Induced Mutagenesis
All Scopes (Crops, Handling, & Livestock)

	Identification of Methods

Common Physical Methods:

Irradiation:

- Ionizing, particle
  - Neutron
  - Alpha
  - Beta
  - Ion beam
  - Cosmic
- Ionizing, non-particle
  - X-ray
  - Gamma
  - Cosmic
- Non-ionizing
  - UV

Common Chemicals Used:

Sodium azide (NaN_3)

Nitrous acid (HNO_2)

Base analogs:

- 5-Bromouracil: 5-Bromopyrimidine-2,4(1H,3H)-dione

Intercalating agents:

- Ethidium bromide: 3,8-Diamino-5-ethyl-6-phenylphenanthridin-5-ium bromide
- Acridine orange: N,N,N',N'-Tetramethylacridine-3,6-diamine
- Proflavine: Acridine-3,6-diamine

Alkylating mutagens:

- Ethyl methanesulfonate (EMS)
- Diethyl sulfate (DES)
- N-methyl-N-nitrosourea (MNU)
- 1-ethyl-1-nitrosourea (ENU)
- N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

Antimitotics:

- Colchicine: N-[(7S)-1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide
- Oryzalin: 4-(Dipropylamino)-3,5-dinitrobenzenesulfonamide

Trade Names of Chemicals Used:

Sodium azide

- Natriumazide

Colchicine

- Mitigare
- Colcrys

Oryzalin

- Surflan

CAS Numbers for Chemicals Used:

Sodium azide – 26628-22-8

Nitrous acid – 7782-77-6

5-Bromouracil – 51-20-7

Ethyl methanesulfonate (EMS) – 62-50-0

Diethyl sulfate (DES) – 64-67-5

N-methyl-N-nitrosourea (MNU) – 684-93-5

1-ethyl-1-nitrosourea (ENU) – 759-73-9

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) – 70-25-7

Ethidium bromide – 1239-45-8

Acridine orange – 494-38-2

Proflavine – 92-62-6

Colchicine – 64-86-8

Oryzalin – 19044-88-3

	Summary of Induced Mutagenesis Methods

Background on where induced mutagenesis fits into the USDA Organic Regulations

Induced mutagenesis refers to a collection of methods used to create mutations in organisms. Researchers identify beneficial mutations within mutated entities and use those mutations in further breeding of organisms with desired traits.

As a set of methods, and not a specific substance, induced mutagenesis does not appear on the National List of Allowed and Prohibited Substances (hereafter referred to as the "National List"). The USDA organic regulations identify allowed and prohibited substances, methods, and ingredients in organic production and handling (7 CFR 205.105). While induced mutagenesis is not mentioned specifically, in order for a product *"to be sold or labeled as '100 percent organic,' 'organic,' or 'made with organic (specified ingredients or food group(s)),' the product must be produced and handled without the use of excluded methods…"* (§ 205.105(e)). Excluded methods are defined at § 205.2, as follows:

83
84   A variety of methods used to genetically modify organisms or influence their growth and
85   development by means that are not possible under natural conditions or processes and are
86   not considered compatible with organic production. Such methods include cell fusion,
87   microencapsulation and macroencapsulation, and recombinant DNA technology
88   (including gene deletion, gene doubling, introducing a foreign gene, and changing the
89   positions of genes when achieved by recombinant DNA technology). Such methods do not
90   include the use of traditional breeding, conjugation, fermentation, hybridization, in vitro
91   fertilization, or tissue culture.
92
93   **NOP Policy Memos related to excluded methods:**
94   In April 2011, the National Organic Program (NOP) issued Policy Memo 11-13, *Clarification of Existing*
95   *Regulations Regarding the Use of Genetically Modified Organisms in Organic Agriculture* (NOP, 2011). In
96   February 2013, the NOP issued Policy Memo 13-1 *Cell Fusion Techniques Used in Seed Production* (NOP,
97   2013). Neither of these mention induced mutagenesis.
98
99   **NOSB recommendations related to induced mutagenesis:**
100   • In 2013, the National Organic Standards Board (NOSB) began evaluating the definition of
101   "excluded methods" in the context of recombinant DNA biotechnology (NOSB, 2016a).
102   • In 2016, the NOSB's Materials/GMOs Subcommittee drafted a discussion document that
103   included a "To Be Determined (TBD)" chart of technologies (NOSB, 2016a). The NOSB was
104   unclear whether these technologies should be considered excluded methods. The terminology
105   chart in this discussion document included "Induced Mutagenesis" and "TILLING" as *TBD*
106   methods, among others. The document did not define these terms.
107   • Later in 2016, The NOSB issued the 2016 *Formal Recommendation on Excluded Methods Terminology*
108   (NOSB, 2016b). This recommendation established definitions for several terms found within the
109   § 205.2 excluded methods annotation, but did not mention either induced mutagenesis or
110   TILLING, specifically. The recommendation created principles and criteria for use in the
111   evaluation of new technologies and terminologies and created a new excluded methods
112   terminology chart for use in future determinations. Unlike the "TBD" chart, this chart included
113   the NOSB's view on whether each technology was an excluded method or not.
114   • In 2019, the NOSB issued a Formal Recommendation (and an updated excluded methods chart)
115   on induced mutagenesis that stated "induced mutagenesis developed through *in vitro* nucleic
116   acid techniques meets the criteria to be determined as an excluded method"(NOSB, 2019).[1] The
117   recommendation also stated that "induced mutagenesis developed through exposure to UV light,
118   chemicals, irradiation, or other stress-causing activities" should remain on the most current "To
119   Be Determined" chart for future discussion and review.
120   • In 2022, the NOSB issued the most current formal recommendation on excluded methods
121   determinations (NOSB, 2022). Both induced mutagenesis (excluding *in vitro* methods) and
122   TILLING remained on the included "TBD list."
123
124   **Scope of this report**
125   This technical report is intended to support the NOSB's review of the induced mutagenesis methods that
126   remain on the 2022 "TBD list," including mutagenesis achieved through the use of UV light, chemicals,
127   irradiation, or other stress-causing activities (NOSB, 2022). This report addresses five focus questions
128   requested by the NOSB Materials/GMOs Subcommittee. However, we also felt that it was appropriate to
129   report on another item on the 2022 "TBD list," TILLING, because of its relationship to induced
130   mutagenesis. The NOSB notes that TILLING is "a type of mutagenesis combined with a new screening
131   procedure" (NOSB, 2022).
132

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[1] *In vitro* means "in the laboratory [or outside of the living organism]" (NIH National Cancer Institute, 2011a). Within the context of induced mutagenesis, *in vitro* may refer to vegetative plant culture methods (i.e., growing plantlets in a laboratory on growth media) or to *in vitro* fertilization of cells. The term is also used when discussing *in vitro* nucleic acid techniques (i.e., the insertion of nucleic acids into cells or organelles) (NOSB, 2022). *In vitro* nucleic acid techniques are considered excluded methods.

133   TILLING or Targeting Induced Local Lesions IN Genomes, is a plant breeding methodology that
134   combines chemical mutagenesis, generally using ethyl methanesulfonate, with a screening protocol
135   (McCallum et al., 2000). Briefly, chemical mutagenesis methods are those that introduce specific
136   chemicals that act directly or indirectly on DNA while screening protocols are methods used to detect
137   useful phenological variants. The screening protocol is a variant of marker-assisted selection (MAS) (see
138   Inset 2, below). As noted in the NOSB's most recent excluded methods chart, MAS is an allowed method
139   (NOSB, 2022). As a combination of induced mutagenesis and MAS, TILLING falls within the scope of this
140   technical report and is reviewed alongside the other induced mutagenesis methodologies that do not
141   involve *in vitro* nucleic acid techniques. Transposons are another method listed on the NOSB's excluded
142   methods chart which may also be considered chemically induced mutations; however, this technical
143   report will not review the use of transposons in full.

145   This report includes a basic overview of DNA, as relevant to induced mutagenesis. For individuals
146   interested in exploring additional information on genetics, the following open-source resources may be
147   useful:
148   • *Introduction to Genetics* by Natasha Ramroop Singh (Singh, 2009)
149   • *Genetics, Agriculture, and Biotechnology* by Walter Suza and Donald Lee (Suza & Lee, 2021)

151   **Characterization of Induced Mutagenesis Methods**

153   **What is induced mutagenesis?**

155   Genetic mutations, which include any heritable changes to deoxyribonucleic acid (DNA) within living
156   organisms, are naturally occurring phenomena that underpin evolution and the diversity of living
157   organisms that exist today. Inset 1 below includes several definitions that are relevant to the discussion of
158   genetic mutations and mutagenesis. Figure 1 provides a visual depiction of the terms and processes
159   covered in Inset 1.

161   In the context of agriculture, random genetic mutations led to suitable animal and plant species that were
162   appropriate for domestication (Gepts, 2002; Mba, 2013). In modern agriculture, plant and livestock
163   breeders utilize a suite of techniques to capture and expand the genetic diversity of crop and livestock
164   species. Many of these techniques fall into the category of induced mutagenesis.

166   *Causes of mutations*
167   Genetic mutations that are observed in plants and other organisms are associated with changes along
168   DNA strands known as "lesions." There are many kinds of lesions that can occur, either as a result of
169   natural or induced factors (Curtis, 2011). Common DNA lesions that lead to mutations include the
170   following (Curtis, 2011; Spampinato, 2017):
171   • oxidized pyrimidines (i.e., oxidation of cytosine, thymine, or uracil bases)[2]
172   • oxidized purines (i.e., oxidation of adenine or guanine bases)
173   • base alkylation (i.e., the addition of an alkyl group to a base)
174   • base deamination (i.e., the removal of an amine group from a base)
175   • single-strand breaks (SSB) (i.e., a break along one strand of DNA double helix)
176   • double-strand breaks (DSB) (i.e., a break along both strands of DNA double helix)
177   • cross links (i.e., bonding between base pairs that are not located directly across from one another
178   on opposite DNA strand; can be interstrand or intrastrand)

180   *Repair mechanisms*
181   The production of DNA lesions induces a repair response within plant cells. Different repair mechanisms
182   are relied upon within a cell, depending on the type of DNA lesion that is under repair and other
183   environmental factors (Curtis, 2011; Manova & Gruszka, 2015; Spampinato, 2017). While spontaneous

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[2] Oxidation refers to one side of an oxidation-reduction (or redox) chemical reaction, in which the oxidized substance loses an
electron to the reducing substance (National Cancer Institute, 2011).

184 lesions in DNA occur frequently, it is the natural error rate in DNA repair mechanisms that leads to
185 mutations that persist in the genome and into subsequent generations (Spampinato, 2017).
186
187   **Inset 1: Important genetic terms defined**

DNA: A double-stranded helix molecule found inside cells, which contains the genetic information necessary for the development and function of an organism. Hydrogen bonds connect purine and pyrimidine nucleotide base pairs, forming a double helix structure.

Nucleotide: A molecule that is a component of DNA and RNA, comprised of a nitrogen-containing nucleobase, a phosphate group, and a sugar. The sugar in DNA is deoxyribose while the sugar in RNA is ribose.

Nucleobase: A nitrogen-containing molecule that is a component of a nucleotide. In DNA these bases are adenine (A), cytosine (C), guanine (G), and thymine (T). DNA bases pair together to join two strands of the double helix. Under normal circumstances in DNA, adenine will pair with thymine (A-T), and cytosine will pair with guanine (G-C). In RNA, thymine is replaced with the nucleobase uracil (U). Nucleobases are frequently called bases.

Purines: One of two categories of nucleobases found in DNA and RNA, which includes adenine (A) and guanine (G).

Pyrimidines: One of two categories of nucleobases found in DNA and RNA, which includes cytosine (C), thymine (T), and uracil (U).

DNA polymerase: A category of enzymes that are responsible for forming new copies of DNA during the process of DNA replication. During the DNA replication process, one double-stranded DNA molecule is copied into two identical DNA molecules. This process is essential for cell division. Some DNA polymerases are able to correct errors, while others lack this ability or show reduced error correction.

Transcription: The cellular process in which DNA is transcribed into RNA.

RNA: A nucleic acid that contains information copied from DNA. While RNA has many functions, many of these relate to making proteins within cells.

Translation: The cellular process in which genetic information carried by RNA is used to communicate to the cell how to link amino acids together to form proteins (polypeptides). RNA sequences are read (by ribosomes) in segments of three nucleotides at a time, called a codon, which correspond to one amino acid. Changes in a single nucleotide may result in changes to the amino acid chain and subsequent protein formation.

Protein: Proteins are molecules made up of amino acids and are the basis of body structures. Proteins are found in enzymes, cytokines, and other living tissues.

188
189 Sources: (National Cancer Institute, 2011, 2012)
190
191 Common repair mechanisms include (Curtis, 2011; Manova & Gruszka, 2015; Spampinato, 2017):
192   • base excision repair (BER)
193   • nucleotide excision repair (NER)
194   • single-strand break repair (SSBR)
195   • double-strand break repair (DSBR)
196   • photoreactivation of UV-induced damage
197   • direct repair
198   • mismatch repair (MMR)
199
200 The mechanisms of these repair pathways are fully discussed by Curtis (2011), Manova & Gruszka (2015),
201 and Spampinato (2017), and will not be covered in detail within this report. For the context of induced
202 mutagenesis, it should be understood that only a fraction of DNA lesions will evade repair mechanisms
203 or be repaired incorrectly (Manova & Gruszka, 2015). These "missed" or "incorrect" repairs lead to
204 changes in the nucleobase sequence of DNA. If these changes occur in regions of DNA that are actively
205 used by an organism to survive (*e.g.*, coding regions, promoter regions, regulatory genes, etc.), they are

206 considered mutations (Curtis, 2011; Spampinato, 2017).[3] In addition to nucleobase changes, double-strand
207 breaks in DNA may lead to larger chromosomal rearrangements during the DNA repair process
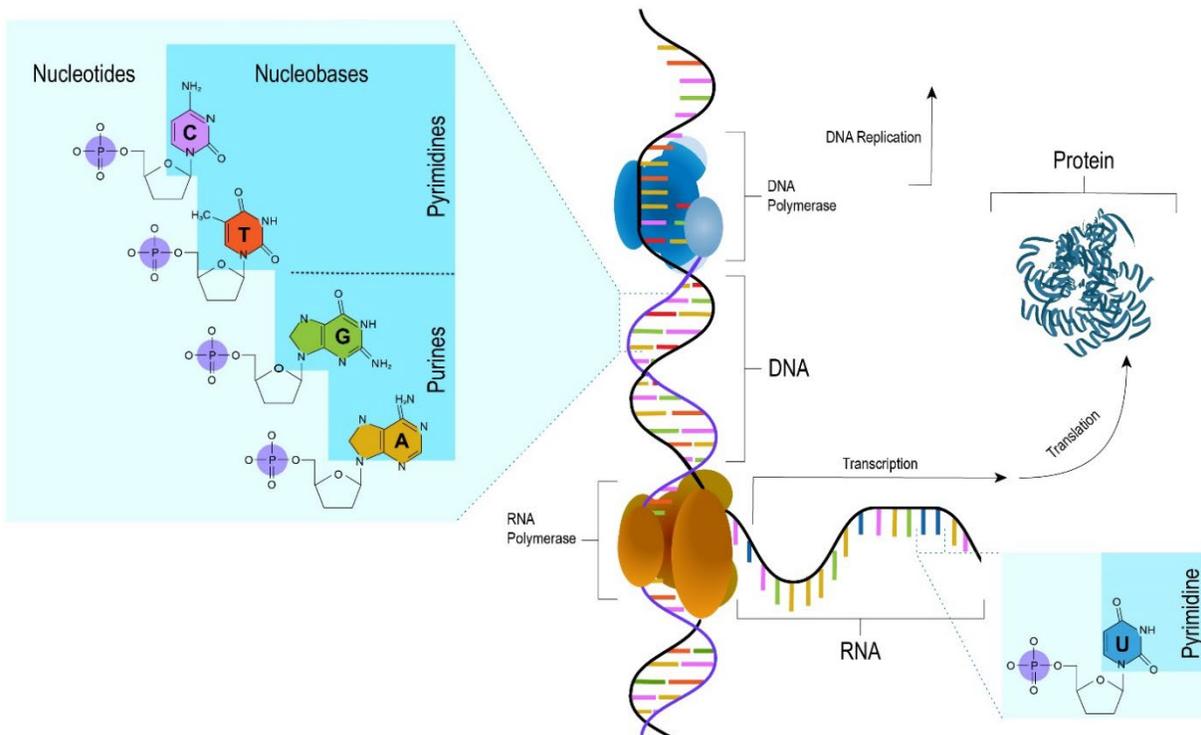208 (Lundqvist et al., 2011).
209

210
211   **Figure 1: DNA structure and basic processes.**
212

213 *Types of mutations*
214 Common mutations and rearrangements include (Lundqvist et al., 2011):
215   • point mutations (single-base changes within DNA)
216   • insertions and deletions[4]
217   • inversions (section of DNA is broken in two locations and reattached in place at a 180° rotation)
218   • translocations (section of DNA is broken in two locations and reattached at another location on
219   the same or different chromosome)
220

221 *Induced mutations*
222 Researchers induce mutations through several physical and chemical methods to capture the genetic
223 diversity generated by natural misrepair processes and increase the rate at which mutations appear
224 (Jankowicz-Cieslak et al., 2017; Wiel et al., 2010). Induced mutagenesis is primarily used to produce
225 beneficial mutations for crop breeding, but the methods are also utilized to produce yeasts and other
226 microorganisms with specific characteristics (Yu et al., 2020). Livestock breeding programs generally do
227 not use *in vivo* induced mutagenesis methods; however, livestock breeders have begun to utilize the *in*
228 *vitro* methods, which are beyond the scope of this report (Liu et al., 2013; Ruan et al., 2017).[5]
229

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[3] Within the DNA structure, some regions of nucleotides encode genes or gene-relevant information. These regions, called coding regions, are transcribed by RNA polymerase, and ultimately become proteins. Other regions, called non-coding regions, are not transcribed (Suza & Lee, 2021). Mutations in coding regions are more likely to result in phenotypic changes in an organism since they are transcribed.

[4] Insertions and deletions are also known as INDELs. They can involve the addition or removal of one-to-many nucleotides, and lead to shifts in the codon "reading frame," known as frameshift mutations.

[5] *In vivo* means "in the body [or living organism]" (NIH National Cancer Institute, 2011b). Within the context of induced mutagenesis, *in vivo* methods are those which result in the genetic changes taking place within the living organism.

230 Induced mutants, in the context of plants, may include either of the following:
231   • new plant varieties produced through the direct use of physical or chemical mutagenesis,
232   followed by vegetative propagation or additional generations of seed propagation.
233   • new plant varieties developed through the use of one or more existing mutant varieties, which
234   are used as parents in subsequent cross breeding.
235
236 *Traditional plant breeding methods vs. induced mutagenesis*
237 The USDA organic regulations allow traditional plant breeding techniques per the NOP definition of
238 excluded methods at 7 CFR 205.2. These include methods that plant breeders have historically used for
239 germplasm improvement.[6]
240
241 Traditional plant breeding methods include (Wiel et al., 2010):
242   • phenotypic selection of open-pollinated populations (i.e., landraces)[7].
243   • phenotypic selection of self-pollinating crop varieties (i.e., purelines).
244   • cross-pollination between two plants of the same species to produce a desired hybrid (i.e., F1
245   hybrid production).
246   • cross-pollination between two plants of the same species to produce a hybrid, followed by
247   selection of desired germplasm in subsequent populations.
248   • cross-pollination between two plants within the same genus (i.e., a wide cross or interspecific
249   cross) to produce a hybrid, followed by selection of desired germplasm in subsequent
250   populations.
251   • cross-pollination between two plants within the same family (i.e., a wide cross or intergeneric
252   cross) to produce a hybrid, followed by selection of desired germplasm in subsequent
253   populations.
254   • the use of wild plant relatives of crop species in the aforementioned cross-pollinations
255   • the use of genetic or genomic information to inform selection (i.e., marker-assisted selection) (see
256   Inset 2).
257
258 Many of the techniques described above expand the genetic diversity of crop species and are valuable to
259 achieving specific phenotypic goals.
260
261 As with traditional plant breeding, induced mutagenesis is also utilized in the pursuit of increased
262 genetic diversity, but differs from traditional plant breeding processes only during the mutation stage
263 itself (Jankowicz-Cieslak et al., 2017). Briefly, the mutation of plant propagules occurs first, followed by 5-
264 6 years of phenotypic selection on the population derived from the original mutant.[8] This is analogous to
265 the traditional plant breeding process, in which plant breeders select desirable phenotypes for 5-8 years
266 following a cross-pollination event (Jankowicz-Cieslak et al., 2017).
267
268 *TILLING*
269 A notable variation on induced mutagenesis is the method known as Targeted Induced Local Lesions IN
270 Genomes, or TILLING. McCallum et al. (2000) developed this methodology by combining traditional
271 chemical mutagenesis with a screening protocol. A number of screening protocols may be used in
272 combination with the initial mutagenesis, including Li-Cor genotyping, high-performance liquid
273 chromatography, and high-throughput sequencing (Till et al., 2006). Using the genetic information
274 obtained in the lab, plant breeders are able to select plants with desirable mutations from a population
275 (McCallum et al., 2000).
276

---

[6] Germplasm refers to living genetic resources that are maintained for future research and education (Byrne et al., 2018). Germplasm banks exist to preserve crop plants, crop wild relatives, microorganisms, as well as livestock genetics.

[7] Unlike genotypes, which refers to the genetic makeup that an organism has, phenotype references the physical, biochemical, or otherwise observable traits of an organism (National Cancer Institute, 2011). Examples include flower color in plants, or the capacity for nitrogen fixation in a microorganism.

[8] Propagules are vegetative sections of plant tissue that are capable of growing into a new plant through the process of vegetative propagation (Jankowicz-Cieslak et al., 2017).

277   TILLING is considered a "reverse genetics" technique. Researchers or breeders begin with information
278   about a gene of interest and work backward to identify plants in a population with that gene (McCallum
279   et al., 2000). TILLING differs from traditional "forward genetics," in which researchers observe a
280   phenotype of interest within a population and then seek to identify the responsible gene. Researchers
281   may choose to use forward or reverse genetics, depending on the type of knowledge (i.e., knowledge of
282   genes or knowledge of phenotype) that they have at the beginning of a project. Figure 2 depicts how
283   these different approaches might look in the context of chemical mutagenesis. In the forward genetics
284   model, a traditional screening process follows chemical mutagenesis. In this process, researchers look for
285   interesting phenotypes in a population and then seek to identify relevant genes. In the reverse genetics
286   model, DNA extraction, amplification, and screening follow the mutation event to determine which
287   mutants hold a gene of interest.
288
289   With the TILLING protocol, breeders can accelerate the breeding process by making selections in earlier
290   generations and with less land resources than phenotypic selection can provide independently
291   (McCallum et al., 2000; Wiel et al., 2010).
292
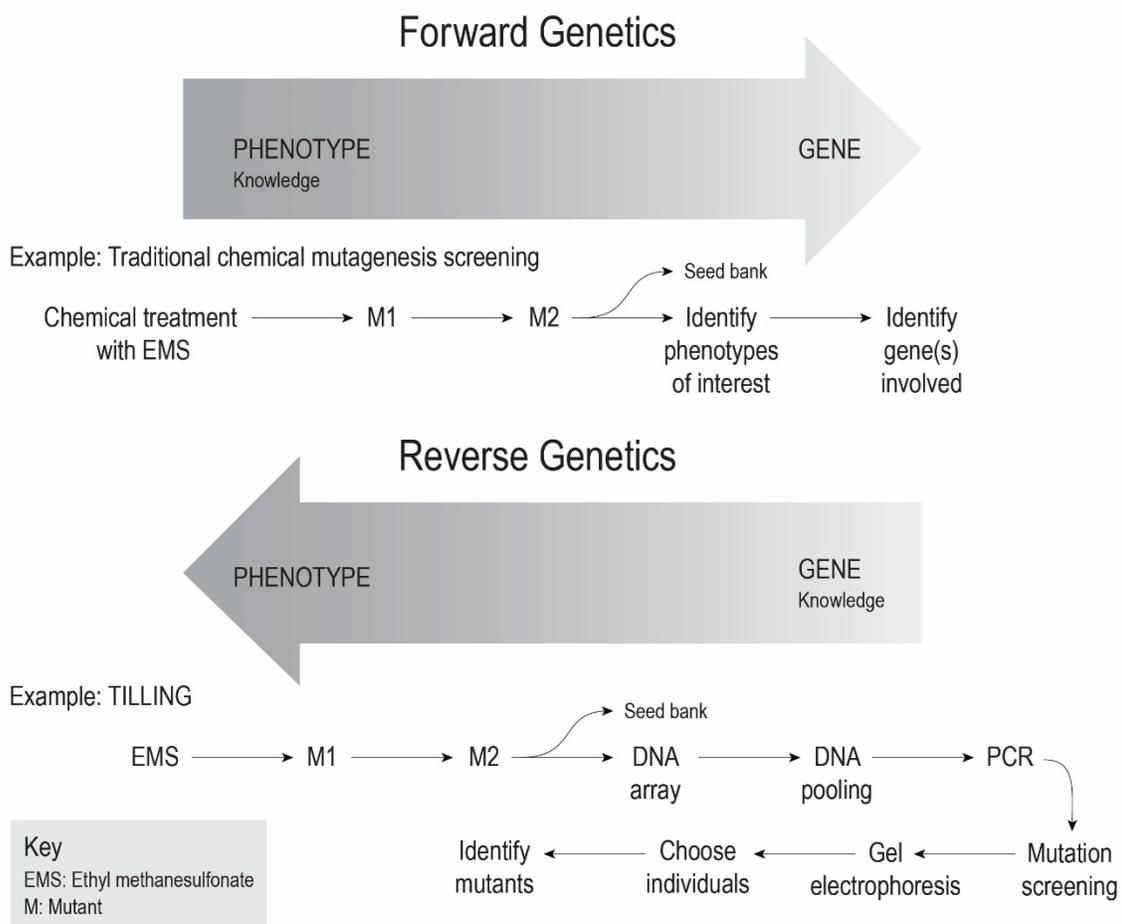
293
294   **Figure 2: Roadmap for forward and reverse genetics following chemical mutagenesis. M1 is the first**
295   **generation produced after mutation, while M2 is the second generation.**
296

Induced mutagenesis vs. new breeding techniques (NBTs)
298   Induced mutagenesis broadly refers to intentional alterations to the DNA of a living organism. Induced
299   mutations fall into *in vivo* and *in vitro* categories. Many *in vitro* methods (but not *in vivo*) are also
300   considered new breeding techniques (NBTs), which generally include breeding technologies that have
301   emerged since 2001 (Holme et al., 2019).
302

303　*In vivo* mutagenesis occurs within the target organism. *In vivo* methods include the application of both
304　physical and chemical mutagens to living tissues to induce heritable mutations. Researchers have used
305　these methods to create new plant varieties since the 1920s (Joint FAO/IAEA Centre of Nuclear
306　Techniques in Food and Agriculture, 2023). *In vitro* mutagenesis methods involve the design and
307　development of a mutation outside of living tissues, followed by the insertion of the mutation. *In vitro*
308　nucleic acid techniques include NBTs such as targeted genetic modification, synthetic biology, cisgenesis,
309　intragenesis, and agro-infiltration (Holme et al., 2019).
310
311　Induced mutagenesis that is derived from *in vitro* nucleic acid techniques is an excluded method (NOSB,
312　2022). Mutations resulting from *in vivo* pressures, such as exposure to UV light, chemicals, irradiation, or
313　other stress-causing activities, are not currently considered to be excluded methods and are the subject of
314　this report (NOSB, 2022).
315
316　**What methods are used to induce mutations?**
317
318　Physical or chemical methods can induce genetic mutations. Scientists have explored numerous methods,
319　but only a selection of these methods have been, or currently are, commonly used (Jankowicz-Cieslak et
320　al., 2017).
321
322　Physical and chemical mutagens damage DNA, indirectly or directly. Direct action by physical mutagens
323　may affect one or both of the DNA strands, resulting in a single-strand break (SSB) or double-strand
324　break (DSB) (Ma et al., 2021). Indirect action by physical mutagens results in the creation of free radicals
325　within the cell, which in turn act on the DNA strands (Ma et al., 2021). Chemical mutagens act directly on
326　DNA and manifest as nucleobase insertions and transitions (switching one nucleobase to another),
327　interference with transcription and replication, deamination, and, less frequently, strand breaks (Mba,
328　2013).[9]
329
330　Figure 3 provides a visual overview of common physical and chemical mutagenesis methods, with
331　categorizations based on similarities between the modes of action of the methods.
332
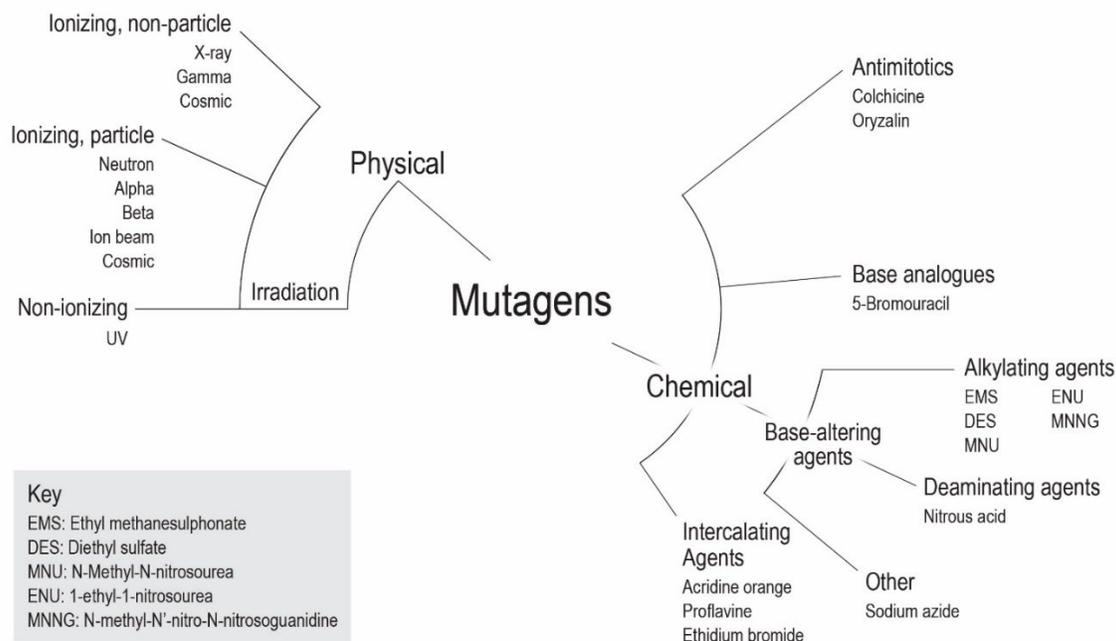
333
334　**Figure 3: Common physical and chemical mutagenesis techniques.**

---

[9] Deamination refers to the removal of an amino group. In the context of DNA, deamination refers to the replacement of the
nucleobase cytosine with the nucleobase uracil. After this occurs, uracil can pair with the adenine nucleotide, resulting in DNA
transition mutations (Mba, 2013).

335

336　*Common physical methods for inducing mutations*
337　The physical methods used for inducing genetic mutations involve irradiation, or radiation exposure
338　(Wiel et al., 2010). To induce mutations, microorganisms or plant tissues are exposed to either ionizing or
339　non-ionizing radiation.

340

341　Ionizing irradiation relies on high-energy frequencies of the electromagnetic spectrum. These frequencies
342　can dislodge electrons from atoms they come in contact with, creating ionic compounds (Mba, 2013). In
343　the context of induced mutagenesis, this electron dislocation may result in direct damage to DNA strands
344　or the creation of reactive oxygen species (ROS) within the cell, which proceed to act on DNA (Ma et al.,
345　2021). The most commonly used forms of ionizing radiation are gamma-rays and X-rays, although
346　particle, ion beam, and cosmic irradiation are also effective (Jankowicz-Cieslak et al., 2017; Mba, 2013).

347

348　The term "particle radiation" refers to radiation released by subatomic particles, such as neutrons, alpha
349　particles, and beta particles, all of which have a history of use as mutation inducers (Mba, 2013). Ion-
350　beam radiation is also used as a particle-based, physical mutagen in crop breeding (Ishikawa et al., 2012;
351　Yamaguchi, 2018). Ion-beam radiation relies on the acceleration of ions using particle accelerators
352　(Yamaguchi, 2018). Cosmic irradiation, which occurs outside of the Earth's atmosphere, exposes plant
353　tissues to numerous sources of radiation simultaneously, including both particle and non-particle forms
354　(Mba, 2013).

355

356　Non-ionizing irradiation can also induce mutations, although to a lesser degree than ionizing forms. UV
357　light is the primary source of non-ionizing radiation (Mba, 2013). UV light acts directly on DNA strands
358　to create mutations (Strzałka et al., 2020).

359

360　*Common chemical methods for inducing mutations*
361　Chemically induced mutations occur in plant tissues and microorganisms following exposure to certain
362　chemical agents. The chemical agents used for these purposes include:
363　　　• base analogs (substitutes)
364　　　• intercalating agents (chemicals that insert themselves into DNA)
365　　　• base altering agents (including deaminating and alkylating agents)
366　　　• antimitotics (chemicals that interfere with cell division)

367

368　Base analogs, such as 5-bromouracil, incorporate into the DNA strand and induce transition mutations
369　within the DNA (Mba, 2013).[10] Researchers do not frequently use base analogs to induce mutations;
370　however, two studies were identified that cite their use in the induction of mutations in plants and
371　microorganisms (Jafri et al., 2011; Khare & Arora, 2010).

372

373　Intercalating agents, such as ethidium bromide and acridine orange, insert themselves between the
374　nucleobases of a DNA strand and cause the strand to stretch abnormally (Mba, 2013). This stretching
375　process prompts DNA polymerase to insert an additional base, causing a frameshift mutation.[11]

376

377　The mode of action of base altering agents (i.e., deamination, alkylation, or indirect action) is used to
378　categorize these agents. Deaminating agents act directly on the amine groups of both adenine and
379　cytosine nucleobases (Michalczuk, 2022). Specifically, these chemicals remove amino groups from the
380　nucleobases, replacing them with hydroxyl groups (Zimmermann, 1977). These chemical changes result
381　in mismatches during DNA synthesis and lead to nucleobase substitutions, also known as point
382　mutations (Michalczuk, 2022; Zimmermann, 1977).

383

---

[10] Transition mutations refer to single base substitutions in which a purine is replaced by the other purine, or a pyrimidine is replaced by the other pyrimidine. In transversion mutations, another type of single base substitution, a purine is replaced by a pyrimidine or vice-versa (Mba, 2013). See Inset 1 for additional information on purines and pyrimidines.
[11] Frameshift mutations occur following an insertion or deletion (INDEL) of one or more base pairs. Depending on the number of inserted or deleted base pairs, this can dramatically change the amino acid sequence encoded by the genes, and alter the resulting protein (Mba, 2013).

384 Alkylating agents include several mutagens that function by binding alkyl groups onto nucleobases
385 (Michalczuk, 2022). Following alkyl group attachment, the nucleobase either degrades to leave a gap in
386 the DNA strand, or misrepairs to create a point mutation (Mba, 2013; Michalczuk, 2022). Ethyl
387 methanesulfonate is the most commonly used chemical mutagen in mutation breeding work and falls
388 into the category of alkylating agents (Jankowicz-Cieslak et al., 2017; Mba, 2013).
389
390 Other chemical mutagens, such as sodium azide, indirectly act on DNA strands and repair mechanisms
391 (Owais & Kleinhofs, 1988). Although the specific mechanism of mutagenic action is not known, sodium
392 azide induces point mutations, primarily transition mutations (Gruszka et al., 2012).
393
394 Antimitotics comprise a unique group of chemical mutagens that do not interfere with DNA directly but
395 lead to chromosome doubling or polyploidy through the prevention of mitosis (Trojak-Goluch et al.,
396 2021). The most commonly used antimitotics are colchicine and oryzalin, which appear under several
397 brand names (Trojak-Goluch et al., 2021).
398
399 **How and why are these methods used in agricultural production, generally?**
400
401 Induced mutagenesis creates novel mutations that are useful to agricultural production.
402
403 *Plants and induced mutagenesis*
404 The method breeders and researchers use to induce mutations depends on whether the crop is
405 propagated vegetatively or with seed, as well as the type of plant tissue treated (Shu, Forster et al., 2011).
406
407 In seed-propagated plants, the seed is the ideal tissue to treat because it can be sorted and prepared for
408 treatment (e.g., dried to ideal seed moisture content, scarified) beforehand (Shu, Forster et al., 2011).
409 Researchers can treat vegetatively propagated crops in their vegetative or seed forms, depending on
410 which method maximizes the desired mutagenic results. Researchers treating vegetative tissue consider
411 how to avoid or accommodate the occurrence of chimeras, organisms in which adjacent cells have
412 differences in their genetic codes. Strategies for navigating chimeras include (Shu, Forster et al., 2011):
413 • inducing mutations on single plant cells from adventitious buds[12]
414 • taking cuttings from plants showing desired traits (only possible when the phenotype is readily
415   visible)
416 • inducing mutations within *in vitro* cultures, such as callus tissue growing on agarose
417
418 After mutagenic treatment in seed-propagated crops, the M_1 (first generation) seedlings are grown to
419 produce M_2 (second generation) seed. The M_2 seed is grown, and phenotypic selection for desired
420 characteristics occurs. Phenotypic selection involves identifying desirable plants based on traits of
421 interest, such as plant height, flower color, and yield, while eliminating those that do not meet the target
422 criteria for the trait (Shu, Forster et al., 2011).
423
424 Vegetatively propagated crops generally do not undergo selection in multiple generations. Instead, the
425 M_1V_1 (i.e., the first vegetative plants that emerges from a parent that has been subjected to mutagenesis) is
426 grown until vegetative cuttings can be taken to produce the M_1V_2 "generation" (Suprasanna &
427 Nakagawa, 2011). Since no sexual reproduction has occurred, use of the term "generation" here differs
428 from how the term is used in seed-propagated crops. Selection may begin in the M_1V_2 generation and
429 continue through the M_1V_4 generation, as the propagated tissue becomes more consistent in appearance
430 and the incidence of chimeras disappears. The timeline for this process may extend longer, depending on
431 the crop. For example, following induced mutagenesis, tulips may undergo selection through the M_1V_8,
432 as breeders wait for the development of a full tulip bulb that contains the desired mutation (Suprasanna
433 & Nakagawa, 2011).
434

---

[12] Adventitious buds are buds that appear in any atypical location on a plant (i.e., not the leaf apex). This can include buds growing from stems, trunks, roots, etc. (Merriam-Webster, 2023).

435 Although the methods differ for tissue generation, seed-propagated and vegetatively propagated crops
436 undergo the same selection process. Figure 4 provides a visualization of this process, in which
437 undesirable phenotypes are identified in the field or greenhouse and removed from the population.
438
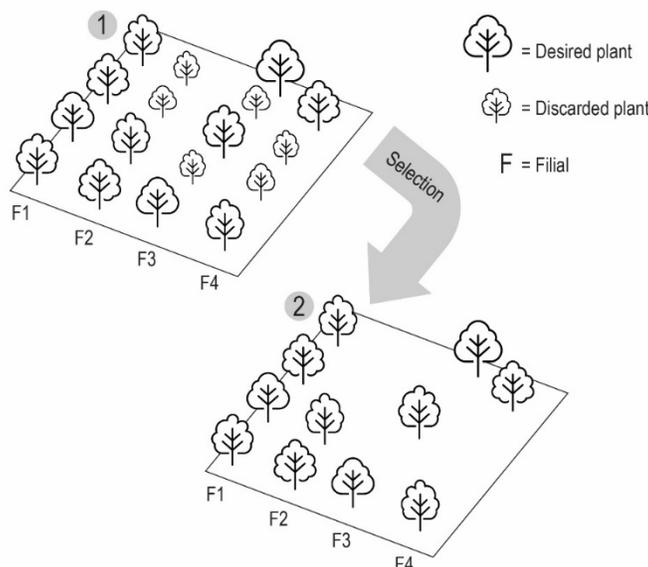
439
440 **Figure 4: Phenotypic selection of desired plant varieties. F_1, F_2, F_3, and F_4 represent a series of different**
441 **generations (offspring) produced from controlled reproduction.**

442
443 The phenotypic selection process repeats during the M_3 generation (similar to F_3, except that the M_3
444 ancestor was subject to mutagenesis), after which the mutant variety will progress through the traditional
445 breeding process (Shu, Forster, et al., 2011). This process involves successive rounds of selection and
446 stabilization of the desired traits. In some instances, a mutant variety is produced through this process
447 and released to the public. In other cases, a mutant variety is developed and used as a parent in another
448 cross to produce a desired variety. When a mutant variety is used as a parent, the offspring are
449 considered mutant varieties.
450
451 *Marker assisted selection (MAS)*
452 The timeline for developing new varieties through phenotypic selection can be extremely long. Many
453 plant breeders choose to incorporate some form of marker-assisted selection (MAS, see Inset 2) into their
454 breeding process. MAS can significantly reduce the time required to develop a desired variety and is not
455 considered an excluded method for use in organic production and handling (NOSB, 2022).
456

457   **Inset 2: Marker-assisted selection (MAS)**

> Molecular markers (or simply "markers") are used to track genetic variation in plants and other organisms. Markers are often variations in DNA called "polymorphisms" that are associated with a gene of interest. These markers can vary in size from a single nucleobase to several hundred base pairs. To identify markers within an organism, researchers extract DNA from tissues such as leaves and analyze them using specific techniques tailored to the marker of interest.
>
> Frequently, the analysis involves the use of polymerase chain reaction (PCR) machines to amplify regions of interest in the DNA provided. This may be followed by running the PCR product through an agarose gel, using electrical current, to determine the presence or absence of a marker.
>
> Genetic studies have identified many markers that are linked to desirable phenotypic traits, as they are often located in, or otherwise interact with, relevant genes. This information can help identify which organisms possess or lack specific genes of interest.
>
> For plant breeders, identifying markers within a population of varieties can reduce the selection timeline. By growing plants to a stage where sufficient tissue can be collected for DNA extraction, breeders can efficiently identify desirable traits and select for them.
>
> Common molecular markers that are used in plant breeding applications include:
> - single-nucleotide polymorphisms (SNPs)
> - restriction fragment length polymorphisms (RFLPs)
> - amplified fragment length polymorphisms (AFLPs)
> - random amplified polymorphic sequences (RAPD)
> - cleavable amplified polymorphic sequences (CAPS)
> - single-strand conformation polymorphisms (SSCP)

458
459   Source: (Hasan et al., 2021)

460
461   *Targeting Induced Local Lesions IN Genomes (TILLING)*
462   MAS can be incorporated into the breeding process for mutant varieties as well. One version of this is
463   Targeting Induced Local Lesions IN Genomes, or TILLING (McCallum et al., 2000). In the TILLING
464   protocol, ethyl methanesulfonate, a chemical mutagen, induces mutations. The molecular markers
465   associated with these mutations are tracked using chromatography (specifically denaturing high-
466   performance liquid chromatography, or HPLC), a molecular screening tool (McCallum et al., 2000).

467
468   Figure 5 compares the timelines for both traditional breeding and induced mutagenesis breeding, as well
469   as the impact of MAS and TILLING on those respective timelines.

470
471   *Importance of genetic diversity*
472   Plant breeders rely on genetic diversity to develop new crop varieties that meet various metrics of
473   interest. Common goals of plant breeding programs might include the development of varieties that:
474   - are higher yielding.
475   - are resistant to specific diseases.
476   - can withstand environmental stressors, such as salinity, temperature extremes, or water stress.
477   - have specific quality metrics, such as unique flower color or leaf pigmentation.
478   - contain higher concentrations of desirable phytochemicals, such as specific vitamins and
479   nutrients.
480   - contain lower concentrations of undesirable phytochemicals, such as anti-herbivory compounds.
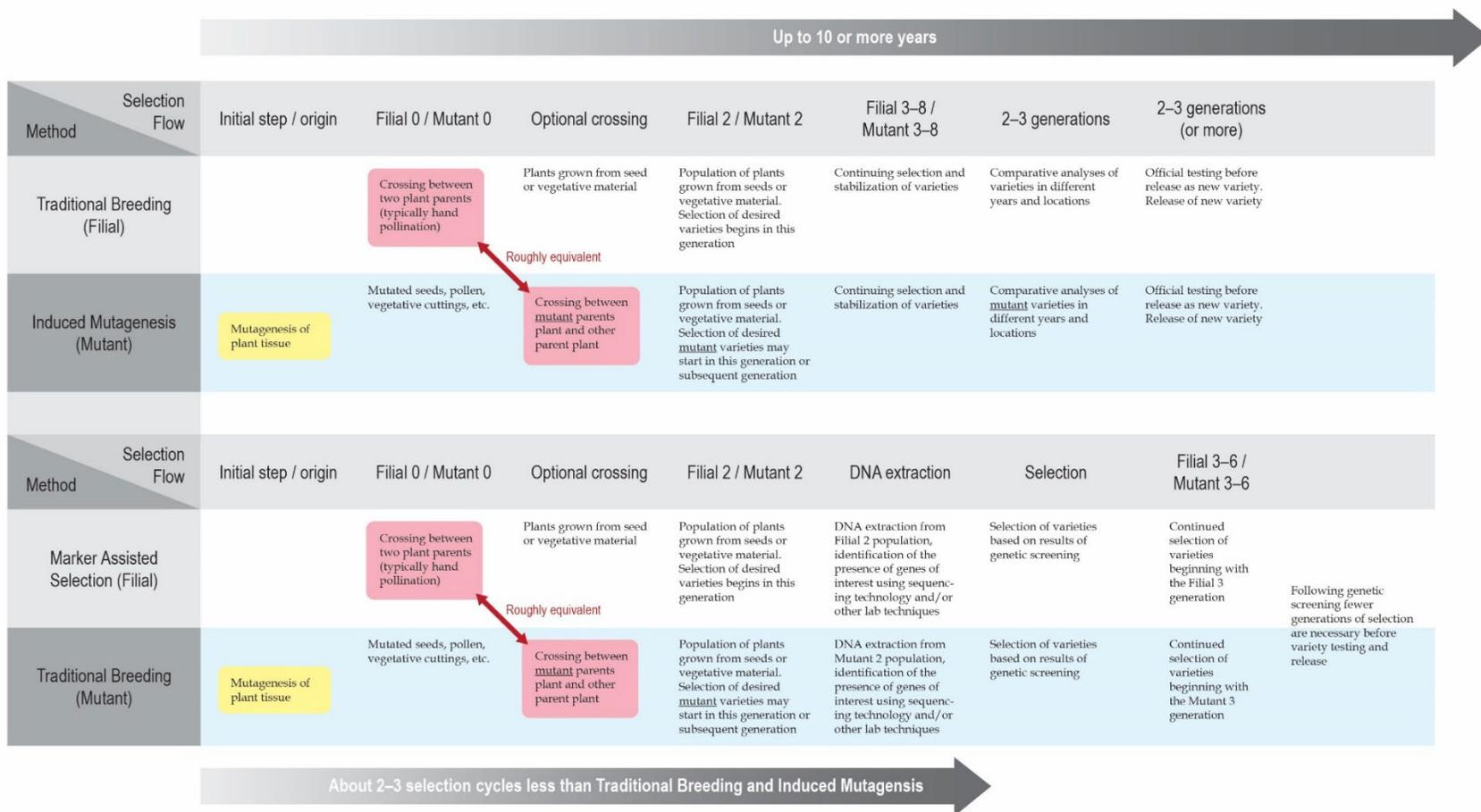
481
482   **Figure 5: Breeding timelines for traditional breeding, induced mutagenesis, MAS, and TILLING.**
483

484 Crop germplasm is cataloged and maintained in the United States through the USDA-ARS National Plant
485 Germplasm System (NPGS). The NPGS is a major source of genetic resources, including existing
486 varieties, breeding materials, landraces, and crop wild relatives (Byrne et al., 2018). Although this system
487 contains enormous genetic diversity, breeders have difficulty identifying which samples in the NPGS
488 may be useful to them (Byrne et al., 2018). Furthermore, breeders are frequently concerned about
489 incorporating new germplasm into their breeding program because doing this may result in the
490 introduction of undesirable genetic qualities, along with the trait of interest (Byrne et al., 2018; Mba,
491 2013). Thus, inducing mutations can be the most efficient (and sometimes only), means of expanding
492 genetic diversity within a crop without the use of *in vitro* nucleic acid techniques (Mba, 2013).

494 Some crop traits are under the control of several genes (such as yield or stress tolerance), while others
495 may be under the control of one or two genes (such as flower color and some types of disease resistance)
496 (M. M. Hasan et al., 2015). Traits controlled by single genes are generally the target of mutagenesis, as it is
497 difficult to achieve multiple, desired mutations within a single mutagenesis event (Shu, Forster, et al.,
498 2011).

500 Shu et al. (2011) provides several examples of how breeders might decide on how to use induced
501 mutagenesis within their work. If a breeder seeks to develop a disease-resistant version of an existing
502 variety, they would induce mutations in the variety and look for disease resistance in the mutant
503 populations. If the breeder aims to develop a new commercial variety of a crop with increased salinity
504 tolerance, they can choose one of the following approaches:
505   • mutate a variety that is high-yielding but salt-sensitive.
506   • mutate a variety that is lower-yielding but salt-tolerant.

508 Since it is easier to induce mutations that improve agronomic performance than it is to induce mutations
509 to improve salt tolerance, the lower-yielding but salt-tolerant variety would be a better target for
510 mutation breeding (Shu, Forster, et al., 2011).

512 As of March 2023, the Mutant Variety Database, maintained by the International Atomic Energy Agency,
513 contained 3,402 registered mutant varieties (Joint FAO/IAEA Centre of Nuclear Techniques in Food and
514 Agriculture, 2023). Among these, 48% of the varieties are cereals, 20% of the varieties are flowers or
515 ornamental crops, and 14% of the varieties are legumes and pulses. The database also lists oilseed crops,
516 vegetables, fiber crops, fodder, tree fruit, and other crops. Additional details on the existing registered
517 mutants, and the types of mutations that exist in these varieties, can be found in the *Historic Use* section of
518 this report.

520 *Microorganisms and induced mutagenesis*
521 In addition to the mutagenesis of plants, researchers induce mutations in microorganisms. The food
522 industry relies extensively on microorganisms to serve as fermentation agents, additives, preservatives,
523 and flavor enhancers (Yu et al., 2020). Naturally occurring or "wild type" microorganisms fall short of
524 industry standards. Specific issues include low yield, low stability, and undesired by-products when
525 using wild type microorganisms (Yu et al., 2020). Researchers also utilize mutagenesis to understand how
526 specific microorganisms interact with their environment, by creating mutant isolates for comparative
527 studies (Khare & Arora, 2010).

529 Mutagenized microbial and fungal isolates are grown on a growth medium such as King's B (KB) broth
530 or agarose gel and are screened for desired traits within the laboratory environment (Aleem et al., 2018;
531 Khare & Arora, 2010). It may take several generations of culture on a growth medium for a microbial or
532 fungal mutant to reach a stable genetic state, with some researchers reporting the need for 10+

533 generations (Wang et al., 2010). The ubiquity of mutant microorganisms is unknown, as they are not
534 tracked within the Mutant Variety Database.
535

536   **What are the approved legal uses of Induced Mutagenesis under other federal regulations?**
537   Describe the status of induced mutagenesis under applicable Federal Regulations (i.e., EPA, FDA, USDA
538   (including APHIS or FSIS), NIEHS, etc.)
539
540   *U.S. EPA*
541   The Environmental Protection Agency (EPA) considers genetic traits that are associated with pest
542   resistance (i.e., insect, weed, or disease resistance) and that are incorporated into plant genomes using *in*
543   *vitro* nucleic acid techniques to be pesticides, or "plant-pesticides." These pesticides are also known as
544   plant-incorporated protectants (PIPs). Under this classification, specific pest resistance traits are subject to
545   EPA regulation under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food,
546   Drug, and Cosmetic Act (FFDCA), and the Food Quality Protection Act (FQPA). As stated earlier, *in vitro*
547   nucleic acid techniques are already considered excluded methods, and not the subject of this report.
548
549   Per 40 CFR 174.25, the PIP traits may be exempt from the requirements of registration under FIFRA if the
550   genetic material responsible for the production of the pesticidal substance is from a plant that is sexually
551   compatible with the recipient plant, or it has never been derived from a source that is not sexually
552   compatible with the recipient plant. Additionally, per § 174.705, in order for a PIP-containing organisms
553   to be exempt from FIFRA registration requirements, any residues of inert ingredients (in other words,
554   non-PIP nucleic acids) must not be present at levels that would be considered injurious to human health.
555   Under these exemptions, plants that are produced through the use of chemically or physically induced
556   mutagenesis may be exempt from FIFRA requirements provided they meet the inert ingredients
557   regulation.
558
559   The EPA also regulates biopesticides comprised of microorganisms, which are labeled as microbial
560   pesticides. These microbial pesticides may be products of induced mutagenesis or genetic engineering. As
561   noted at § 158.2100, each new isolate of a microbial pesticide is a new active ingredient and must be
562   registered independently from other similar registered microbial pesticide active ingredient. Microbial
563   pesticides that have been modified through *in vitro* nucleic acid techniques may be subject to additional
564   data or information requirements.
565
566   *U.S. FDA*
567   In May 1992, the Food & Drug Administration (FDA) released the Guidance Document *Statement of Policy*
568   *– Foods Derived from New Plant Varieties* (U.S. FDA, 1992). Under this guidance, physical and chemical
569   mutagenesis are considered to be traditional breeding methods. The guidance outlines that that all new
570   plant varieties should be evaluated for safety and nutritional aspects, regardless of plant breeding
571   methods used.
572
573   The FDA notes that some products of mutagenesis, specifically enzymes, may be subject to regulation as
574   food additives, while others may be considered Generally Recognized as Safe (GRAS) under the Federal
575   Food, Drug, and Cosmetic Act (U.S. FDA, 2010). Per the document *Guidance for Industry: Recommendations*
576   *for Submission of Chemical and Technological Data for Food Additive Petitions and GRAS Notices for Enzyme*
577   *Preparations*, the agency requests that developers of enzyme products of mutagenesis provide information
578   about the identity, proposed use, intended technical effects, analysis methods for use in food, and all
579   safety reports conducted in regard to a new enzyme additive (U.S. FDA, 2010).
580
581   *USDA AMS*
582   The USDA Agricultural Marketing Service (AMS) maintains the National Bioengineered Food Disclosure
583   Standard (7 CFR part 66), which states that the labeling of bioengineered food is only required for *in vitro*
584   bioengineering. Food certified organic under the National Organic Program is exempt from regulation
585   under this standard, per 7 CFR 66.5(e).
586
587   *USDA APHIS*
588   Per 7 CFR part 340 (Movement of Organisms Modified or Produced through Genetic Engineering), the
589   USDA Animal and Plant Health Inspection Service (APHIS) is responsible for regulating genetically

590 engineered organisms; however, this does not extend to organisms altered through the mutagenic use of
591 chemicals or radiation, which the agency does not consider to be genetically engineered organisms.
592

593 **Status**

594
595 **Historic Use:**
596
597 Plant breeders as well as food and agriculture researchers have used induced mutagenesis widely
598 (including in organic production), throughout the past century (Mba et al., 2011). At the end of the 19th
599 century, the discoveries of X-rays, radioactivity, and radioactive elements provided a foundation for
600 understanding the mutagenic potential of radiation-based physical mutagens. The development of
601 chemical weapons during World Wars I and II led to increased knowledge of chemical mutagens, and
602 scientists began using these mutagens on a commercial scale in the 1950s and 1960s (Mba et al., 2011).
603
604 We were unable to identify any public databases that link seed or planting stock specifically used in
605 organic agriculture with its history of development. Identifying varieties of seed or planting stock used in
606 organic production, developed with induced mutagenesis is laborious. The USDA's Organic Integrity
607 Database does not provide detailed varietal information, and many staple crops are referred to in generic
608 terms, so surveying specific certified organic crop varieties is difficult. However, as described in the
609 *Summary of the Petition* section of this report, seed and planting stock from *in vivo* induced mutagenesis
610 techniques are currently allowed in organic production. These techniques are also widely used to
611 produce conventional seed and planting stock, which are also allowed in organic production in some
612 circumstances, per 7 CFR 205.204(a). Because of these facts, we assume that some of the seed and planting
613 stock used in organic production is derived from induced mutagenesis. As described below, there are
614 numerous registered mutant varieties.
615
616 *Historic use of induced mutagenesis on agricultural plants*
617 The use of physical and chemical mutagens to induce beneficial mutations in agricultural crops dates
618 back to the 1930s, when a mutant tobacco variety was developed through X-ray radiation (Jankowicz-
619 Cieslak et al., 2017). Since then, the Joint FAO/IAEA Centre of Nuclear Techniques in Food and
620 Agriculture has tracked the development and release of mutant varieties. The Mutant Variety Database
621 (MVD), their online database, contains information on both historic and modern mutant varieties (Joint
622 FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, 2023). As of the writing of this report,
623 there were 3,402 registered mutants listed in the MVD.
624
625 Data from the MVD provide insights into temporal and regional trends for induced mutagenesis. The
626 number of new mutant registrations increased dramatically in the 1970s and 1980s, with nearly half of the
627 new mutants produced in the past 90 years falling into this period (see Figure 7). The registration of new
628 mutants tapered slightly in the 1990s and 2000s and fell dramatically in the 2010s. This decrease is
629 attributed to an increased focus on transgenic methods in genetic modification (Michalczuk, 2022).
630 Regional differences indicate that most induced mutagenesis has occurred on the Asian continent, with
631 China and Japan registering 38.7% of all mutants within the database (see Figure 8). Mutants registered
632 by researchers in the United States comprise just 4% of the database.
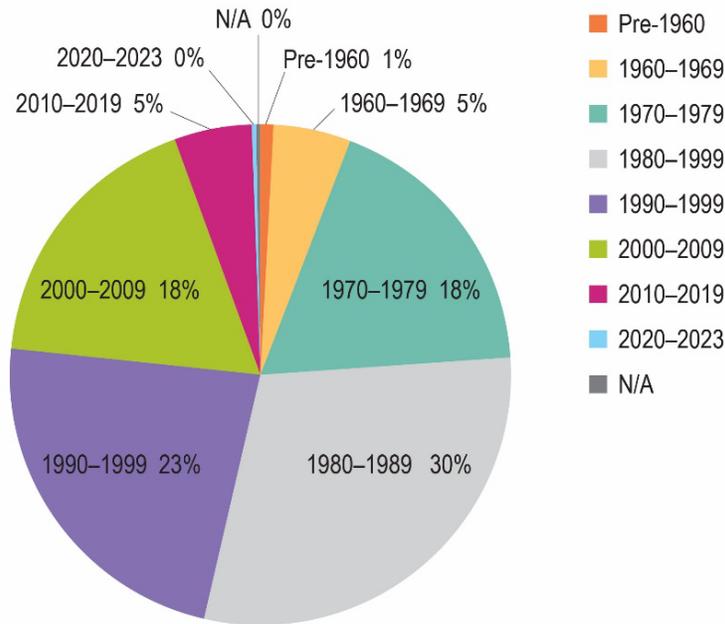
Date of registration of mutant varieties

633
634   **Figure 6: Date of initial registration of mutant varieties in the MVD.**
635

Number of registered mutants

636
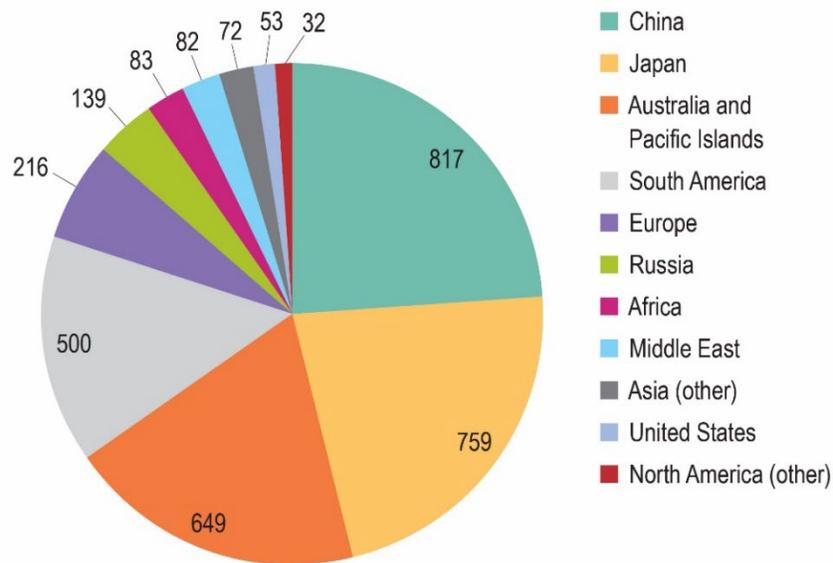637   **Figure 7: Country of origin of registered mutants.**
638

639   The MVD contains mutants from a diversity of crop types, including:
640   • cereals
641   • flowers
642   • legumes and pulses
643   • oilseed crops
644   • vegetables
645   • fiber crops
646   • fodder
647   • tree fruits
648
649   Cereals comprise the majority of registered mutants, at 48% of the database (see Figure 9). Cereals are
650   followed by flowers in ubiquity, at 20% of the database. Registered mutants include varieties developed
651   directly with induced mutagenesis or developed using mutant varieties as parents within a breeding
652   project. The majority of the registered mutants in the MVD are a result of the direct use of an induced
653   mutant; however, 29% of the mutants in the database are the product of using one or two mutants as
654   parents in the generation of a new variety (see Figure 10).
655
656   *Induced mutagenesis in barley*
657   The application of sodium azide leads to mutations in barley, particularly A/T to G/C transition
658   mutations (Olsen et al., 1993). In recent years, researchers have utilized sodium azide and other chemical
659   mutagens to develop mutant populations of barley for use in research and breeding applications. One
660   such example is the TILLMore population, which includes 1,605 unique mutants with phenotypic
661   variation in leaf characteristics, heading date, plant height, tillering, and disease resistance (Talamè et al.,
662   2008). Another example is the HorTILLUS population, which is comprised of 3,481 mutants with
663   variation in plant height and architecture, time of flowering, spike characteristics, and awn
664   characteristics, among other traits (Szurman-Zubrzycka et al., 2018). Both populations showed a
665   predominance of A/T to G/C mutations, and both were developed using a TILLING approach to
666   mutagenesis and molecular screening (Szurman-Zubrzycka et al., 2018; Talamè et al., 2008).
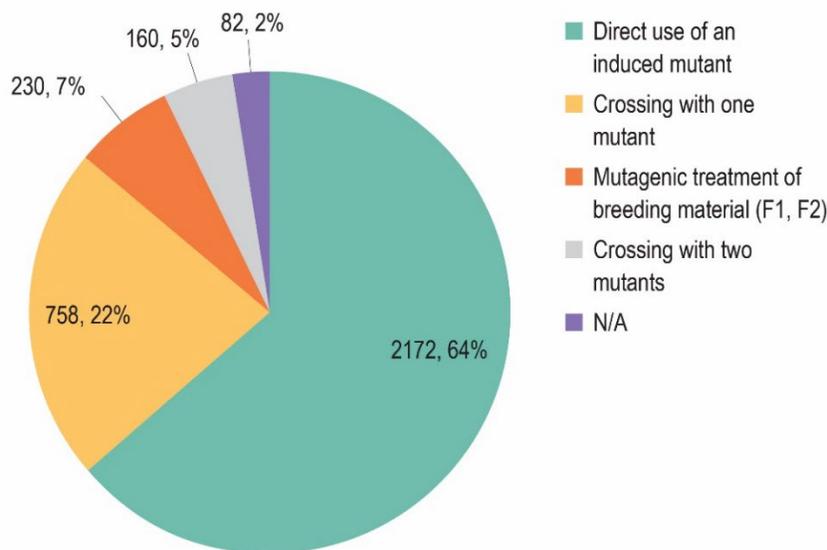667
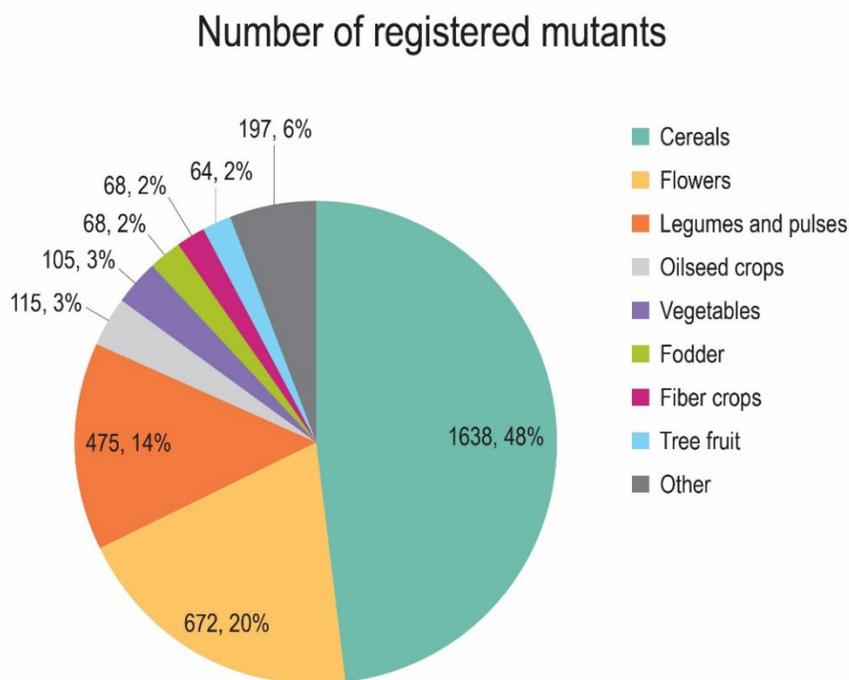
668
669   **Figure 8: Crop categorization of registered mutants.**
670

Development method of registered mutant

Figure 9: Origin of mutation of registered mutants in MVD.

There are over 300 registered barley mutants in the MVD. Recent and/or notable releases include the following varieties:

- "Haneumamochi" was released in 2019 in Japan. The variety was developed by treating the parent variety "Fiber Snow" with sodium azide. It contains a new "waxy" allele and has an improved endosperm composition with lower amylose and higher amylopectin (Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, 2023).
- "Diamant" was released in the Czech Republic in 1965. The variety was developed through the use of X-ray radiation. The mutant shows higher yield, shorter height, good grain and malting quality, and lodging resistance (Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, 2023). Mlčochová et al. (2004) noted that over 120 European spring barley varieties have "Diamant" in their breeding history. The MVD lists 68 of these.
- "Jotun," is a mutant variety that was developed in Norway in 1973 (Mickelson & Rasmusson, 1994). This variety is noted for a unique mutation leading to a semi-dwarf plant stature. Although this variety is noted in several papers, and as parent in the development of other mutant varieties such as "UC 829," it is not listed in the MVD (Mickelson & Rasmusson, 1994; Xu et al., 2017).

Induced mutagenesis in ornamental crops

Induced mutagenesis is used in flowers and other ornamental crops to generate new and interesting phenotypes (Yamaguchi, 2018). Mutations have been achieved through numerous methods including ion-beam radiation, colchicine exposure, and X-ray radiation (Manzoor et al., 2019; Reznik et al., 2021; Yamaguchi, 2018).

Flowers and ornamental crops comprise a substantial portion of the varieties registered in the MVD, with 672 varieties registered in the flowers category alone. Some notable releases include:

- The snapdragon, "Madame Butterfly," was developed using X-ray radiation and was released in the United States in 1966 (Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, 2023). This snapdragon showed a unique, open-flower morphology. There are a number of newer varieties that have "Madame Butterfly" in their parentage, including "Madame Butterfly Bronze," "Madame Butterfly Pink," and "Madame Butterfly Mix."

703   • The lilac, "Prairie Petite," was developed using particle radiation and was released in the United
704   States in 1995. The mutations induced included dwarfness and a change in leaf morphology. This
705   variety continues to be valuable to ornamental crop breeders (Lattier & Contreras, 2017).
706
707   Among the ornamental plants in the MVD, chrysanthemums are the most ubiquitous, with 285 unique
708   registered mutants. Furthermore, 19 registered ornamental plants in the MVD have undergone ploidy
709   manipulation using colchicine or oryzalin. Manzoor et al. (2019) note that ploidy manipulation is valuable
710   for generating diversity in the ornamental plant industry, and that colchicine can alter the ploidy levels of
711   floral crops such as lily, phlox, gladiolus, petunia, and marigold.
712
713   *Induced mutagenesis in rice*
714   As a major cereal crop, rice is the target of a significant amount of breeding work, some of which involves
715   induced mutagenesis. Physical methods for mutagenesis, including ion beam and gamma-ray radiation,
716   are more frequently utilized in rice mutagenesis than chemical methods (Ishikawa et al., 2012; F. Li et al.,
717   2019).
718
719   Rice breeders and researchers have developed a population of mutant rice from the variety IR64, the most
720   widely grown *indica* rice in southern Asia (Leung et al., 2001; Wu et al., 2005). As of 2005, the population
721   held over 60,000 mutant varieties developed through both physical and chemical methods (Wu et al.,
722   2005). Of these, 15,000 unique mutants have been evaluated using field trials and TILLING methods and
723   have been distributed to other breeders and researchers. Mutants in the population vary on dozens of
724   traits, including hull type, hull color, disease resistance, leaf morphology, leaf color, awn presence, and
725   tillering, among others (Leung et al., 2001; Wu et al., 2005).
726
727   Rice is the predominant cereal among listings in the MVD, with 874 registered varieties. Some notable
728   releases include:
729   • Offspring of the variety "Koshihikari," which was developed in Japan in 1956, and is the most
730   widely grown *japonica* variety in Japan (Ishikawa et al., 2012). While the initial "Koshihikari"
731   variety was developed without the use of mutagenesis, many subsequent varieties were
732   developed using induced mutagenesis and "Koshihikari" as a parent. Although there are many
733   advances beyond the original variety, all of the newer varieties are categorized as Koshihikari-
734   type rice (Kobayashi et al., 2018). The MVD lists 23 mutant varieties of Koshihikari-type rice,
735   while other sources list 29 mutant varieties (Kobayashi et al., 2018).
736   • Offspring of the variety "Hitomebore," which was developed in Japan in 1981, and is generally
737   grown in regions that are too cold for "Koshihikari" (Kobayashi et al., 2018; F. Li et al., 2019). The
738   MVD lists 10 varieties with "Hitomebore" as a parent. The most recent variety was released in
739   2019.
740
741   *Other examples of induced mutagenesis*
742   Beyond these examples of induced mutagenesis, there are a number of other important crop traits that
743   have been developed using mutation breeding. These include:
744   • red flesh in grapefruit (Louzada & Ramadugu, 2021)
745   • drought tolerance genes in tomato (Çelik et al., 2021)
746   • black spot (caused by *Alternaria alternate)* disease resistance in pear (Saito, 2016)
747   • dwarf height and lodging resistance in Durum wheat (Scarascia-Mugnozza et al., 1993)
748   • salt tolerance in barley (Forster, 2001)
749
750   *Historic use of induced mutagenesis on microorganisms in agriculture*
751   Researchers have been using induced mutagenesis to develop new microbial strains on an industrial scale
752   since the mid-1950s (Alikhanian, 1962). Initially, scientists used this method to enhance antibiotic
753   production in *Penicillium* bacteria (Alikhanian, 1962). Today, it is commonly used to improve agricultural
754   microorganisms' yield, stability, or by-products (Yu et al., 2020).
755

756 Aleem et al. (2018) used gamma ray radiation to develop mutant isolates of the fungus *Aspergillus oryzae*,
757 known as koji. Some of these koji mutants showed hyperproduction of α-amylase enzymes, which are
758 essential to the digestion of starches in the textile, brewing, and food industries. One mutant koji isolate
759 produced 125.8% more α-amylase than the non-mutant isolate, at a production rate that was
760 approximately 3x faster (Aleem et al., 2018).
761
762 Researchers developed a mutant library of the cyanobacteria *Spirulina platensis* to identify strains with
763 improved biomass for use as fermentation feedstock (Fang et al., 2013). The mutant library, developed
764 using atmospheric and room temperature plasma (ARTP), produced multiple strains with higher
765 carbohydrate content and increased growth rates. *Evaluation Question #1* includes more details on the
766 ARTP technique.
767
768 Pigment-producing microorganisms, such as those in the *Nannochloropsis* and *Chlorella* genera, are also
769 targets of mutation breeding (Aruldass et al., 2018). Using UV radiation, scientists have effectively
770 induced mutations that lead to increased carotenoid production. Microbially-derived pigments have
771 various industrial applications, including use as food colorants, and in the textile and leather industries
772 (Aruldass et al., 2018).
773
774 **Organic Foods Production Act, USDA Final Rule:**
775
776 The Organic Foods Production Act of 1990 (OFPA) does not mention induced mutagenesis or any other
777 genetic engineering terms. Induced mutagenesis does not appear on the National List, nor do the USDA
778 organic regulations at 7 CFR part 205 reference it. The term "mutagenesis" does not appear under the
779 definition for excluded methods at § 205.2.
780
781 **International Acceptance**
782
783 *Canadian General Standards Board Organic General Principles and Management Standards and Permitted*
784 *Substances List*
785 The Canadian General Standards Board Organic General Principles and Management Standards,
786 CAN/CGSB-32.310, does not mention induced mutagenesis; however, the 32.310 standard does provide a
787 definition of "genetic engineering" which clarifies that polyploidy induction does not fall under the
788 description of genetic engineering. Polyploidy can be induced through the use of chemical mutagens, and
789 therefore maybe be considered a result of induced mutagenesis. Neither induced mutagenesis nor
790 induced polyploidy appear on the Permitted Substances List (PSL), CAN/CGSB-32.311.
791
792 *CODEX Alimentarius Commission, Guidelines for the Production, Processing, Labelling and Marketing of*
793 *Organically Produced Foods (GL 32-1999)*
794 The Codex guidelines (GL 32-1999) include a provisional definition of *Techniques of genetic*
795 *engineering/modification*, which define the following as genetic engineering techniques: recombinant DNA,
796 cell fusion, micro and macro injection, encapsulation, gene deletion, and gene doubling. Traditional
797 techniques, such as conjugation, transduction and hybridization are specifically excluded from the
798 definition of genetic engineering techniques. Neither induced mutagenesis nor induced polyploidy are
799 mentioned in the guidelines.
800
801 *European Economic Community (EEC) Council Regulation, EC No. 834/2007 and 889/2008*
802 EC Regulation No. 834/2007 points to the definition of genetically modified organisms provided by
803 Directive 2001/18/EC of the European Parliament. This directive specifically exempts the following
804 techniques from consideration as genetic modification: in vitro fertilization, conjugation, transduction,
805 transformation, and polyploidy induction. There is no explicit mention of induced mutagenesis within
806 the Directive or EC Regulation No. 834/2007.
807
808 *Japan Agricultural Standard (JAS) for Organic Production*
809 The Japanese Agricultural Standard for Organic Products of Plant Origin and the Japan Agricultural
810 Standard for Organic Processed Foods both reference recombinant DNA technology. The use of living

811 organisms produced using recombinant DNA technology is prohibited by the Japanese Agricultural
812 Standard.
813
814 Within the Japanese Agricultural Standard, recombinant DNA technology includes those processes that
815 produce recombinant DNA. This involves the cutting and rejoining of DNA using enzymes in *in vitro*
816 environments, followed by insertion of this DNA into living cells. There is no mention of induced
817 mutagenesis or induced polyploidy.
818
819 *IFOAM – Organics International*
820 According to the IFOAM Norms, organic systems do not use genetically modified organisms or their
821 derivatives, nor does organic processing use irradiation (ionizing radiation). The IFOAM Norms define
822 genetic engineering techniques as those that use recombinant DNA, cell fusion, micro and macro
823 injection, and encapsulation. The Norms note that techniques such as conjugation, transduction, and
824 natural hybridization are not considered to be genetic engineering. The definition of irradiation (ionizing
825 radiation) includes the use of high-energy emissions from radio-nucleotides for the purpose of inducing
826 mutations for selection and breeding.
827
828 In reference to breeding for organic varieties, section 4.8.4 of the Norms notes that technical interventions
829 into the genome of the plants are not allowed, and that ionizing radiation is considered a technical
830 intervention. Chemical mutagenesis is not specifically mentioned.
831

832 **Evaluation Questions Specific to Organic Crop or Livestock Production**

833
834 **Evaluation Question #1: Describe the most prevalent processes used to induce mutations. Further,**
835 **describe how these mutations chemically change their host organisms.**
836
837 Scientists and breeders can induce mutations using physical or chemical methods. X-ray and gamma-ray
838 radiation, both forms of ionizing radiation, are the most common physical methods used (Mba, 2013)
839 while ethyl methyl sulfonate, sodium azide, N-methyl-N-nitrosourea, and colchicine are commonly used
840 chemical mutagens (Holme et al., 2019; Manzoor et al., 2019; Wiel et al., 2010).
841
842 In addition to X-ray and gamma-ray radiation (see Table 1, below), scientists and breeders use particle
843 (e.g., neutron, alpha, and beta), ion beam, cosmic, and UV radiation (Mba, 2013). Additional chemical
844 mutagens include base analogs, intercalating agents, and base altering agents (Mba, 2013). Scientists may
845 also use antimitotic chemicals to induce chromosome doubling (Trojak-Goluch et al., 2021).
846
847 **Table 1: Common physical and chemical mutagens and their action.**

Mutagen	Mode of action	Types of mutations produced	Citation
Ionizing radiation (including X-ray, gamma, particle, and ion beam irradiation)	Direct action forms ions within DNA strand; Indirect action adds –OH groups or removes H atoms from DNA strands.[13]	Direct action produces strand breaks (primarily double). Indirect action produces base deletions/substitutions and strand breaks (single and double).	(Becker & Sevilla, 1993; Kazama et al., 2011; F. Li et al., 2019; Ma et al., 2021; Mba et al., 2011; Pacher & Puchta, 2017; Ren et al., 2014; Riviello-Flores et al., 2022; Roldán-Arjona & Ariza, 2009)
Cosmic ray irradiation	Direct action forms ions and lesions within DNA strand; Indirect action adds –OH groups or removes H atoms from DNA strands; Microgravity conditions limit DNA repair mechanisms.	Direct action produces strand breaks. Indirect action produces base deletions/substitutions and strand breaks (single and double).	(Ferrari & Szuszkiewicz, 2009; Ma et al., 2021; Tepfer & Leach, 2017)

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[13] See section *What methods are used to induce mutations* for an explanation of direct vs. indirect action.

Mutagen	Mode of action	Types of mutations produced	Citation
UV irradiation	Direct action forms lesions within DNA strand; Indirect action adds –OH groups or removes H atoms from DNA strands.	Direct action produces dimers, which frequently lead to base substitutions and frameshift mutations during the process of DNA repair. Indirect action produces base deletions/substitutions and strand breaks (single and double).	(Kielbassa et al., 1997; Nakamura et al., 2021; Strzałka et al., 2020)
Atmospheric and room temperature plasma (ARTP)	Indirect action by chemically active species generated by plasma jet stream. These species include ROS and RNS. Indirect action adds –OH groups or removes H atoms from DNA strands.	Indirect action produces base deletions/substitutions and strand breaks (single and double).	(Arjunan et al., 2015; Fang et al., 2013; G. Li et al., 2008; Wang et al., 2010; Zhang et al., 2014)
Base analogs	Direct incorporation into DNA during replication, taking the place of a nucleobase. This leads to mispairing and mutations during transcription.	Direct action through nucleobase replacement leads to transversion mutations and small INDELs.	(Jafri et al., 2011; Jones & Neely, 2015; Khare & Arora, 2010; Leitão, 2011)
Intercalating agents	Integrate between nucleobases within DNA strand, causing a stretching effect.	Direct action through incorporation leads to the production of frameshift mutations.	(Leitão, 2011; Mba, 2013; Sayas et al., 2015).
Indirect base altering agents	Indirect action on DNA through the production of an intermediary metabolite which leads to the production of dimers and slows down DNA repair mechanisms	Dimer formation and lack of repair leads to individual base substitutions, with the majority of substitutions reported as transition mutations.	(Akinyosoye et al., 2021; Arenaz et al., 1989; Gruszka et al., 2012; Olsen et al., 1993; Owais & Kleinhofs, 1988; Talamè et al., 2008)
Deaminating base altering agents	Removal of amine groups from nucleobase and replacing them with –OH groups.	Direct action leads to base substitutions during DNA repair.	(Leitão, 2011; Michalczuk, 2022; Zimmermann, 1977)
Alkylating base altering agents	Addition of ethyl or methyl group to nucleobase, resulting in base degradation.	Direct action leads to base substitutions during DNA repair.	(Leitão, 2011; Mba, 2013; Michalczuk, 2022)
Antimitotics	Depolymerization of microtubules during the process of mitosis.	Direct action leads to production of cells with more than two sets of chromosomes.	(Ebrahimzadeh et al., 2018; Ganga & Chezhiyan, 2002; Manzoor et al., 2019; Ravelli et al., 2004; Trojak-Goluch et al., 2021)

848
849 *X-ray radiation (physical)*
850 X-rays are the oldest known tool for inducing mutations (Stadler, 1928). They are high-energy
851 electromagnetic waves produced by running a high-voltage current between a cathode and a heavy metal
852 anode (Mba et al., 2011). X-ray machines housed within a metal cabinet induce mutagenesis while
853 preventing radiation from traveling beyond the target area (Mba et al., 2011).
854
855 Successful radiation rates range from 100 to 3,000 Gy, with the ideal dose allowing for an approximate
856 survival rate of 20-50% (Arena et al., 2017; Arunyanart & Soontronyatara, 2002; Hung & Johnson, 2008;

857 Kikuchi et al., 2009; Reznik et al., 2021).[14] Doses may also be tuned to a specific duration, to maximize the
858 number of mutations without sacrificing the survival rates of treated tissues (Arunyanart &
859 Soontronyatara, 2002; Reznik et al., 2021). Tolerance for a radiation dose varies by tissue type, and the
860 optimum dose varies greatly across plant species (Riviello-Flores et al., 2022; Tanaka et al., 2010).
861
862 X-rays are capable of penetrating plant tissue up to a few centimeters, and interact with DNA to cause
863 point mutations or small chromosome deletions through ionizing action (Holme et al., 2019; Kikuchi et
864 al., 2009; Mba et al., 2011).[15] The mutations are produced either by the direct or indirect action of X-rays.
865 For radiation such as X-rays, 30-50% of damage is due to direct effects of the radiation on DNA, and 50-
866 70% is due to indirect effects (Becker & Sevilla, 1993). Direct action by radiation leads to the formation of
867 ions within the DNA strand, and subsequent strand breaks (Becker & Sevilla, 1993).
868
869 Indirect action produces reactive oxygen species (ROS) that damage DNA through oxidative attacks,
870 generally by the addition of an –OH (hydroxyl) group to double bonds, or through the removal of a
871 hydrogen atom from the deoxyribose sugar in the DNA backbone (Pacher & Puchta, 2017; Riviello-Flores
872 et al., 2022; Roldán-Arjona & Ariza, 2009). Oxidative attacks can remove a hydrogen atom from DNA's
873 sugar-phosphate backbone, creating a deoxyribose radical (Roldán-Arjona & Ariza, 2009). This radical
874 can cause single-strand or double-strand breaks in the DNA by interacting with nearby molecules. X-ray-
875 produced ROS may also harm other cellular macromolecules, resulting in additional reactive by-products
876 that can damage DNA (Roldán-Arjona & Ariza, 2009).
877
878 Most X-ray induced mutations result in the loss-of-function mutation of a specific gene, due to the
879 tendency towards single base changes and small deletions (Kikuchi et al., 2009). One study by Kikuchi et
880 al. (2009) reported that there were between 30-40 chromosome breaks per cell following the X-ray
881 radiation of hexaploid wheat. This study also found that X-ray irradiation produced wheat mutants with
882 more growth habit aberrations compared to other forms of irradiation, despite having similar numbers of
883 chromosome breaks (Kikuchi et al., 2009).
884
885 *Gamma ray radiation (physical)*
886 Gamma rays, like X-rays, are a form of ionizing electromagnetic radiation (Mba, 2013). Gamma rays and
887 X-rays overlap on the electromagnetic spectrum; however, gamma rays are higher energy overall (Mba,
888 2013). Researchers often use gamma rays produced from radioisotopes of cobalt-60 and cesium-137 (Mba,
889 2013; Michalczuk, 2022).[16] Gamma radiation can be applied acutely or chronically, with acute radiation
890 applied in small machines (called gammacells), and chronic radiation applied in larger gamma
891 greenhouses or fields (Ahmad et al., 2018; Mba, 2013). Optimum total gamma radiation doses for
892 mutation induction range from 10 to 2400 Gy, depending on the plant species and tissue treated (Riviello-
893 Flores et al., 2022).
894
895 Gamma rays induce mutations by directly and indirectly damaging DNA strands, causing base
896 modifications and strand breaks (Riviello-Flores et al., 2022). Rice mutants produced through gamma
897 radiation have an average of 57 single-strand breaks, 17.7 base deletions, and 5.9 base insertions (F. Li et
898 al., 2019). Compared to X-rays, gamma rays have a lower impact on plant growth habits, although some
899 damage is still observed (Hung & Johnson, 2008; Riviello-Flores et al., 2022).
900
901 *Particle radiation (physical)*
902 Particle radiation, also known as corpuscular radiation, encompasses the use of subatomic particles to
903 induce ionizing radiation (Mba, 2013; Mba et al., 2011). The most commonly utilized particles are alpha

---

[14] Gray (Gy) is the international system (SI) unit of measurement of absorbed radiation. This measurement can be used for any type
of radiation. It does not describe biological effects, only the absorbed energy per unit mass of tissue (U.S. NRC, 2021).
[15] Ionizing radiation includes highly energetic forms of radiation with variable abilities to pass through materials (e.g., wood, air,
water, and living tissue). Ionizing radiation deposits energy within the materials it passes through, causing molecular bonds to
break and electrons to be displaced (U.S. NRC, 2023).
[16] Radioisotopes are radioactive isotopes of an element. Isotopes are versions of an element with variable numbers of neutrons in
their nuclei. Radioactive isotopes tend to be unstable and have excess energy (ANSTO, 2023).

904 and beta particles, although neither particle source is relied on frequently for inducing mutations in
905 plants (Mba et al., 2011).
906
907 Alpha particles are comprised of two protons and two neutrons. They are emitted during the decay of
908 radioisotopes (i.e., radium, uranium, americium) (Mba et al., 2011; Ren et al., 2014). Alpha particles lose
909 energy very quickly, causing them to penetrate into tissues less than either X-rays or gamma rays (Mba et
910 al., 2011). One team of researchers found that alpha particle radiation at doses ranging from 1 to 100 Gy
911 can penetrate only 22 µm into plant tissue (Ren et al., 2014). Despite their lower penetration, the high
912 ionizing capacity of alpha particles makes them strongly radioactive, with doses of about 40 Gy
913 producing signs of genetic damage (Mba et al., 2011; Ren et al., 2014). Researchers report abnormal
914 growth in plants following the use of alpha particles. At low doses (<40 Gy), alpha particles appear to
915 stimulate germination and root length, while higher doses appear to suppress shoot growth or lead to
916 plant death (Ren et al., 2014).
917
918 Beta particles are high-energy electrons (or positrons) that are also emitted during the decay of
919 radioisotopes, such as phosphorus-32 or sulfur-35 (Mba et al., 2011). These particles are ionizing and can
920 cause direct and indirect damage to DNA, through similar mechanisms to other forms of ionizing
921 radiation. Historic varieties of cotton and rice were produced using beta particle radiation; however, like
922 alpha particles, beta particles have low penetrability and therefore limited applications in plant
923 mutagenesis (Mba et al., 2011).
924
925 *Ion-beam radiation (physical)*
926 Over the past 30 years, ion-beam radiation has been used to induce mutations in plants. This ionizing
927 radiation relies on particle accelerators to deposit radioactive ions in a localized region of tissue (Kazama
928 et al., 2011). Commonly used ions include ^{12}C, ^{14}N, ^{40}Ar, and ^{20}Ne, among others (Ma et al., 2021; Mba et
929 al., 2011). Ion-beam radiation has higher mutagenic effectiveness than other forms of ionizing radiation,
930 as calculated by dividing the frequency of mutations by the radiation dose (Li et al., 2019).
931 Ion-beam radiation is harnessed using particle accelerators, such as cyclotrons or synchrotrons, which
932 propel beams of particles toward target tissues (Kazama et al., 2011; Ma et al., 2021). These accelerators
933 may be classified as low-, medium-, or high-energy, although scientists do not typically use low-energy
934 accelerators to induce mutations (Ma et al., 2021). Researchers change the energy levels (measured in
935 electronvolts or eV per micrometer), ion type, and radiation dose to produce the desired number and
936 type of mutations (Kazama et al., 2011).
937
938 Ma et al. (2021) report energy ranges between 0.5-640 keV/µm have been successfully used to induce
939 mutations in plants. Kazama et al. (2011) explored energy level and dosage combinations for generating a
940 maximum number of mutants, finding that a 300-400 Gy dose at 30 keV/µm was the best combination in
941 the model plant *Arabidopsis thaliana*. In general, researchers prefer lower energy levels when the goal is to
942 produce small deletions or substitutions within DNA and prefer higher energy levels for producing
943 larger genetic changes (Ma et al., 2021). Researchers also change the ion type (e.g., argon instead of
944 carbon) to meet specific mutation goals, such as more complicated genetic changes (Ma et al., 2021).
945
946 As with other types of ionizing radiation, ion beams induce genetic mutations through direct and indirect
947 damage (Kazama et al., 2011; F. Li et al., 2019). Direct damage occurs through the ionization of DNA
948 substructures, while indirect damage results from ROS generation within the cell and subsequent damage
949 to DNA strands (F. Li et al., 2019). Most mutations produced through ion-beam radiation are base
950 substitutions or insertions/deletions (INDELs) that are less than 100 basepairs (bp) in size (Kazama et al.,
951 2011; F. Li et al., 2019). Large chromosomal rearrangements are also reported to occur more frequently
952 following ion-beam radiation than after gamma radiation (Li et al., 2019).
953
954 *Cosmic radiation (physical)*
955 Cosmic rays refer to the radiative forces produced by astrophysical sources outside Earth's atmosphere
956 that cannot penetrate the atmosphere (Ferrari & Szuszkiewicz, 2009). They are composed of 89% protons,
957 10% alpha-particles, ~1% heavier nuclei (i.e., atoms with high atomic numbers that are missing some

958   electrons), and include UV radiation produced by the sun (Ferrari & Szuszkiewicz, 2009; Tepfer & Leach,
959   2017). Researchers have successfully harnessed cosmic radiation to produce valuable mutations in crop
960   plants, but using this type of radiation in plant breeding programs is extremely limited by cost
961   considerations and the availability of retrievable satellites (Mba, 2013).

963   Cosmic radiation is either galactic (GCR), solar (SCR), anomalous (ACR), or ultra-high energy (UHECR),
964   with GCR and SCR being the most prevalent in induced mutagenesis (Ferrari & Szuszkiewicz, 2009; Ma
965   et al., 2021). GCR originates from sources within the Milky Way galaxy, but beyond the bounds of our
966   solar system. It includes radiation from stellar flares, stellar coronal mass ejections, supernova explosions,
967   and black hole jets. SCR is derived from solar events, like flares, and includes UV radiation (Ferrari &
968   Szuszkiewicz, 2009).

970   The energy level of cosmic radiation sources that reach the Earth's atmosphere can vary significantly.
971   Although high-energy particles do enter the low-orbit region, such as UHECR particles, these are far less
972   common and less relevant to cosmic ray mutagenesis (Ferrari & Szuszkiewicz, 2009; Ma et al., 2021).
973   Because the energy level of cosmic radiation is low, compared to other radiation sources used in
974   mutagenesis, researchers use chronic exposure to induce mutations in plants (Ferrari & Szuszkiewicz,
975   2009; Ma et al., 2021; Tepfer & Leach, 2017).

977   Tepfer & Leach (2017) exposed seeds of *Arabidopsis thaliana* and tobacco to cosmic radiation outside of the
978   International Space Station. The first radiation exposure, which lasted 558 days and had an estimated 296
979   mGy of total radiation exposure, resulted in a 23% survival rate of seeds. Despite observations of
980   abnormal growth in the first generation of plants, none of the seedlings had mutations that persisted into
981   subsequent generations (Tepfer & Leach, 2017). The second radiation exposure, which lasted 682 days
982   and had an estimated 461 mGy of total radiation exposure, produced structural and functional damage to
983   DNA (Tepfer & Leach, 2017). Through comparison to simulated cosmic radiation exposures, Tepfer &
984   Leach (2017) determined that most of the lethal effects of cosmic radiation were due to high-energy UV
985   radiation, and that non-UV cosmic radiation had a minor impact on DNA and plant growth.

987   Microgravity can exacerbate DNA mutations induced by cosmic ray exposure by suppressing the DNA
988   repair system (Ma et al., 2021; Moreno-Villanueva et al., 2017). Studies of microgravity during both space
989   flight and simulation studies suggest that within a microgravity environment, the DNA repair of double-
990   strand breaks and lesions is subdued, leading to a higher persistence of mutations in exposed tissues
991   (Moreno-Villanueva et al., 2017).

993   *UV radiation (physical)*
994   Although less commonly used than other forms of radiation, UV light can induce mutations in animal
995   and plant cells (Kielbassa et al., 1997; Roldán-Arjona & Ariza, 2009). It has a spectrum falling between
996   100-400 nm on the electromagnetic spectrum. UV light is emitted by the sun and most UV rays are
997   absorbed by the atmosphere, except for UV-A (320-400 nm) and some UV-B (290-320 nm) light (Diffey,
998   2002). A broader spectrum of UV rays is found beyond the Earth's atmosphere, as noted in greater detail
999   in the cosmic radiation section above (Ferrari & Szuszkiewicz, 2009). To induce mutations, UV light of
1000 varying energy levels can be produced using UV lamps and chambers (Nakamura et al., 2021).

1002 Nakamura et al. (2021) found that doses of 500 and 1000 J/m2 of UV-C radiation significantly increased
1003 mutation frequencies in *Arabidopsis thaliana*, while a 3000 J/m2 dose resulted in severe alterations of plant
1004 growth rate and form. Induced mutations were mainly base substitutions and some base deletions,
1005 wherein transition mutations are more frequent. Point mutations, including base substitutions and some
1006 INDELs, are directly produced by UV light-induced dimers (Nakamura et al., 2021). The UV photons can
1007 break bonds between paired strands of DNA, and cause nucleobases to repair with neighboring
1008 nucleobases on their DNA strand, forming what is known as a dimer (Roldán-Arjona & Ariza, 2009;
1009 Strzałka et al., 2020). Typically, dimers are recognized by DNA repair mechanisms, which in turn can
1010 become the source of point mutations when repairing the dimer lesions (Roldán-Arjona & Ariza, 2009;
1011 Strzałka et al., 2020).

1012
1013 Indirect alteration of DNA can occur via the UV-induction of ROS, primarily by lower energy UV light
1014 (Kielbassa et al., 1997; Roldán-Arjona & Ariza, 2009).
1015
1016 *Atmospheric and room temperature plasma (physical)*
1017 Over the past 15 years, researchers have identified and expanded the use of nonthermal atmospheric
1018 pressure plasma to induce mutations in microorganisms (Fang et al., 2013; Wang et al., 2010).[17] The
1019 specific approach, known as atmospheric and room temperature plasma (ARTP), relies on an ionized
1020 stream of plasma produced by metallic electrodes and a radio frequency power supply (Wang et al.,
1021 2010). This system can be run at room temperature and pressure, making it lower cost and more
1022 convenient than other plasma technology (Li et al., 2008; Wang et al., 2010).
1023
1024 While studying the applications of ARTP on microorganisms, Li et al. (2008) identified that it is the
1025 chemically activated species found within the plasma jet that act directly on DNA, and not the UV
1026 radiations, heat, charged particles, or electrical field that are also produced. Plasma treatment of
1027 microorganisms ranging from 30-120 seconds is sufficient to generate DNA strand breaks without fully
1028 degrading genetic material (Li et al., 2008). The genetic damage induced by ARTP is primarily attributed
1029 to the ability of plasma to generate ROS and reactive nitrogen species (RNS), which act on DNA (Arjunan
1030 et al., 2015). One study estimated that the mutation rate following ARTP treatment of fungal spores was
1031 30% (Wang et al., 2010). Twenty-one percent of those mutations produced desirable phenotypes (Wang et
1032 al., 2010).
1033
1034 In one example of the mutagenic potential of ARTP systems, a helium-plasma source was used to
1035 generate mutant strains of *Streptomyces avermitilis* (Wang et al., 2010). Following plasma jet treatment of
1036 the spores, the mutant populations were screened and cultured for 15 generations. From this, researchers
1037 identified a strain capable of producing antiparasitic avermectins (pesticidal compounds) at a rate that
1038 was 40% higher than the wild-type predecessor (Wang et al., 2010).
1039
1040 *Base analogs (chemical)*
1041 Chemical mutagens called base analogs can be incorporated into DNA during replication due to their
1042 physical similarity to the four nucleobases (Leitão, 2011). Base analogs cause frequent mispairing and
1043 mutations during DNA transcription (Leitão, 2011). Commonly used base analogs to induce plant and
1044 microbial mutations include 5-bromouracil (5-BU), 5-bromo-2'-deoxyuridine (BUdR), and 2-aminopurine
1045 (2AP) (Jafri et al., 2011; Mba, 2013). 2AP is effective in bacterial cells, while 5-BU and BUdR are preferred
1046 for plant mutagenesis. Base analogs produce transversion mutations and small INDELs (Leitão, 2011).
1047
1048 As a tool in plant breeding, Jafri et al. (2011) found that even small doses of 5-BU were effective for
1049 inducing mutations in chicory (*Cichorium intybus*), with a 0.02% concentration being ideal for inducing the
1050 most mutations while avoiding the deleterious effects that higher concentrations had on plant growth
1051 later on. However, higher doses of the mutagen induced pollen sterility in the M_1 generation and caused
1052 a subsequent reduction in seed production. Beyond this work in chicory, the use of base analogs in plant
1053 breeding is limited; although Leitão (2011) notes that there is one malting barley variety "Fuji Nijo II"
1054 produced through a combination of gamma radiation and BudR treatment.
1055
1056 The base analog 2AP fluoresces under certain environmental conditions, making it an excellent tool for
1057 testing DNA structure and dynamics (Jones & Neely, 2015). Thus, base analogs have numerous potential
1058 applications in plant and microbial research and industries.
1059

---

[17] Plasma is an ionized gas composed of charged particles, radicals, photons (visible and UV), and electromagnetic fields. The forms
of plasma that can be generated at ambient pressures and temperatures (i.e., atmospheric and room temperature plasma) can be
thermal or nonthermal. Nonthermal ARTP plasma is capable of generating biologically active chemical agents, including reactive
oxygen species (ROS) and reactive nitrogen species (RNS) (Arjunan et al., 2015).

1060 *Intercalating agents (chemical)*
1061 Intercalating agents are chemicals that can insert themselves between nucleobases in DNA, causing a
1062 stretching effect (Leitão, 2011; Mba, 2013). These chemicals are typically used as dyes in biological
1063 studies, such as to visualize DNA (Leitão, 2011; Sayas et al., 2015).
1064
1065 Examples of intercalating agents include ethidium bromide, acridine orange, Gelred, and proflavine
1066 (Leitão, 2011; Mba, 2013; Sayas et al., 2015). The direct incorporation of these compounds into a DNA
1067 strand can cause frameshift mutations (Leitão, 2011; Mba, 2013).
1068
1069 Although intercalating agents are known to be mutagenic in yeasts and bacteria, their potential for
1070 inducing mutations in plants is not well established (Leitão, 2011; Sayas et al., 2015).
1071
1072 *Base-altering agents (chemical)*
1073 Base-altering agents cause point mutations in DNA by changing individual nucleobases. These chemicals
1074 are categorized based on their modes of action, such as indirect action, deamination, or alkylation.
1075
1076 Sodium azide (NaN3) can act as an indirect base-altering agent by reducing the efficiency of DNA repair
1077 in plants, animals, and bacteria (Gruszka et al., 2012; Owais & Kleinhofs, 1988). Sodium azide is
1078 metabolized within the cell, forming a metabolite known as L-azidoalanine (Gruszka et al., 2012). Once L-
1079 azidoalanine forms within the cell, it alters the formation of standard nucleobases, though the exact
1080 mechanisms for this are still unknown (Owais & Kleinhofs, 1988; Talamè et al., 2008). Errors during the
1081 repair of these alterations lead to the production of point mutations, primarily transition mutations
1082 (Olsen et al., 1993). L-azidoalanine also inhibits an enzyme that is essential for energy synthesis within
1083 the cell, slowing down DNA repair overall and exacerbating errors during its repair (Gruszka et al., 2012).
1084 Not all plants and animals contain the cellular components necessary for the conversion of sodium azide
1085 to L-azidoalanine, so the use of sodium azide is limited to specific crops (Arenaz et al., 1989; Gruszka et
1086 al., 2012). Barley and African yam beans are among the successful targets of mutagenesis with this
1087 chemical (Akinyosoye et al., 2021; Olsen et al., 1993).
1088
1089 Nitrous acid was first used in molecular genetics research in the mid-1900s (Zimmermann, 1977). It
1090 removes the amine group (i.e., deamination) from nucleobases and replaces it with a hydroxyl group,
1091 leading to base substitutions during DNA repair and transcription (Michalczuk, 2022; Zimmermann,
1092 1977). However, its instability requires quick mutagenic treatments, making it more commonly used for
1093 improving fungi and bacteria rather than plant materials, which require longer exposure to mutagens
1094 (Leitão, 2011).
1095
1096 Alkylating agents are base-altering agents that add ethyl or methyl groups to nucleobases in DNA (Mba,
1097 2013; Michalczuk, 2022). The most commonly used alkylating mutagen is ethyl methanesulfonate (Leitão,
1098 2011; Michalczuk, 2022). Ethyl methanesulfonate primarily targets guanine, modifying it into a form that
1099 DNA polymerase reads as adenine, causing a base substitution (Michalczuk, 2022). Ethyl
1100 methanesulfonate is typically used at concentrations of 10-100+ millimolar (mM) (Leitão, 2011). In
1101 comparison, other alkylating agents like N-methyl-N-nitrosourea and 1-ethyl-1-nitrosourea are used at
1102 much lower concentrations of 5-6 mM and 0.2-1mM, respectively (Leitão, 2011).
1103
1104 Some other alkylating agents include Diethyl sulfate and N-methyl-N'-nitro-N-nitrosoguanidine,
1105 although these are less commonly used as mutagens (Leitão, 2011; Mba, 2013).
1106
1107 *Antimitotics (chemical)*
1108 Polyploids are organisms with more than two sets of chromosomes (Woodhouse et al., 2009). Polyploids
1109 occur naturally in some plants, along with some fish, amphibians, and other organisms (Woodhouse et
1110 al., 2009). Inducing polyploidy can be achieved through exposure to natural stressors or antimitotic
1111 chemicals, which disrupt the normal process of mitosis (Trojak-Goluch et al., 2021).
1112

1113 Antimitotic chemicals, such as colchicine and oryzalin, are commonly used to induce polyploidy in plant
1114 breeding (Manzoor et al., 2019; Trojak-Goluch et al., 2021). Colchicine is an alkaloid extracted from the
1115 seeds and roots of the autumn crocus, while oryzalin is a synthetic herbicide (Trojak-Goluch et al., 2021).
1116 These chemicals depolymerize microtubules during mitosis, inhibiting their formation and attachment to
1117 chromosomes during cell division (Ravelli et al., 2004; Trojak-Goluch et al., 2021).[18] As a result, daughter
1118 cells may have either no chromosomes or double the number of chromosomes (Manzoor et al., 2019).[19]
1119 The latter is a polyploidy, while the former is not viable.

1121 The ideal application rate of colchicine is 0.1%-0.8% in an aqueous solution, while oryzalin can be
1122 effective at much lower concentrations, such as 0.005% (Ebrahimzadeh et al., 2018; Ganga & Chezhiyan,
1123 2002). Although colchicine is more commonly used, oryzalin is reportedly more effective in inducing
1124 tetraploids at lower concentrations (Ganga & Chezhiyan, 2002; Manzoor et al., 2019). Antimitotics can be
1125 applied to shoots, buds, roots, callus tissue, or pre-germinated seeds to achieve the desired results
1126 (Trojak-Goluch et al., 2021).

1128 Inducing polyploidy with antimitotic compounds can be valuable in creating plant varieties with
1129 improved traits, such as disease resistance or increased yield (Comai, 2005). However, using antimitotics
1130 may result in low survival rates and poor growth for the resulting polyploid plants, and it may be
1131 challenging to control the level of induced polyploidy (Bretagnolle & Thompson, 1995).

1133 **Evaluation Question #2: Are excluded methods used in the production of physical energy sources or**
1134 **chemicals that are used to induce mutations?**

1136 Numerous physical and chemical methods induce mutations (see Table 2, below). In the following
1137 section, we will discuss whether excluded methods are used in the production of these substances. We do
1138 not discuss whether induced mutations themselves are the result of excluded methods.

1140 None of the physical or chemical methods used to induce mutations are explicitly included in the
1141 definition of excluded methods at 21 CFR 205.2:
1142   A variety of methods used to genetically modify organisms or influence their growth and
1143   development by means that are not possible under natural conditions or processes and are not
1144   considered compatible with organic production. Such methods include cell fusion,
1145   microencapsulation and macroencapsulation, and recombinant DNA technology (including gene
1146   deletion, gene doubling, introducing a foreign gene, and changing the positions of genes when
1147   achieved by recombinant DNA technology). Such methods do not include the use of traditional
1148   breeding, conjugation, fermentation, hybridization, in vitro fertilization, or tissue culture.

1150 According to their most recent recommendation on the topic, the NOSB (2022) considers inducing
1151 mutations through exposure to the following to be "TBD" with respect to excluded methods status:
1152   • UV light
1153   • chemicals
1154   • irradiation
1155   • other stress

1157 *Physical methods*
1158 Physical methods primarily rely on ionizing radiation from radioisotopes and X-ray facilities (Ahmad et
1159 al., 2018; Ma et al., 2021; Mba, 2013; Michalczuk, 2022). In certain applications, ionizing radiation is
1160 prohibited in organic production and handling, per 7 CFR 205.105(f). This section of the organic

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[18] Microtubules are microscopic structures found within cells and are essential to cellular division. During cellular division, the microtubules attach to the centers of chromosomes and pull one side of the chromosomes (i.e., a chromatid) to opposite sides of the cell. The cell then divides, forming two daughter cells, and the single chromatid is replicated so that two paired chromatids (i.e., a chromosome) exist in each new daughter cell (Suza & Lee, 2021).
[19] Daughter cells are the product of cellular division in non-reproductive tissues, a process known as mitosis. Two genetically-identical cells are formed from the reproduction and division of the one initial parent cell (Trojak-Goluch et al., 2021).

1161 regulations relies on the FDA's definition of ionizing radiation at 21 CFR 179.26. The definition states that
1162 ionizing radiation is limited to:
1163   (a) Energy sources. Ionizing radiation is limited to:
1164   (1) Gamma rays from sealed units of the radionuclides cobalt-60 or cesium-137.
1165   (2) Electrons generated from machine sources at energies not to exceed 10 million
1166   electron volts.
1167   (3) X rays generated from machine sources at energies not to exceed 5 million electron
1168   volts (MeV), except as permitted by paragraph (a)(4) of this section.
1169   (4) X rays generated from machine sources using tantalum or gold as the target material
1170   and using energies not to exceed 7.5 (MeV).
1171
1172 The subsequent limitations on the uses of ionizing radiation are described in 21 CFR 179.26(b). However,
1173 these uses are all related to food disinfestation and foodborne pathogen control. In the preamble to the
1174 Final Rule (65 FR 80548), the NOP clarifies that it is only the uses that are allowed by the FDA at
1175 21 CFR 179.26 that are prohibited by the Final Rule in organic production. Therefore, 7 CFR 205.105(f)
1176 does not strictly prohibit the use of ionizing radiation (such as gamma rays) to induce mutations for plant
1177 breeding and microbial strain development.
1178
1179 Atmospheric and room temperature plasma treatments (ARTP, see *Evaluation Question #1*, above) do not
1180 directly involve radiation to induce mutations, but instead rely on ionized plasma streams generated by
1181 running radio waves through metallic electrodes (Wang et al., 2010). These treatments do not clearly fall
1182 under any current definitions for ionizing radiation or excluded methods.
1183
1184 Cosmic ray radiation contains ionizing and non-ionizing radiation; however, it differs from other forms
1185 of ionizing radiation in that it is not produced through human action (Ferrari & Szuszkiewicz, 2009;
1186 Tepfer & Leach, 2017).
1187
1188 UV radiation is non-ionizing. In the context of induced mutagenesis, UV radiation is artificially produced
1189 through the use of UV lamps and chambers, despite being a naturally-occurring form of radiation
1190 produced by the sun (Kielbassa et al., 1997; Roldán-Arjona & Ariza, 2009).
1191
1192 Chemical mutagenesis involves the use of synthetic chemicals, including ethyl methanesulfonate, 1-ethyl-
1193 1-nitrosourea, N-methyl-N-nitrosourea, and colchicine. (Leitão, 2011). None of these chemicals are
1194 produced in a manner that could be considered nonsynthetic, according to Guidance NOP 5033-1:
1195 *Decision Tree for Classification of Materials as Synthetic or Nonsynthetic* (NOP, 2016).
1196
1197   **Table 2: The origins of commonly used physical and chemical mutagens.**

Mutagen	Source	Citations
X-ray irradiation	X-rays are produced by running a high voltage current between a cathode and a heavy metal anode, typically within the confines of a metal cabinet or other machine housing.	(Mba et al., 2011)
Gamma irradiation	Gamma rays are produced during the radioactive decay of radioisotopes like cobalt-60 and cesium-137. They can be harnessed and applied using gammacells, gamma greenhouses, or gamma fields.	(Ahmad et al., 2018; Mba, 2013; Michalczuk, 2022)
Particle irradiation	Subatomic particles used in particle radiation are produced during the radioactive decay of a range of radioisotopes.	(Mba et al., 2011; Ren et al., 2014)
Ion beam irradiation	Ion-beam radiation uses particle accelerators, like cyclotrons, to move radioactive ions towards a target. The particles used are typically radioactive ions, such as ^{12}C, ^{14}N, ^{40}Ar, and ^{20}Ne.	(Kazama et al., 2011; Ma et al., 2021; Mba et al., 2011)
Cosmic ray irradiation	Cosmic rays are radiative forces produced by natural astrophysical sources. In order to use them in mutagenesis, plant tissues must be taken beyond Earth's atmosphere and exposed.	(Ferrari & Szuszkiewicz, 2009; Tepfer & Leach, 2017)

Mutagen	Source	Citations
UV irradiation	UV light is naturally emitted by the sun; however, UV lamps and chambers can produce UV light of varying energy levels to induce mutations.	(Kielbassa et al., 1997; Roldán-Arjona & Ariza, 2009)
Atmospheric and room temperature plasma (ARTP)	Radio frequencies are run between bare-metallic electrodes at ambient temperature and pressure, producing a plasma stream that contains biologically active chemical species.	(Arjunan et al., 2015; Fang et al., 2013; G. Li et al., 2008; Zhang et al., 2014)
5-Bromouracil (5-BU)	To produce 5-BU for use as a chemical mutagen, the RNA nucleobase uracil is mixed with a solvent and catalyst. This mixture is heated and exposed to a brominating agent before cooling. Raw crystals of 5-BU are produced as the solution cools.	(Jafri et al., 2011; Leitão, 2011; Yuanqing et al., 2015)
Ethidium bromide	Synthesized from reaction between acridine and ethyl bromide, which produced ethidium. This intermediary is then brominated by exposing ethidium to bromine in the presence of a catalyst.	(Graves et al., 1977; Leitão, 2011).
Sodium azide (NaN$_3$)	Ammonia is reacted with molten sodium at 350ºC, producing sodium amide. Sodium amide is then reacted with N$_2$O at 230ºC. Sodium azide is isolated from the resulting products through dissolution in water and evaporation.	(Owais & Kleinhofs, 1988; PubChem, 2023f)
Nitrous acid (HNO2)	Nitrous acid may be formed by the reaction of a strong acid with an inorganic nitrite. Sodium nitrite is most commonly used in the production of nitrous acid.	(PubChem, 2023d; Zimmermann, 1977)
Ethyl methanesulfonate (EMS)	EMS is produced through the reaction of methanesulfonic anhydride, the acid anhydride of methanesulfonic acid, and ethyl alcohol.	(Leitão, 2011; PubChem, 2023a)
N-methyl-N-nitrosourea (MNU)	MNU is produced through the reaction of sodium nitrite and aqueous methylurea nitrate.	(PubChem, 2023e)
1-ethyl-1-nitrosourea (ENU)	ENU is formed from the reaction of N-ethylurea with nitrous acid.	(PubChem, 2023c)
Colchicine	Colchicine is extracted from seeds and roots of autumn crocus (*Colchicum autumnale* L.) using ethanol, water, ether, and/or chloroform. The purified crystals of colchicine are produced with repeated washes with chloroform. Some chloroform remains complexed with the colchicine in the crystalline form.	(Manzoor et al., 2019; PubChem, 2023b)
Oryzalin	Oryzalin is produced from the reaction of 4-amino-3,5-dinitrobenzenesulfonyl chloride with ammonium hydroxide at temperatures between 100-200ºC.	(Eli Lilly and Company, 1975)

1198

1199 **Evaluation Question #3: Describe the ecotoxicity and mode of action of traits created by induced**
1200 **mutations within the environment.**

1201

1202 Induced mutagenesis methods are used many generations prior to when producers plant their crops. The
1203 chemicals and physical energy sources used during breeding are not used directly on organic seed,
1204 plants, or tissues that are consumed. Induced mutations exist within plants and microorganisms as part
1205 of the genetic information encoded by DNA (Mba, 2013). Mutagenesis produces traits that are either
1206 unknown in the available crop germplasm, or that are known to exist but are difficult to access or
1207 incorporate. We did not find any reports of ecotoxicity related to specific traits produced using induced
1208 mutagenesis. Non-ecotoxic interactions between induced traits and other components of the
1209 agroecosystem are covered in *Evaluation Question #6*.

1210

1211 Furthermore, the decomposition of nucleic acids outside of living organisms does not indicate that DNA
1212 (as a chemical) poses a high risk of persistence in the environment (Keown et al., 2004). Soil texture and
1213 pH influence the breakdown of nucleic acids. Specifically, nucleic acids will bind more tightly to clay

1214 particles in soil when the soil pH is below 5.0, resulting in slower degradation of the nucleic acids. At
1215 higher pH, generally above 6.0, nucleic acids are rapidly degraded and incorporated into the microbial
1216 biomass. Low pH does not eliminate nucleic acid degradation; however, researchers found that
1217 approximately 50% of introduced nucleic acids were degraded within 90 days in acidic soils (Keown et
1218 al., 2004).
1219
1220 **Evaluation Question #4: Describe any environmental contamination that could result from production**
1221 **or use of various methods used to induce mutations.**
1222
1223 The toxicity of the various physical and chemical mutagens used to induce mutagenesis are detailed in
1224 *Focus Question #4.* This section will discuss the environmental contaminants that can result from the
1225 manufacture and use of these chemicals in mutagenic applications.
1226
1227 *Physical mutagens – radioactive*
1228 Many of the physical mutagenesis methods rely on the use of radioactive ions and subatomic particles to
1229 induce mutations. Common isotopes used in these methods include:
1230 • the radioisotopes cobalt-60 and cesium-137, for gamma radiation
1231 • the radioisotopes radium, americium-241, phosphorus-32 and sulfur-35, for particle radiation
1232
1233 Radioactive particles can pose a long-term risk to aquatic and soil environments, depending on their
1234 solubility and mobility characteristics (see Table 3). The bioavailability of these particles influences their
1235 risk of bioaccumulation within plants, animals, and the human food system.
1236
1237   **Table 3: Movement and accumulation of common radioisotopes in the environment.**

Radioisotope	Radiation emitted	Solubility	Mobility	Bioavailability	Half-life	Sources
Cesium-137 (^{137}Cs)	Gamma radiation and particle radiation	Very high	Immobile in lake sediments and clay soils, but readily displaced from sediment by salt water.	High, readily taken up due to chemical similarity to potassium; 40% bioavailability after 3 years, 8% bioavailability after 7 years.	30.2 years	(M. A. Ashraf et al., 2014; Wasserman et al., 2001).
Cobalt-60 (^{60}Co)	Gamma radiation	Low	Lower mobility in lake sediments, with moderate to high mobility in marine environments. Mobility is highly influenced by environmental pH.	Low, no detectable bioavailable levels 5 years after contamination.	5.3 years	(Ashraf et al., 2019; Bennett et al., 1998; Mahara & Kudo, 1981; Wasserman et al., 2001)
Radium-223 (^{223}Ra)	Particle radiation	High, particularly under acidic conditions	Low to moderate mobility in soil depending on soil type, with slowest movement in clay soils and fastest movement in organic soils. Lower soil pH increases radium mobility.	High, readily taken up by plants, animals, and microbiota due to chemical similarity to calcium. Have potential to bioaccumulate in food chain.	11.4 days	(Smith & Amonette, 2006)

Radioisotope	Radiation emitted	Solubility	Mobility	Bioavailability	Half-life	Sources
Americium-241 (^{241}Am)	Particle radiation	Low, particularly in marine environments. Solubility may increase if ^{241}Am complexes with organic matter.	Low in soils and sediments. Mobility may increase if ^{241}Am complexes with organic matter.	Moderate to high. Readily accumulated in plant roots and leaves, seaweed, and shellfish. Low accumulation in fish.	432.2 years	(Malátová & Bečková, 2022)
Phosphorus-32 (^{32}P)	Particle radiation	Low to no solubility in water.	Low mobility in soils.	High, due to similarity to non-radioactive phosphorus. Bioaccumulation is tissue specific.	14.3 days	(Vernon et al., 2018, 2020)
Sulfur-35 (^{35}S)	Particle radiation	Low to no solubility in water.	Low mobility when found in organic forms, moderate to high mobility in inorganic forms.	High, due to similarity to non-radioactive sulfur.	87.4 days	(Collins & Cunningham, 2005)

1238
1239 The mobility of radioactive particles may decrease if substances bind to the radioisotopes. In one study,
1240 Seaman et al. (2001) found the addition of vermiculite and illite effective in immobilizing ^{137}Cs in soils
1241 and sediments. However, the effects of radioisotopes complexing with other compounds are variable. For
1242 example, the solubility of Americium-241 may be reduced or enhanced through complexing with soil
1243 organic matter (Malátová & Bečková, 2022).
1244
1245 The risk of radioactive contamination is highest when transporting radioactive substances or following
1246 large-scale incidents at nuclear power plant facilities (Ashraf et al., 2014; Ashraf et al., 2019; Michalczuk,
1247 2022). The risk associated with use in commercial facilities is considered low and is generally confined to
1248 the transport of radioactive materials (Ashraf et al., 2019; Bennett et al., 1998).
1249
1250 The charged ion particles used in heavy ion-beam radiation do not produce residual radioactive waste in
1251 the manner described above for various radioisotopes; however, the ion beam accelerators and
1252 surrounding facilities do retain low levels of radioactivity that must be managed during
1253 decommissioning (Opelka et al., 1979). In addition to assessing and removing radioactive concrete within
1254 accelerator facilities, Opelka et al. (1979) outline nine synchrotron components that must be individually
1255 decommissioned and managed for radioactivity.
1256
1257 *Physical mutagens – non-radioactive*
1258 Though non-radioactive physical mutagens, including X-ray radiation, cosmic ray radiation, ion-beam
1259 radiation, UV radiation, and ARTP do not produce radioisotope contaminants, the machines required to
1260 use these methods require substantial physical and energetic inputs.
1261
1262 Researchers generate UV radiation using fluorescent UV tubes, xenon arc lamps, metal-halide lamps, and
1263 mercury vapor lamps (Heikkilä et al., 2009). These lamps contain mercury, which must be recycled in
1264 specialized facilities to avoid contributing to toxic contamination via landfills and incinerators (Heikkilä
1265 et al., 2009). Similarly, X-ray machines are known sources of lead, beryllium, and polychlorinated
1266 biphenyls (PCBs), which require special disposal to avoid environmental contamination (ATSDR, 2021;
1267 Rogers, 1947).
1268
1269 Cosmic ray irradiation requires exposing plant tissues to natural radiation beyond the atmosphere. Thus,
1270 mutagenesis relies on the launch of satellites and other vehicles that transport plant tissues to these
1271 locations. Propellants used in space launches release numerous atmospheric pollutants known to deplete

1272 stratospheric ozone, including chloride radicals (Cl_x), nitrogen oxides (NO_x), and hydroxyl (OH) radicals
1273 (Dallas et al., 2020). Furthermore, the formation of pollutant-dense clouds (i.e., ground clouds) at space
1274 shuttle launch sites leads to very acidic rain within a 0.23 km² region around the launch pad (Dallas et al.,
1275 2020). Damage to ecosystems within launch sites includes the loss of plant species, a reduction in soil pH,
1276 and large acid depositions in surrounding waterways (Dallas et al., 2020).
1277
1278 *Chemical mutagens*
1279 Intercalating agents, like ethidium bromide and acridine orange, are known pollutants that are found in
1280 aquatic ecosystems following improper treatment and disposal of research lab waste (Nayak & Pal, 2018;
1281 Salah El-Din et al., 2021; S. Singh & Singh, 2018). Within aquatic ecosystems, intercalating agents induce
1282 genetic abnormalities in a number of organisms, including sea urchins, Nile tilapia, mice, bacteria, and
1283 flies in the *Drosophila* genus. Researchers have identified remediation tools to remove these agents from
1284 waterways, including the use of *Spirulina platensis* and *Abelmoschus esculentus* seed powder (Nayak & Pal,
1285 2018; Salah El-Din et al., 2021; S. Singh & Singh, 2018).
1286
1287 In addition to its use as a mutagen, sodium azide was historically used as an agricultural herbicide and
1288 pesticide and is currently used in vehicular airbags (Arenaz et al., 1989; Tat et al., 2021). These uses are
1289 responsible for the direct and indirect (i.e., via waste streams) introductions of the substance into the
1290 environment (PubChem, 2023f). Sodium azide degrades through hydrolyzation in soil and aquatic
1291 environments, forming free metal and nitrogen gas. Given its tendency to react and degrade, sodium
1292 azide is unlikely to bioaccumulate (PubChem, 2023f).
1293
1294 Nitrous acid is a very unstable substance that rapidly degrades in sunlight (Zimmermann, 1977). Nitrous
1295 acid can be found in the atmosphere, particularly at night, due to a buildup of automobile emissions and
1296 the reaction of nitrogen dioxide with water (Sakugawa & Cape, 2007). One study explored the effect of
1297 atmospheric nitrous acid on Scots pine trees, finding that two months of fumigation with nitrous acid gas
1298 resulted in decreased photosynthetic capacity in plants (Sakugawa & Cape, 2007). This work was the first
1299 to discover that the biological effects of nitrous acid expanded beyond mutagenesis and had broader
1300 implications for the health of plants.
1301
1302 The use of alkylating agents (e.g., ethyl methanesulfonate, N-methyl-N-nitrosourea, and 1-ethyl-1-
1303 nitrosourea) as research chemicals may result in their release into the environment through laboratory
1304 waste streams (PubChem, 2023c, 2023e, 2023a). Degradation of these compounds occurs in the
1305 atmosphere through the action of sunlight-produced –OH radicals, and the half-life for this degradation
1306 ranges from 3 to 17 days. Some alkylating agents, such as N-methyl-N-nitrosourea and 1-ethyl-1-
1307 nitrosourea, degrade in sunlight. Ethyl methanesulfonate, the most common chemical mutagen, is not
1308 susceptible to degradation by sunlight. In soil, alkylating agents have very high mobility. Hydrolysis is
1309 the primary degradation fate for these agents in water and soil. The potential for bioaccumulation of
1310 these compounds in aquatic organisms is reportedly low (PubChem, 2023c, 2023e, 2023a).
1311
1312 Environmental pollution by the antimitotic colchicine is primarily due to the use of the chemical as a
1313 medicine in the treatment of gout. Furthermore, colchicine is expected to be susceptible to degradation by
1314 sunlight and holds a low risk for bioconcentration in aquatic organisms (PubChem, 2023b). No
1315 environmental contamination data related to the use of colchicine as a mutagen was found in the
1316 available literature.
1317
1318 Similarly, environmental pollution associated with the use of the antimitotic oryzalin is a result of its use
1319 as a residential and agricultural herbicide. Within the environment, oryzalin has a moderate to high
1320 potential for bioconcentration in aquatic organisms. Microbial degradation and sunlight account for most
1321 of the breakdown of oryzalin in the environment (PubChem, 2023g).
1322
1323 As noted in *Evaluation Question #3*, the use and disposal of mutant crops and microorganisms is not
1324 known or assumed to pose a significant risk for ecotoxicity or environmental contamination.
1325

Evaluation Question #5: Describe any known chemical interactions between traits caused by induced mutations and other substances used in organic crop or livestock production or handling. Describe any environmental or human health effects from these chemical interactions.

We did not find any reports of known chemical interactions between traits caused by induced mutagenesis and other substances used in organic production. However, there is some risk that the development of pesticide-resistant crops and microorganisms may lead to increased use of pesticide chemicals. This increased use may in turn lead to pesticide overuse, more pesticide resistant weeds and pathogens, and unintended effects to non-target organisms. This is similar to what is seen in other genetically engineered conventional crop-pesticide systems, such as glyphosate and 2,4-D (dicamba)-resistant crops (Schütte et al., 2017).

Mutagen-induced herbicide tolerance and the use of herbicides
Herbicide resistance (HR) is a common goal of modern crop breeding, as it allows the use of broad-spectrum herbicides to control weeds in crop fields (Newhouse et al., 1992; Prakash et al., 2020). While HR is a major goal of transgenic crop production, historical breeding work developed HR through germplasm screening and induced mutagenesis. Notable HR mutations have been created using induced mutagenesis in soybean, sunflower, wheat, and other crops (Newhouse et al., 1992; Prakash et al., 2020; Johnson et al., 2002). For example, *Clearfield* technology was developed for a variety of crops including wheat, corn, rice, canola and sunflower using chemical mutagenesis (Johnson et al., 2002). These crops are resistant to the conventional herbicide Beyond™ (Johnson et al., 2002).

Some herbicides may be used in organic agriculture, provided they meet the requirements of § 205.206 *Crop pest, weed, and disease management practice* standard. Common organic herbicides include those with the following active ingredients:
- nonsynthetic plant extracts (e.g., clove oil, cinnamon oil, citrus oil)
- nonsynthetic fatty acids (e.g., capric acid, caprylic acid, pelargonic acid)
- citric acid
- vinegar

If crops are developed with resistance to organic-compliant herbicides, increased application of herbicides could occur. However, we did not find evidence of crops developed for resistance to these materials. This may be due to the fact that these are generally non-selective herbicides (Shaffer, 2022). While the risk of HR in weeds is inseparable from herbicide use, many nonsynthetic herbicides have complicated mechanisms of action with multiple molecular targets in a plant. The complexity of these mechanisms reduces the risk of resistance developing, as there is a more complicated biosynthetic barrier to overcome (Perotti et al., 2020).

Mutagen-induced disease control resistance in microorganisms and the use of organic disease control pesticides
Beneficial microorganisms are frequently utilized in organic crop production to control disease. When disease control by beneficial microorganisms is incomplete, crop producers may use integrated pest management (IPM) strategies that combine the use of disease-control pesticides and microorganisms (Hatvani et al., 2006). To combine these means of control, the development of beneficial microorganisms that can tolerate some degree of pesticide applications is necessary.

Researchers have used induced mutagenesis to develop fungicide-tolerant strains of beneficial fungi (Hatvani et al., 2006). Hatvani et al. (2006) used UV-radiation to develop mutant *Trichoderma* fungi capable of enduring treatment with several classes of fungicides.

As with herbicide-resistant crops, there are similar risks with developing disease control-tolerant beneficial microorganisms, which allow for increased use of pesticides. There are known negative effects of organic-compliant fungicides such as copper products on non-target species. See the *Copper Products (Fixed Coppers and Copper Sulfate)* technical report for more information (NOP, 2022).

1380 **Evaluation Question #6: Describe any effects that induced mutations have on biological or chemical**
1381 **interactions in the agro-ecosystem, including gene flow into soil organisms, crops, livestock, and wild**
1382 **populations.**
1383
1384 Induced mutations within plants and microorganisms invariably produce characteristics that interact
1385 with the broader agroecosystem. Interactions that may need additional consideration include:
1386 • the impact of mutant floral traits on pollinator activity
1387 • the risk of gene flow between crops and wild crop relatives
1388 • the impact of mutant microbiota on plants and the broader microbiome
1389
1390 *Impact of mutant floral traits on pollinators*
1391 Plant breeders utilize mutagenesis to produce novel flower characteristics, either for ornamental plant
1392 breeding or agronomic goals (Datta & Teixeira da Silva, 2006). Alterations to the floral structure can
1393 significantly impact pollinator interactions (Owen & Bradshaw, 2011).
1394
1395 In an exploratory study of pollinator interactions with wild-type and chemically-mutated flowers of
1396 *Mimulus lewisii*, researchers found that bumblebees visited mutant flowers at 29-80% of the visitation rate
1397 documented in wild-type flowers (Owen & Bradshaw, 2011). Researchers attributed the reduction in
1398 pollinator visitation to the loss of an essential "landing platform" provided by the lower petals of *M.*
1399 *lewisii* and a change in petal color pattern. They also note that even minor changes, like changes to the
1400 color contrast in flowers, can significantly reduce pollinator visitation.
1401
1402 Tantray et al. (2017) used the chemical mutagen ethyl methanesulfonate to produce black cumin (*Nigella*
1403 *sativa*) with a self-pollination mechanism instead of the natural cross-pollination. Self-pollination in black
1404 cumin ensures full pollination of each ovary, leading to a higher seed set in the crop. The researchers
1405 documented numerous changes to the floral biology associated with the conversion to complete self-
1406 pollination, including color change and a change in the angles of flower structures (Tantray et al., 2017).
1407 With this mutation, the resulting black cumin varieties no longer need or attract pollinators.
1408
1409 While changes to floral biology are agronomically valuable, the associated loss of pollinator visitation
1410 may be problematic. Biesmeijer et al. (2006) note that reductions in the abundance of insect-pollinated,
1411 outcrossing plants are significantly associated with pollinator declines. When the floral structures of crop
1412 plants change but self-pollination is not the goal, a negative feedback loop may still inevitably form. As
1413 noted by Huang & D'Odorico (2020), reducing pollinator visitation (e.g., through mutagenesis, drought,
1414 planting density) may shift populations of plants to become more reliant on self-pollination mechanisms.
1415 As plant populations shift towards self-pollination, pollinator density further declines (Huang &
1416 D'Odorico, 2020).
1417
1418 *Gene flow between crops and wild crop relatives*
1419 Gene flow encompasses the movement of genes from one location to another, typically through the
1420 movement of reproductive cells (such as drifting pollen), or migration of populations or individuals
1421 (Beckie et al., 2019). In plants, this can involve movement of genes through pollen, seeds, or plant
1422 propagules, depending on the primary pollination and reproductive mechanisms of the species.
1423 Herbicide resistance (HR) and other traits may be able to spread from crops into wild relatives and feral
1424 plants through gene flow (Beckie et al., 2003, 2019).
1425
1426 If herbicide-resistant plants or other pesticide-resistant organisms are developed for organic use, then
1427 these traits may be able to move into other populations through gene flow. However, we did not find
1428 literature indicating that herbicide resistance exists in organic production systems, which typically rely
1429 on physical methods and non-selective herbicides. The spread of HR from conventional crops into wild
1430 crop relatives is well-documented (Beckie et al., 2003; Gealy et al., 2003; Vrbničanin et al., 2017).
1431

1432   *Agro-ecological interactions of mutant microbiota*
1433   The mutagenesis of plant-associated microbiota can lead to the production of favorable plant growth
1434   promoting (PGP) strains. To date, mutagenesis has been used to induce characteristics in the microbiota
1435   that lead to improved drought tolerance, vigor, nutrient acquisition, and disease resistance in host crop
1436   plants. These mutations are explored in the following paragraphs.
1437
1438   Kumari et al. (2016) explored the effect of a mutant rhizobacterium (*Pseudomonas simiae*), developed using
1439   the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine, on drought tolerance. One mutant strain of
1440   the rhizobacterium had strong PGP capabilities, including the ability to significantly reduce water stress
1441   in mung beans, compared to the wild-type strain. This drought tolerance was accompanied by reduced
1442   ethylene levels within the roots of treated mung beans. Ethylene levels generally increase in response to
1443   drought stress and will inhibit root growth and nodulation in legumes. Furthermore, the mutant
1444   rhizobacteria induced the upregulation of a drought and salinity stress tolerance gene within mung beans
1445   (Kumari et al., 2016).
1446
1447   In another study, researchers used UV-radiation to develop *Bacillus* sp. bacteria with PGP action (Shahid
1448   et al., 2022). The researchers used wheat, growing under heavy metal stress conditions to test the effects
1449   of the mutant bacteria. Two resulting mutant strains increased phosphate solubilization and ammonia
1450   production within the wheat rhizosphere. However, the authors found that both mutant strains also *lost*
1451   the function of a critical stress-reducing enzyme, ACC deaminase. Despite the contradictory gain and loss
1452   of these functions, the mutant *Bacillus* strains were found to significantly increase plant growth
1453   characteristics in wheat grown in chromium-contaminated soils (Shahid et al., 2022).
1454
1455   Using gamma radiation, researchers induced mutations in isolates of the fungus *Trichoderma harzianum*
1456   (Abbasi et al., 2016). These mutant strains had biocontrol capabilities against soilborne plant pathogens
1457   like *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Isolates of the mutant *T. harzianum* controlled pathogen
1458   growth and had a higher colonization rate compared with the wild type fungus. The authors attribute the
1459   antagonistic activity against the pathogens to several secreted compounds. However, more work was
1460   necessary to identify a specific mechanism behind the antagonism (Abbasi et al., 2016).
1461
1462   Many nutrients are made available to plants through the action of microbiota, including phosphorus,
1463   nitrogen, and potassium. To improve the nutrient acquisition abilities of crops, researchers have
1464   developed mutant microbiota that show high nutrient acquisition or digestion activity. Using the
1465   chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine, Lynn et al. (2014) produced strains of
1466   *Enterobacter* with a 50% increase in phosphate solubilizing activity compared to wild-type strains. In
1467   another study, researchers used N-methyl-N'-nitro-N-nitrosoguanidine to induce mutations in unnamed
1468   rhizobacteria, increasing potassium solubilization by 84.8 to 127.9% over wild type bacteria (Parmar &
1469   Sindhu, 2019).
1470
1471   **Evaluation Question #7: Describe and summarize any reported effects of induced mutations upon**
1472   **human health.**
1473
1474   We found no evidence to suggest that genetic mutations derived from induced mutagenesis pose a risk to
1475   human health. There are risks associated with occupational or accidental exposure to the radiation or
1476   chemicals used to induce mutations.
1477
1478   *Risk of radiation exposure*
1479   The risk of radiation exposure associated with induced mutagenesis is most relevant for researchers and
1480   equipment operators who work with radioactive materials.
1481
1482   We found no studies related to occupational exposure of researchers working on induced mutagenesis
1483   projects. However, one study on medical personnel found that annual radiation doses ranged from 3.05
1484   to 28.25 mSv (millisieverts), far below the annual dose limit of 500 mSv (Alkhorayef et al., 2020). Despite
1485   this, Alkhorayef et al. (2020) note that brain cancer, cataracts, and non-malignant diseases have been
1486   reported by medical personnel who are routinely exposed to ionizing radiation. Another study found

1487 that occupational exposure to radiation among medical personnel led to significant increases in DNA
1488 damage (Dobrzyn´ska et al., 2014). The consensus in the available literature is that there is no safe level of
1489 exposure to ionizing radiation, and exposure, for medical or research purposes, does pose a risk to
1490 workers (Alkhorayef et al., 2020; Dobrzyn´ska et al., 2014; Prasad et al., 2004).
1491
1492 Accidental loss and subsequent improper disposal constitute another layer of risk that is specifically
1493 associated with medical or research settings (Ashraf et al., 2014). Numerous instances have been reported
1494 over the past 40 years in which radiation machines used in medical, research, or industrial applications
1495 have been stolen and/or improperly handled. In many instances the machines have been scrapped and
1496 subsequently recycled into new, contaminated building materials. These incidents include the following:

- Equipment containing cobalt-60 was recycled into steel rebar, which was used in the construction of 200 residential and non-residential buildings in Taipei, Taiwan in 1982. Residents had a higher incidence of leukemias and thyroid cancer, as well as reduced fertility (Lin et al., 2010).
- A discarded radiation therapy machine containing cobalt-60 was salvaged and the radioactive metal was recycled into five tonnes of radioactive steel in Ciudad Juárez, Mexico in 1983. The contaminated steel was distributed and used throughout Mexico, the U.S., and Canada, and approximately 4,000 individuals were exposed to cobalt-60 radiation (Van Etten et al., 1984).
- A cesium-137 radiation therapy machine was stolen and scrapped in Goiânia, Brazil in 1987. Cesium-137, as well as scrap from the protective housing it was encased in, were removed from the machine and distributed to numerous individuals, resulting in 20 cases of radiation sickness and 4 deaths (da Cruz et al., 1994).
- An expired cobalt-60 radiation therapy unit was stolen and subsequently scrapped in Samut Prakan, Thailand in 2000. Exposure among junkyard workers led to 10 cases of radiation sickness and three deaths (Xiaohua et al., 2018).
- A cobalt-60-containing gammacell 220, used in research applications, was accidentally scrapped in New Delhi, India in 2010. Exposure to the contaminated scrap metal led to 7 cases of radiation sickness and 1 death (Bagla, 2010).
- Research equipment containing cesium-137 was improperly stored in a residential area in Serpong, Indonesia in 2020. To date, hundreds of drums of contaminated soil and all vegetation has been removed from the area (Setiawan et al., 2021).

Chemicals in drinking water
Another potential concern is the exposure of humans to chemicals used in induced mutagenesis, either occupationally or through drinking water. *Focus Question* #2 discusses the toxicity of these chemicals with regard to human health.

Data from the Toxics Release Inventory, maintained by the U.S. EPA, included release reports for two chemical mutagens in 2021: sodium azide and diethyl sulfate (U.S. EPA, 2023). This inventory tracks releases of certain toxic chemicals to the environment. In total, four facilities reported releasing sodium azide and seventeen facilities reported releasing diethyl sulfate. A total of 18,155 lbs. of sodium azide and 5043 lbs. of diethyl sulfate were released in 2021 (U.S. EPA, 2023). The companies associated with these chemical releases appear to be chemical producers and waste disposal facilities and are not clearly associated with plant breeding. In addition to its use in mutagenesis, sodium azide is an herbicide, a propellant in airbags, and a chemical intermediate in the production of other chemicals and personal care products (PubChem, 2023f). Diethyl sulfate is primarily used to make dyes but is also used to produce agricultural chemicals, household products, pharmaceuticals, and personal care products (PubChem, 2023h).

No other release reports or water pollution reports were found for other chemical mutagens.

Evaluation Question #8: Describe any alternative practices that would make the use of induced mutagenesis unnecessary.

Traditional plant breeders utilize natural plant diversity to develop crop varieties (Breseghello & Coelho, 2013; Oregon State University, 2019). Instead of utilizing induced mutagenesis, collections of plant genetic resources can be the source of the desired genotypic variation.

Plant genetic resources, such as the National Plant Germplasm System (NPGS), protect and maintain enormous diversities of crop varieties and wild relatives (Byrne et al., 2018). In the United States, the NPGS has a collection of over 575,000 varieties from 15,116 species of crops and crop relatives. While this collection represents enormous genetic diversity, plant breeders may not always have the knowledge and time to fully utilize these resources. Byrne et al. (2018) note that if the NPGS incorporated more detailed genetic information into their resources, breeders might use the collection more effectively.

Allele "mining" involves seeking out superior, naturally-occurring genetic variants (Kumar et al., 2010). Two mining methods are Eco-TILLING and sequence-based allele mining. These methods expedite the process of identifying natural variations in germplasm resources.

While TILLING is a molecular method used to search for induced mutations, Eco-TILLING is a method that researchers use to search for known, specific, naturally occurring genetic variations (Till et al., 2006). Using this molecular method, researchers can rapidly identify numerous natural genetic mutations (including SNPs and INDELs) for plant breeding work. The use of molecular techniques can dramatically accelerate the process of searching for desired characteristics within a germplasm pool (Till et al., 2006).

A similar strategy is sequence-based allele mining, in which researchers amplify DNA from genotypes of interest and sequence this DNA to identify variations (Kumar et al., 2010). This approach is less complex, more efficient, and more flexible for detecting numerous types of mutations. Furthermore, Kumar et al. (2010) note that sequence-based allele mining typically costs less per data point than the screening strategies used in Eco-TILLING, like Li-Cor genotyping or denaturing high-performance liquid chromatography.

Numerous studies support the use of allele mining methods to improve crops. A selection of achievements made using these approaches includes:
- Two genes capable of conveying resistance to RNA viruses to the five cultivated *Capsicum* species (Ibiza et al., 2010) have been identified.
- Six candidate genes were identified related to rice grain shape, which is a major determinant of grain yield and quality (Yang et al., 2019).
- Reserachers isolated thirty candidate genes associated with important agronomic traits in common bean, like days to flower, days to maturity, growth habit, canopy height, lodging, and seed weight (Moghaddam et al., 2016).
- Allele mining helped identify a gene associated with frost tolerance and a reduced vernalization requirement in barley (Guerra et al., 2022).

Evaluation Questions Specific to Organic Handling or Processing

Evaluation Question #9: Describe whether products of induced mutagenesis are used to improve flavors, colors, textures, or nutritive values that would otherwise be lost, and how these products are used in products to improve any of these food/feed characteristics.

Many traits achieved with induced mutagenesis are relevant to the organoleptic qualities of crops and food microorganisms. Frequently, mutant traits do not replace a characteristic that would be lost but increase the overall occurrence of that appearance. Examples of mutant traits relevant to sensory qualities or processing practices are covered below.

1592 *Examples of mutant traits in crops*
1593 Gamma radiation in mandarins reduced seed number in citrus fruit, resulting in a 70-92% reduction in
1594 seed number (Goldenberg et al., 2014). This treatment also enhanced color in early-season varieties but
1595 had variable effects on nutritional quality. However, there is evidence that mutagenesis can increase
1596 vitamin C content and antioxidant activity, as indicated by increases in some but not all mutant
1597 mandarins (Goldenberg et al., 2014).
1598
1599 In sorghum leaves, ethyl methanesulfonate-mediated mutagenesis was used to develop a variety that
1600 accumulates a red pigment, a natural source for red food color (Petti et al., 2014). After the identification
1601 of 567 red pigment-containing mutants, mutant lines were backcrossed for three generations to bring the
1602 red producing trait into the wild type seedling. The final mutant variety contained up to 10.1 mg of red
1603 pigment per gram of dry leaf tissue, which is higher than other existing sources of natural red pigment
1604 such as red cabbage (Petti et al., 2014).
1605
1606 In another study, Gomez et al. (2017) used gamma radiation to improve the yield, agronomic
1607 performance, and quality of barley varieties adapted to growing in the high Andean region of Peru.
1608 Mutant lines were grown and screened until the M_8 generation, at which point 64 lines were identified as
1609 having yields 20-105% higher than the parent variety. The researchers also identified high-yielding
1610 mutant lines with increased micronutrient content (i.e., phosphorus, zinc, manganese, iron, and copper)
1611 (Gomez et al., 2017).
1612
1613 *Examples of mutant traits in microorganisms*
1614 In a study on soy sauce, ARTP-mediated mutagenesis was used on yeast to induce salt tolerance and
1615 desirable aroma production (Li et al., 2021). Researchers identified a strain of interest with the desired
1616 ester-based aroma and mutagenized it to produce salt tolerant mutants. Sixty-seven salt tolerant mutants
1617 were identified in the screening process, and three of these were selected for the highest ester production.
1618 Survival rate was improved over wild type yeasts, as well as glucose metabolism and ethanol production,
1619 the latter of which is important for flavor and as a precursor to additional flavor in soy sauce (Li et al.,
1620 2021).
1621
1622 In another study, researchers used the chemical proflavine to mutate *L. lactis* subspecies *Lactis* biovar
1623 diacetylactis (Liu et al., 2020). One resulting strain had a single insertion mutation within the *ldh* gene.
1624 Compared to the parent strain, this isolate showed an increased ability to digest lactose in dairy waste,
1625 producing the compounds acetoin and diacetyl instead of lactate. Acetoin and diacetyl are responsible for
1626 the butter aroma found in dairy products and can be used as flavor additives in dairy products (Liu et al.,
1627 2020).
1628
1629 Researchers also mutagenize microorganisms to produce higher quantities of vitamins to be used in the
1630 fortification of organic food. Balabanova et al. (2021) reference twenty-five genera of bacteria that produce
1631 cobalamin (i.e., vitamin B12); however, they note that natural microbial yields can be very low. The
1632 researchers also note that UV light, ARTP, and chemical mutagenesis of several genera contributed to 10
1633 to 20-fold increases in cobalamin production over non-mutant yields (Balabanova et al., 2021). Similar
1634 studies found that chemical mutagenesis may be used to increase the production of folate and riboflavin
1635 (Averianova et al., 2020; Park et al., 2011).
1636
1637 **Evaluation Question #10: Describe any effect or potential effect on the nutritional quality of the food**
1638 **or feed when induced mutagenesis is used (7 CFR 205.600(b)(3)).**
1639
1640 Mutagenesis can influence the nutritional quality of food or feed or may inadvertently induce changes.
1641 These changes may be to the benefit or detriment of nutritional quality. Examples of intentional and
1642 unintentional effects of mutagenesis on the nutritional quality of food and feed are covered below.
1643
1644 *Intentional changes to nutritional quality*
1645 As noted in *Evaluation Question #9*, Gomez et al. (2017) used gamma radiation to develop a population of
1646 mutant lines of barley. In the process of developing barley with the desired agronomic and quality

1647 characteristics, they identified increases in several micronutrients, including phosphorus, zinc,
1648 manganese, iron, and copper. The researchers note that these micronutrients may serve as a source of
1649 bioavailable minerals in food.
1650
1651 Wijekoon et al. (2020) developed a protocol for using ethyl methanesulfonate treatment to mutagenize
1652 alfalfa and sainfoin (a forage crop), intending to improve lipid content. Mutants of both crops showed a
1653 3-5% increase in total shoot lipid content. After screening, ten alfalfa mutants and eight sainfoin mutants
1654 were selected for increased total shoot lipid content and lack of morphological deficiencies (Wijekoon et
1655 al., 2020).
1656
1657 In another study, researchers used the chemicals ethyl methanesulfonate and N-methyl-N'-nitro-N-
1658 nitrosoguanidine to mutagenize four probiotic bacteria strains, to produce isolates showing β-
1659 galactosidase (β-gal) overproduction (Ibrahim & O'Sullivan, 2000). The enzyme β-gal digests lactose, the
1660 primary carbohydrate found in milk, and thus improves dairy product quality for lactose-intolerant
1661 individuals. Following screening, researchers found 75 mutants with increased β-gal. Some strains
1662 consumed 2-3 times more lactose. These products may be added to any dairy products as probiotic
1663 cultures to improve lactose malabsorption (Ibrahim & O'Sullivan, 2000).
1664
1665 *Unintentional changes to nutritional quality*
1666 While developing a mutant trait for red pigment accumulation in sorghum leaves, Petti et al. (2014) found
1667 mutants that over-accumulated red pigment contained a high quantity of several polyphenols. Thus, the
1668 new sorghum variety has the potential for use as a source of bioactive dietary phenols (Petti et al., 2014).
1669
1670 As noted in *Evaluation Question #9*, Goldenberg et al. (2014) used gamma radiation to reduce seed
1671 prevalence in mandarin fruit. They also identified variable effects on nutritional quality, with some fruit
1672 showing no change in vitamin C and antioxidant levels and others showing significantly higher or lower
1673 vitamin C and antioxidant activity (Goldenberg et al., 2014).
1674
1675 In the process of developing salt-tolerant soy sauce, Li et al. (2021) identified engineered strains with
1676 lower phenol content but more abundant and diverse flavor volatiles compared to the wild-type strain.
1677 Many phenols have positive implications for food quality and human health (Petti et al., 2014).
1678
1679 Food scientists also fortify foods with essential vitamins from mutant microorganisms (Balabanova et al.,
1680 2021). While several studies cite using mutagenesis to increase vitamin production in numerous microbial
1681 genera, other work by Xie et al. (2021) found that non-mutant microorganisms can be used in vitamin
1682 fortification. Specifically, the authors found that two genera of non-mutant bacteria (*Propionibacterium*
1683 and *Levilactobacillus)* successfully produced 300 nanograms of vitamin B12 per gram of bacterial dry
1684 weight. This quantity is sufficiently high to meet industry production needs, and is equivalent to the
1685 quantities produced by mutant bacteria in other studies (Averianova et al., 2020; Balabanova et al., 2021;
1686 Xie et al., 2021)
1687
1688 **Evaluation Question #11: Describe any alternative practices (or substances) that would make the use**
1689 **of products of induced mutagenesis unnecessary during the handling or processing of organic**
1690 **products.**
1691
1692 In addition to the genetic discoveries discussed in *Evaluation Question #8*, allele mining methods can
1693 improve crops and microorganisms in a manner relevant to processing and handling. This is particularly
1694 true for microorganisms used in food processing.
1695
1696 Mutagenesis and genetic engineering methods have been used to create biofortified crops with higher
1697 macronutrient and micronutrient concentrations; however, this work may also be done using allele
1698 mining methods (Saltzman et al., 2013). In rice, Bollinedi et al. (2020) identified 29 genetic regions
1699 responsible for controlling up to 53.3% of the natural variation in micronutrient concentrations. By
1700 utilizing this information in breeding work, it is possible to improve the grain content of micronutrients
1701 essential to the human diet, like iron and zinc (Bollinedi et al., 2020). Similar work in pearl millet

1702   identified 74 genetic regions associated with micronutrient and protein content (Pujar et al., 2020).
1703   Biofortification through breeding work has the potential to reduce the need for commercial fortification of
1704   food and is particularly valuable to malnourished, rural populations with reduced access to commercially
1705   fortified foods and supplements (Saltzman et al., 2013).
1706
1707   As noted in the *Historic Use* section, Aleem et al. (2018) used gamma-ray mutagenesis to develop new
1708   strains of koji, or *Aspergillus oryzae*. An earlier study by Wicklow et al. (2007) explored the generally
1709   uncharacterized diversity of koji populations in soy sauce production environments, finding over 30
1710   genotypes within 64 cultured samples. Based on these findings, the authors note that preserving and
1711   utilizing the diversity of koji strains, such as the strains they identified, is essential to maintaining natural
1712   sources of competitively superior genotypes for use in agriculture and food processing (Wicklow et al.,
1713   2007).
1714

1715   **Focus Questions Requested by the NOSB**

1716

1717   **1. Does IM use means that are not possible under natural conditions?**

1718

1719   As noted in Evaluation Question #2, the physical and chemical methods used to induce mutations require
1720   human manipulation (as in the case of producing and harnessing radioisotopes to generate ionizing
1721   radiation) or synthetic chemicals. Cosmic ray radiation differs somewhat from the other mutagens in that
1722   it is entirely naturally occurring; however, accessing cosmic ray radiation requires sending plant material
1723   beyond Earth's atmosphere (Ferrari & Szuszkiewicz, 2009).

1724

1725   While mutations occur under natural conditions, the rate is substantially slower than when induced
1726   mutation techniques are used. Furthermore, because different chemicals or techniques can create specific
1727   types of mutations (e.g., mitosis errors, frameshift mutations, base substitutions), breeders can exercise a
1728   limited amount of control.

1729

1730   **2. What are rates of mutation using the various IM methods compared to background levels?**

1731

1732   Spontaneous mutation is a natural process that varies between species. It occurs in somatic (non-
1733   reproductive) tissues during mitosis, as well as during sexual reproduction and meiosis (Schoen &
1734   Schultz, 2019).

1735

1736   Environmental stressors such as temperature and UV exposure can increase spontaneous mutation rates
1737   (Lindgren, 2009; Lu et al., 2021; Schoen & Schultz, 2019). Background mutation rates can also be affected
1738   by cell age and epigenetic factors (Schoen & Schultz, 2019). The successful spread of mutations depends
1739   on the plant developmental stage (i.e., age) and fitness (Schoen & Schultz, 2019).

1740

1741   Recent studies have used whole-genome sequencing to estimate the number of point mutations, generally
1742   produced through the natural deamination of cytosine, that occur in plants and microorganisms (Lynch,
1743   2010; Schoen & Schultz, 2019; Yali & Mitiku, 2022). Table 3 details a number of these estimates, which can
1744   be considered the background levels of mutation.

1745

1746   **Table 4: Spontaneous Mutation Rates of Yeasts, Bacteria, Algae, and Various Plant Species.**

Organism	Genome size	Spontaneous mutation rate, per nucleotide site	Predicted number of mutations across genome, per cell division	Sources
Yeasts	~ 12 Mb	3.3×10^{-10}	4.0×10^{-3}	(Lynch, 2010)
E. coli	~4.5-5.5 Mb	2.6×10^{-10}	$1.2 - 1.4 \times 10^{-3}$	(Lynch, 2010)
Arabidopsis thaliana (germline)	~125 Mb	1.6×10^{-10}	2.0×10^{-2}	(Lynch, 2010)
Green alga (total)	~120 Mb	3.23×10^{-10}	0.04	(Ness et al., 2012)

Organism	Genome size	Spontaneous mutation rate, per nucleotide site	Predicted number of mutations across genome, per cell division	Sources
Oak (estimated somatic)	~730 Mb	5×10^{-8}	36.5	(Schoen & Schultz, 2019)
Eucalyptus (somatic)	~500 Mb	4.13×10^{-8} to 8.25×10^{-8}	20.7 - 41.3	(Orr et al., 2020)
Sitka spruce (somatic)	~21 Gb	2.7×10^{-8}	567	(Hanlon et al., 2019; Orr et al., 2020)

1747
1748 Researchers use mutagens to significantly increase the mutation rate beyond that which occurs naturally.
1749 Several factors can affect the frequency of induced mutations in an organism. Chemical mutagens are
1750 more effective than physical mutagens (Shu, Shirasawa, et al., 2011). Tanaka et al. (2010) note that the
1751 effectiveness of physical mutagens, such as gamma rays and X-rays, is variable. They found that ion
1752 beams are the most effective, followed by gamma rays and X-rays.
1753
1754 The type of tissue treated during mutagenesis can also affect the mutation frequency. For instance,
1755 Tanaka et al. (2010) note that treated floral petals had over two times as many mutations as leaf tissue
1756 treated with the same dosage of gamma-ray radiation. Seeds are frequently favored in chemical
1757 mutagenesis, as treating other tissues may produce more chimeras (Manzoor et al., 2019). When selecting
1758 a tissue type for mutagenesis, researchers consider resilience to the lethal effects of the mutagens. For
1759 example, Tepfer & Leach (2017) found that seeds with thicker seed coats (such as morning glory) were
1760 more resilient to the lethal effects of cosmic radiation than seeds with thinner seed coats (such as
1761 *Arabidopsis*). All of these factors impact the ultimate mutation frequency.
1762
1763 Similar to mutation rate, mutation frequency describes the number of mutations within a given section of
1764 DNA, generally 1,000 kilobases (kb). Unlike rate, which describes the likelihood that a mutation will
1765 occur at a given nucleotide site (typically from one cell division to the next), frequency describes the
1766 number of mutations that exist within a given length of DNA (Shu, Shirasawa, et al., 2011). Mutation
1767 frequency changes with plant species and mutation method (see Table 5).
1768
1769 **Table 5: Mutation frequency and estimated number of legions per genome in M$_2$ populations of**
1770 **selected crop plants.**

Crop	Ploidy Level	Genome Size	Mutagen	Mutation frequency	Predicted number of lesions per genome following mutagenesis
Arabidopsis thaliana	Diploid	125 Mb	EMS*	~1/170 kb	~700
Barley	Diploid	~ 5.3 Gb	EMS*	~1/1,000 kb	~5,300
Barley	Diploid	~ 5.3 Gb	Sodium azide	~1/374 kb	~15,000
Maize	Diploid	2.5 Gb	EMS*	~1/485 kb	~5,100
Rice	Diploid	389 Mb	EMS*	~1/300 kb	~1,300
Rice	Diploid	389 Mb	SA*** + MNU**	~1/300 kb	~1,300
Rice	Diploid	389 Mb	MNU**	~1/135 kb	3,100
Rice	Diploid	389 Mb	Gamma rays	~1/6,190 kb	63
Sorghum	Diploid	735 Mb	EMS*	~1/526 kb	~1,400
Soybean	Paleopolyploid	1.1 Gb	EMS*	1/550-1/140 kb	~2,000-8,000
Soybean	Paleopolyploid	1.1 Gb	MNU**	1/550-1/140 kb	~2,000-8,000
Tomato	Diploid	~950 Mb	EMS*	1/730 kb	~1,300
Wheat	Hexaploid	~16 Gb	EMS*	1/35-1/24 kb	~457,000-666,000
Durum wheat	Tetraploid	~10.8 Gb	EMS*	~1/51 kb	~211,000

1771 ***EMS:** ethyl methanesulphonate; ****MNU:** N-methyl-N-nitrosourea; *****SA:** sodium azide.*

1772
1773   Compared to background levels, induced mutagenesis greatly increases the mutation rate or frequency.
1774   For example, the "normal" mutation rate in *Arabidopsis thaliana* is approximately 0.02 mutations in the
1775   genome per cell division (see Table 4). When subject to ethyl methanesulfonate treatment, the number of
1776   mutations increases to around 700 (see Table 5).
1777
1778   **3. How toxic are chemicals or radiation used in IM?**
1779
1780   *Physical mutagens*
1781   Ionizing radiation and other high-energy electromagnetic radiation, such as UV-C waves, can have
1782   indirect and direct toxic effects on human health and developing fetuses.
1783
1784   In fetuses and children, the effects of radiation may include failure of an embryo to implant, early
1785   miscarriage, stillbirth, congenital malformations, impaired brain function, and fetal growth retardation
1786   (Bakar et al., 2019). Exposure to ionizing radiation can lead to uncontrolled cell division and cancer,
1787   including leukemia, and breast, colon, and lung cancers. High doses of radiation may also lead to acute
1788   radiation sickness, which is characterized by burns, fever, loss of coordination, immunity disorders,
1789   diarrhea, and other symptoms (Bakar et al., 2019).
1790
1791   While all ionizing radiation sources pose a risk to human health, gamma radiation is the most penetrant
1792   and likely to result in negative human health impacts (Bakar et al., 2019). The effects of ionizing radiation
1793   can be dose-dependent or random. In dose-dependent reactions, the total dose, volume of irradiated
1794   tissue, rate, radiation type, and individual-specific characteristics determine the severity of the effects.
1795   Random effects can occur at any radiation dose, including low doses, and tend to have a delayed
1796   appearance following radiation exposure (Bakar et al., 2019).
1797
1798   Prasad et al. (2004) note that radiation-induced cancer may remain latent for 10 to 30 years before
1799   proliferating. They also note that due to the inherent complexity of the relevant influencing factors, no
1800   radiation dose can be considered completely safe (Prasad et al., 2004).
1801
1802   *Chemical mutagens*
1803   Hazardous chemicals, such as those used in mutagenesis, are classified under the Globally Harmonized
1804   System of Classification and Labeling of Chemicals (GHS). This system divides chemicals by the nature
1805   and degree of the hazard they pose and provides a common classification system for hazardous
1806   chemicals.
1807
1808   GHS is voluntary at an international level; however, within the United States, it is incorporated into the
1809   OSHA Hazard Communication/Right to Know Standard at 29 CFR 1910.1200 Subpart Z. Table 6 below
1810   summarizes the GHS Hazard Statement information for the chemicals that are most commonly used to
1811   induce mutagenesis.
1812   .

1813

Table 6: GHS Hazard Statements for Common Chemical Mutagens.

Chemical mutagen	Oral toxicity	Dermal and ocular toxicity	Respiratory toxicity	Mutagenicity and carcinogenicity	Reproductive toxicity	Environmental toxicity
5-BU	H302: Harmful if swallowed.					
Ethidium bromide	H302: Harmful if swallowed.		H330: Fatal if inhaled.	H341: Suspected of causing genetic defects.		
Sodium azide (NaN₃)	H300: Fatal if swallowed.	H310: Fatal in contact with skin. H314: Causes severe skin burns and eye damage.		H370: Causes damage to organs. H372: Causes damage to organs through prolonged or repeated exposure.		H400: Very toxic to aquatic life. H410: Very toxic to aquatic life with long lasting effects.
Nitrous acid (HNO2)	H300: Fatal if swallowed.	H314: Causes severe skin burns and eye damage. H318: Causes serious eye damage.				H400: Very toxic to aquatic life.
Ethyl methanesulfonate (EMS)	H302: Harmful if swallowed.			H351: Suspected of causing cancer. H340: May cause genetic defects.	H361: Suspected of damaging fertility or the unborn child.	
N-methyl-N-nitrosourea (MNU)	H301: Toxic if swallowed.			H350: May cause cancer.	H360: May damage fertility or the unborn child.	
1-ethyl-1-nitrosourea (ENU)	H301: Toxic if swallowed.	H312: Harmful in contact with skin.	H332: Harmful if inhaled.	H350: May cause cancer.	H360: May damage fertility or the unborn child.	
Diethyl sulfate (DES)	H302: Harmful if swallowed.	H312: Harmful in contact with skin. H314: Causes severe skin burns and eye damage.	H332: Harmful if inhaled.	H340: May cause genetic defects. H350: May cause cancer.		
Colchicine	H300: Fatal if swallowed.				H340: May cause genetic defects.	
Oryzalin	H300 Fatal if swallowed.			H351: Suspected of causing cancer. H373: May cause damage to organs through prolonged or repeated exposure.		H410: Very toxic to aquatic life with long lasting effects.

1814
1815

Data sources: (PubChem, 2023a, 2023b, 2023c, 2023e, 2023d, 2023f, 2023g, 2023h)

1816   **4. Can unwanted mutations caused by IM be removed via backcrossing?**
1817
1818   As discussed in *Characterization of Induced Mutagenesis Methods*, the process of developing a mutant plant
1819   variety begins with a mutagenesis event, followed by several generations of selection. Both seed-
1820   propagated and vegetatively propagated crops undergo selection, although the selection process
1821   generally lasts for more generations in seed-propagated crops (Forster et al., 2011). Selection is necessary
1822   to identify plants with stable and desirable mutant traits while minimizing unwanted or deleterious
1823   mutations.
1824
1825   In seed-propagated crops, breeders can separate unwanted mutations from desired mutant traits through
1826   self-pollination or backcrossing (Suprasanna & Nakagawa, 2011). In vegetatively propagated crops, there
1827   is no additional sexual recombination, and removing any unwanted mutations becomes more difficult. To
1828   navigate these difficulties, breeders of vegetatively propagated crops may use lower doses of chemical or
1829   physical mutagens to reduce the incidence of unwanted mutations (Suprasanna & Nakagawa, 2011).
1830
1831   A mutant variety may be developed and used as a parent in a subsequent cross. This process can be
1832   referred to as crossbreeding or backcrossing, depending on the ultimate goal. If the goal is to use the new
1833   mutant variety to create a hybrid plant or a population of plants with new characteristics, the process is
1834   cross-breeding (Suprasanna & Nakagawa, 2011). If the goal is to incorporate single mutant traits into
1835   existing "elite" or regionally-adapted germplasm, the process is backcrossing (Forster et al., 2011).
1836
1837   To backcross a mutant trait into desired germplasm, the mutant parent is the "donor" parent and the best
1838   available variety is the "recurrent" parent (Forster et al., 2011). Following an initial hybridization event,
1839   the progeny is evaluated for the presence of the mutant trait. Progeny that contains the mutant trait and
1840   bear the most similarity to the recurrent parent is then crossed with the recurrent parent once more. This
1841   process is repeated until the desired mutant trait is fully introgressed into the recurrent parent
1842   (Anamthawat-Jónsson, 2001).[20] Backcrossing a trait in this manner is a common plant breeding strategy
1843   that is not limited to use with mutant traits. This process is used when bringing a trait of interest from a
1844   wild crop relative into an established crop variety (Anamthawat-Jónsson, 2001).
1845
1846   TILLING can also be used in a backcrossing regimen, as a means to expedite the identification of induced
1847   mutants with desired traits that could be used as "donor" parents in subsequent backcrossing (McCallum
1848   et al., 2000). The use of TILLING generally takes place in an early generation, such as M_2 or M_3, and
1849   backcrossing uses a mutant parent beyond this generation (Szurman-Zubrzycka et al., 2018; Talamè et al.,
1850   2008).
1851
1852   According to Holme et al. (2019), crop breeders can now add TILLING and backcrossing to traditional
1853   mutation breeding techniques to efficiently acquire specific mutant genes of interest. However, in
1854   contrast with New Breeding Techniques (NBTs) such as CRISPR/Cas9, traditional mutation breeding still
1855   produces off-target mutations, which need to be removed through backcrossing (Holme et al., 2019).
1856   Crossbreeding strategies to introgress a specific trait into the desired parent may require five to ten years
1857   (Dhugga, 2022). In order to eliminate unwanted mutations and produce plants with desirable
1858   characteristics, breeders select and backcross many generations of plant to create commercial varieties
1859   (Lemke et al., 2022). While breeders attempt to select for only the desirable traits, unknown background
1860   mutations can still remain, even after many generations of selection (The Central Committee on Biological
1861   Safety, 2018). Genes for different traits segregate during reproductive events based on how far away from
1862   each other they are (Ackert-Bicknell & Rosen, 2016). Undesirable mutations or traits that are adjacent to
1863   desirable mutations or traits are therefore more difficult to breed out. There is no specific number of
1864   backcrosses breeders use to eliminate unwanted mutations.
1865

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[20] Introgression refers to the introduction of a genetic trait from one variety or species (Parent A) into another variety or species
(Parent B) through the process of hybridization and repeated backcrossing (Anamthawat-Jónsson, 2001). Ultimately, the introduced
genetic trait from Parent A will exist within the genetic background of Parent B, without superfluous genetic information from
Parent A.

1866 Some vegetatively propagated crops do not produce viable seeds, such as commercial banana varieties.
1867 Without the ability to utilize sexual recombination for breeding, it is not possible to use backcrossing to
1868 improve mutant or non-mutant varieties (Suprasanna & Nakagawa, 2011).
1869
1870 Backcrossing is a common technique used to remove unwanted alleles in induced mutant plants, but it is
1871 not typically used for microorganisms. Instead, larger populations of microorganisms can be screened
1872 using high-throughput, lab-based techniques to find the desired mutant that has the fewest undesirable
1873 mutations (Fang et al., 2013; Zhang et al., 2014).
1874
1875 **5. How can one determine whether IM was used in the breeding of a plant variety or animal, using**
1876 **historical records, genetic markers, patent records, etc.?**
1877
1878 The mutation breeding approaches described in this report produce changes in DNA that persist beyond
1879 the natural DNA repair cycle. Although chemical and physical mutagens are capable of causing
1880 mutations, they are not insertions of new or foreign DNA into existing genomes. Unlike the *in vitro*
1881 mutagenesis methods, physical and chemical mutagens produce mutations by interacting with DNA that
1882 exists within a cell (Michalczuk, 2022). Mutations like these also occur naturally (for example, through
1883 natural exposure to UV radiation), although at a lower frequency than observed following mutagenesis
1884 (Forster et al., 2011; Strzałka et al., 2020).
1885
1886 Given the similarity to natural mutations, tracking induced mutations via genetic markers proves
1887 difficult, but not impossible. DNA fingerprinting, the collection of laboratory techniques used to identify
1888 and compare genetic material, can be used to identify plants and microorganisms that have undergone
1889 mutagenesis (Abbasi et al., 2016; M. M. Hasan et al., 2015).
1890
1891 Many crop varieties have been developed using induced mutagenesis, including lettuce, beans,
1892 grapefruit, rice, oats, and wheat (Institute of Medicine & National Research Council, 2004). As noted in
1893 the *Historic Use* section, many of the plant varieties developed using induced mutagenesis are tracked
1894 within the Mutant Variety Database (MVD) (Joint FAO/IAEA Centre of Nuclear Techniques in Food and
1895 Agriculture, 2023). Although this database remains active, the registration of new varieties has dropped
1896 significantly over the past two decades. Furthermore, this database only tracks mutant plant varieties,
1897 and mutant microorganisms are not included. Another option for tracing the genetic lineage of a variety
1898 is using parentage information found within utility patents or Plant Variety Protection (PVP) certificates.
1899 The U.S. Patent and Trademark Office (USPTO) manages utility patents. The USDA Agricultural
1900 Marketing Service manages PVP certificates.
1901
1902 While tracking the origins of specific varieties is possible, we did not find a comprehensive database that
1903 connects plant history with varieties that are specifically used in organic production. Identifying whether
1904 any given variety was produced from induced mutagenesis and used in organic production would be a
1905 laborious process.
1906
1907 The following examples reveal the nature of gaps that exist in the MVD:
1908 • The "Madame Butterfly" snapdragon was developed using induced mutagenesis and is listed in
1909   the MVD. Several spinoff varieties exist that are not registered in the MVD. None of the
1910   "Madame Butterfly" varieties fall under PVP certificates, nor are they described in utility patents.
1911 • The hop variety "Santiam" is registered in the MVD. It was developed using a mutant parent
1912   "Hallertauer mittelfruh" that had been induced into a state of tetraploidy using colchicine.
1913   "Hallertauer mittelfruh" parent was also utilized in the hop variety Newport, but Newport is not
1914   in the MVD (Henning et al., 2004).
1915
1916 In some instances, breeders have not registered new varieties derived from mutagenesis or mutant stock
1917 in the MVD but have registered the varieties for patent protection. An example is the barley variety
1918 "Fritz," which was released by Washington State University in 2016. According to the variety's Plant
1919 Variety Protection (PVP) certificate, there are at least two mutagenized barley parents used to develop

1920 "Fritz." The mutagenized parent varieties are unreleased germplasm and were instead maintained in
1921 research populations. One of the parent varieties was initially mutagenized in 1987, after which it was
1922 maintained due to the presence of a mutant gene that improved disease resistance (Washington State
1923 University, 2017). Neither "Fritz" nor any other of its predecessors have ever been listed in the MVD.
1924
1925 In theory, a complete picture of the lineage of a given variety can be compiled using a combination of the
1926 patent databases, MVD, and variety release notifications. Because these databases are not synchronized,
1927 not all varieties are well described, and not all of the lineage-tracing documents are available for a given
1928 variety, this process would be impossible to rely on without significant time and the cooperation of
1929 international seed companies.
1930
1931 *Recent updates to patent tracking in the seed industry*
1932 An industry-compiled database known as the International Licensing Platform (ILP) Vegetable was
1933 launched in 2014 to improve access to and the use of plant breeding traits in vegetables (International
1934 Licensing Platform Vegetable, 2014). This database allows seed company members to share all of their
1935 patents related to vegetable breeding with other members under protected conditions. As part of this
1936 work, an ILP Patent register is maintained and available to the public; however, these patent listings do
1937 not provide easily accessible information about development methods like induced mutagenesis. While
1938 this platform is currently limited to traits in vegetable seed held by member seed companies, it suggests
1939 one route the seed industry may take to improve the traceability of traits.
1940
1941 In March of 2023, the USDA released a report titled "More and Better Choices for Farmers: Promoting
1942 Fair Competition and Innovation in Seeds and Other Agricultural Inputs." The report outlines several
1943 actions that seek to improve market fairness and may impact the availability of information regarding
1944 seed varieties (including varieties developed with induced mutagenesis) (USDA AMS, 2023). Relevant
1945 actions include:
1946   • the creation of a Farmer Seed Liaison to work between farmers, plant breeders, and the patent
1947   system.
1948   • a new working group between the USDA and USPTO that will work towards improving
1949   stakeholder engagement and enhancing the transparency and quality of the patent system.
1950

1951 **Report Authorship**

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1954 approval of this report:
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1960   • Amy Bradsher, Deputy Director, OMRI
1961   • Doug Currier, Technical Director, OMRI
1962
1963 All individuals are in compliance with Federal Acquisition Regulations (FAR) Subpart 3.11—Preventing
1964 Personal Conflicts of Interest for Contractor Employees Performing Acquisition Functions.
1965

| 1966 | **References** |

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