of the HCB is based entirely on the Scientific Opinion adopted in response to the referral of 2 July 2020 concerning the draft decree amending Article D.531-2 of the French Environment Code by the HCB Scientific Committee on 29 June 2020.	103	The GMO Panel takes note of the comment.
International Seed Federation (ISF)	5. Conclusions	ISF welcomes the very comprehensive report and agrees with its conclusions that the distinction between plants obtained by in vivo or in vitro mutagenesis approaches is not justified and that the same mutation and the derived trait can be potentially obtained using both in vivo and in vitro random mutagenesis and the resulting mutants would be indistinguishable. All this is based on a detailed and thorough analysis of the available scientific evidence as referenced in the report. According to our knowledge, none of the countries differentiates between in-vivo and in-vitro mutagenesis. Therefore, the EFSA analysis is not just scientifically sound but also in line with the policy/regulatory approaches that have been taken in other parts of the world. This fact could be referenced in the introduction of the publication ISF strongly believes that science-based, consistent policies for products of any form of mutation breeding, are imperative to facilitate the development and uptake of	104	The GMO Panel thanks for the comment.



		innovative breeding applications by private and public breeders in developed and developing countries.		
Not Applicable (Submission on Personal Capacity)	5. Conclusions	955: change into: is part of an isolated cell or cultivated tissue 955-956: not clear: "Because mutations are the results of both the molecular mechanisms that cause the alterations to the DNA", should it not be " Because mutations are the results of both the type of physico-chemical damage to the DNA" 960: to change or into and	105	Regarding the comment to line 955, the text has been changed accordingly. Regarding the comment to lines 955-960, the text has been improved. Regarding comment to line 960, the text has been changed accordingly.
OGM dangers/ Groupe International d'Etudes Transdisciplinaires (GIET)	5. Conclusions	<p>The discovery by EFSA that water remains water when applied <i>in vivo</i> or <i>in vitro</i> is appalling. This epistemological incongruity can be explained:</p> <ul style="list-style-type: none"> <li>by the willingness of the European Commission and EFSA to distort the letter and the spirit of the opinion of the French Conseil d'État by developing questions about the techniques instead of addressing the two fundamental questions: can we distinguish between different biological effects of mutagenesis techniques depending on whether they are applied to cells <i>in vivo</i> or <i>in vitro</i>? Do citizen politically want to distinguish the subproducts of these techniques? (92% of European citizens want gene edited food to be labeled or assessed<sup>13</sup>).</li> <li>By the fact that the Commission and EFSA have decided to restrict themselves to mutations linked to changes in nucleotide sequences and not open up what they fear is Pandora's box of mutation effects. That is to say, on the results acquired from research over the last 50 years: epigenome, epitranscriptome and their transmissibility to descendants, alternative splicing, moonlighting and conforming proteins and exaptation, aberrant proteins, nature of the gene, effect of point mutation on the TADs involved in sequence expression, three-dimensional dynamic structure of regulation, interactions between nuclei and organelles and between them, interactions and communication between more or less close cells and distal tissues... in short, to take an interest in more than 90% of what constitutes the transmission and regulation belt of living cells in organized organism. Thus, in the end, we have to be satisfied with the phenotype studies and molecular biology 50 years old. Let us recall that a single nucleotide mutation induces almost unpredictable distal changes in the regulatory genomic 3D structures (Bianco et al., 2018) and that a single insertion shifts the whole origin of reading frame. This enables what is called a knock-out to produce a new and unrelated protein. In such (very classical) experiments the usual scientific motto "all things being equal elsewhere" is forgotten.</li> <li>the lack of independence between EFSA and the European Commission and the constant politicisation of science is a drawback for EFSA that serves a non-scientific agenda.</li> </ul> <p>Efsa's text states : "These processes and the repair mechanisms that are triggered by the mutagens act at the cellular level and are therefore the same irrespective if the cell is part of a cultivated tissue <i>in vitro</i> or any part of a plant <i>in vivo</i>."</p>	106	With the mandate, EFSA was requested to address the different kind of mutation at the DNA level, the differences in molecular mechanisms that cause the DNA alterations, and the difference in random mutagenesis techniques. The opinion describes the mechanisms that cause the DNA damage and the mechanisms triggered by the cells to attempt to restore the original sequence. The consequences of any given mutation on gene expression, such as frame shift or gene knock out, are the same independently of the technique used, and they are the same as the consequences of naturally occurring mutations. The literature considered for this opinion contains recent reports, whose publications have been possible thanks to the advanced knowledge on DNA repair mechanisms achieved in recent years. As described in detail in the opinion, random mutagenesis is an old technology, but the understanding of the mechanisms underlying the genetic alterations caused by the mutagens is relatively new.

		<p>This sentence is a pure concentrate of contradiction. It claims mutagens act at the cell's level (not wrong), and that the repair mechanism too (wrong as argued above at length with Napoléon's oak, and so on) and concludes that it compares "cultivated tissue <i>in vitro</i>" and "any part of a plant <i>in vivo</i>"! According to the definitions reminded by EFSA and given by the Commission, these are two <i>in vivo</i> since there is no need for regeneration of a whole plant. The conclusion contains an internal contradiction that could come from internal debates closed by administratives and not by scientists from EFSA.</p> <p>Efsa states too :</p> <p>"Indeed, the same mutation and the derived trait can be potentially obtained using both <i>in vivo</i> and <i>in vitro</i> random mutagenesis and the resulting mutants would be indistinguishable." That two things <u>can</u> derive one same thing is not an argument to say they are the same. H. Arendt laughs at such ideas: "It is as though I had the right to call the heel of my shoe a hammer because I, like most women, use it to drive nails into the wall" (Past and future 1961).</p> <p>We would like to add one more argument. That two things <u>can</u> give a same consequence is not enough. Is it in the same time-range? If not then they are different. Not raising the question of the time-range is not a scientific consideration.</p> <p>However, this is, one suspects, what the EU's principals and certain lobbies were waiting for.</p>		
International Seed Federation (ISF)	8. References	<p>EFSA states (line 146 ff) 'EFSA, in its Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with a similar function, examines conventional plant breeding techniques relevant for a comparison with Site-Directed Nuclease technique.' ISF recommends also include a reference to EFSA's most recent relevant report on the 'Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis.' This report states 'Overall, the application of SDN-1, SDN-2 and ODM methods results either in random (SDN-1) or predicted (SDN-2 and ODM) mutations of a targeted genomic locus without the insertion of exogenous DNA at the targeted locus'. With this, including this reference would provide the overall context of all kinds of mutagenesis techniques.</p>	107	The EFSA opinion on SDN-1, SDN-2 and ODM has been cited in the text.
CropLife Europe	3.1. Problem formulation	<p>CropLife Europe agrees with the translation of ToRs into scientifically answerable assessment questions in line with the EFSA procedures. In addition, and as indicated further in our specific comments to the sections of the text addressing ToR 3, we believe that it would be helpful to the reader if more information is included on the chemical and physical properties of the DNA molecule and what are the possible changes that can occur to it. This is currently transiently mentioned in section 4.1 but is not providing the need detail. If included, such information would provide a better basis for the description of the molecular mechanisms of mutations.</p>	108	Providing a detailed description of the physical and chemical properties of the DNA goes beyond the ToR as provided by the EC. However, a small amendment has been introduced in the text (section 4.1).
Not Applicable (Submission on Personal Capacity)	3.1. Problem formulation	<p>line 211: to my knowledge, <i>in vivo</i> application of mutagens (in addition to applications to meristems) is mainly done on reproductive structures (e.g. anthers or whole flowers) after which these are used to generate a next sexual generation, which is then used in a selection process. This seems to be missing from the EC questions.</p>	109	GMO Panel takes note of the comment. Please note that the text in line 211 is taken from the background information provided by EC (Section 1).

## **Appendix A – Explanatory text on the EFSA website for the public consultation**

EFSA's GMO Unit has launched an open consultation on its draft scientific opinion on *in vitro* random mutagenesis techniques. In line with the mandate of the European Commission, this Opinion provides a more detailed description of *in vivo* and *in vitro* random mutagenesis techniques and the types of mutations and mechanisms involved, to conclude on whether *in vivo* and *in vitro* random mutagenesis techniques are to be considered different techniques.

Interested parties are invited to submit their comments by the indicated deadline.

Additional data or files to support the comments may be submitted using the relevant function in the digital form.

Comments will not be considered if they:

- are submitted in other languages than English;
- are submitted after the closing date of the consultation;
- are still in 'draft' status on the closing date of the consultation;
- are presented in any form other than what is provided for in the instructions and the relevant function in the tool (e.g. comments made by email will not be considered);
- are made outside the corresponding fields of the form, for instance as part of supporting files uploaded in the tool;
- are not related to the contents of the document or scope of the consultation;
- contain complaints against institutions, personal accusations, irrelevant or offensive statements or material;
- are related to policy or risk management aspects, which are out of the scope of EFSA's activity.

Comments will be assessed in line with the criteria above and taken into consideration if found to be relevant.

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### **Publication of contributions:**

Third-party comments will be made public in their original form without delay after the closing date of the consultation and may be reused by EFSA in a different context. The outcome of the consultation will be made public in conjunction with the publication of the relevant scientific output.

Contributions submitted by individuals in a personal capacity will be published indicating the author's first and family name unless the respondent has requested anonymity. Contributions submitted on behalf of an organisation will be attributed to the organization in question.

More information on the processing of personal data are available in the Privacy Statement.

Submit comments (deadline: **30 June 2021**)

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## Abbreviations

DSB	Double strand break
EC	European Commission
EFSA	European Food Safety Agency
ERA	Environmental Risk Assessment
EU	European Union
GM	Genetic Modification / Genetically Modified
GMO	Genetically Modified Organism
MC	Molecular Characterization
NGO	Non-Governmental Organization
RA	Risk Assessment
ToR	Terms of Reference
WG	Working Group

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