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# TILLING and Eco-TILLING Approaches to discover induced mutations on three cowpea varieties

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## Abstract

**Background:** This study investigates the application of TILLING (Targeting Induced Local Lesions IN Genomes) and Eco-TILLING techniques to identify induced mutations in three cowpea (Vigna unguiculata) varieties. Cowpea, a protein-rich legume, is essential for soil protection and fertility through nitrogen fixation. However, its cultivation is often impeded by various biotic and abiotic stresses, including salinity, drought, and toxic heavy metals. To enhance cowpea's stress tolerance, both conventional and molecular breeding methods are employed.

**Results:** The experiment was conducted at the IAEA Plant Breeding and Genetics Laboratory in Seibersdorf, Austria, using irradiated cowpea seeds from Senegal. TILLING and Eco-TILLING methods were employed to detect mutations in mutant and natural populations using EST markers. The results indicate that mutant populations exhibit mutation frequencies suitable for high-throughput TILLING operations, with mutation densities varying according to irradiation doses and environmental conditions. Gamma-ray-induced mutations generated significant genetic diversity, which is beneficial for varietal selection.

**Conclusion:** This study demonstrates the effectiveness of TILLING and Eco-TILLING methods in improving cowpea's tolerance to abiotic stresses and optimizing its agronomic yield.

**Keywords:** TILLING; Eco-TILLING Mutation; Detection; EST Markers

## 1. Introduction

Cowpea, Vigna unguiculata (L.) Walp, is a high-protein legume belonging to the Fabaceae family [1]. It is known as cowpea in Africa, where it is predominantly produced, accounting for about 64% of the global production, with Nigeria being the largest producer in the region [1, 2]. Cowpea thrives in various agro-ecological zones and plays a significant role in soil protection against erosion and improving soil fertility through nitrogen fixation. Its leaves and stems are an important source of animal feed. The seeds and pods, whether dried or green, are used for human consumption and livestock fodder [3]. Primarily cultivated in tropical regions, cowpea cultivation is often affected by numerous biotic and abiotic stresses, including soil salinity, insects, drought, and toxic heavy metals. Drought is one of the limiting factors for cowpea growth. To increase yield production, plant breeders make considerable efforts to improve cowpea's drought stress tolerance using both conventional and molecular breeding methods. High-efficiency genetic methods are used to study genes involved in salinity and drought stress resistance [4]. Several methods are employed for gene identification, determining their position, and the proteins they encode for a selected trait. An important method among

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these is reverse genetics, a new technique that allows the recognition of gene characteristics by analyzing the phenotypic consequences of certain modified gene sequences [5]. This method starts from a protein or DNA for which there is no genetic data, then works backward to create a mutant gene, resulting in a mutant phenotype [6]. TILLING (Targeting Induced Local Lesions IN Genomes) and Eco-TILLING are commonly used to offer a number of alleles and are effective in mutation detection. TILLING can be applied to species for which genomic sources are limited. TILLING, first applied to Arabidopsis thaliana [7] and Drosophila melanogaster [8], is an effective reverse genetic screening technique used to identify mutations in a specific region of a given population. Eco-TILLING, a variant of TILLING, allows the detection of point mutations related to phenotypic traits in natural populations [6, 9]. These methods have been used on many crops such as cassava [10], banana [11], peanut [12], rice [13], sorghum, and tomato [14]. For legumes, Medicago and Lotus have respectively developed lines of 12,000 and 4,904 M2 [15,16]. Up to 3,000 mutant (M3) bean lines have also been created and tested for their root nodulation capacity [17].

This experiment focused on using DNA obtained from cowpea mutants to discover mutations in the mutant population and natural mutations in the parents and diversity by detecting band polymorphism. TILLING and Eco-TILLING methods were applied to the cowpea mutant population using EST markers 2.

## 2. Materials and Methods

This experiment was conducted at the Plant Breeding and Genetics Laboratory (PBGL) of the IAEA laboratories in Seibersdorf, International Atomic Energy Agency, Vienna, Austria, during 2015/2016. The cowpea seeds used were sourced from Senegal (ISRA) and were first irradiated at PBGL with different doses (table 1) and then multiplied in the field. Non-irradiated seeds were also used as controls. 38 mutant lines of the M2 generation from three cowpea varieties and their parents were tested.

**Table 1** Varieties and Gamma radiation doses

| MOURIDE 280γ | MELAKH 300γ | YACINE 340γ |
|---|---|---|
| Mo495 | Me935 | Ya176 |
| Mo307 | Me842 | Ya493 |
| Mo 348 | Me 203 | Ya632 |
| Mo502 | Me531 | Ya545 |
| Mo488 | Me463 | Ya507 |
| MoM4 | Me435 | Ya706 |
| M0533 | Me732 | Ya4 |
| Mo201 | Me261 | Ya701 |
| Mo363 | Me604 | Ya557 |
| Mo504 | Me355 | Ya720 |
| Mo288 | Me708 | YaT |
| Mo695 | Me611 | |
| MoT | MeT | |

### 2.1. Seeds germination

For the experiment, 10 seeds per mutant line (5 mutant lines) were pre-germinated in Petri dishes, then transplanted into pots and maintained in a greenhouse at 25°C. All mutant lines and control cowpea from Senegal (ISRA) were grown in the greenhouse for one month.

### 2.2. DNA Extraction with Low-Cost method

The Low-Cost method was developed to avoid the toxic organic phase separation used in many low-cost DNA extraction protocols such as the CTAB (Cetyltrimethylammonium bromide) method. It includes several steps: (1) lysis of plant material, (2) binding of DNA to silica powder under chaotropic conditions, (3) washing of bound DNA, and (4) elution

of DNA from the silica powder. This method has been tested on several plant species, and the applicability of such DNA preparations for molecular marker studies in barley has been demonstrated [18].

After germination, the leaf tissue of the mutants was harvested by taking three young leaves from each plant in the plastic pots at one month. These leaves were collected using porous paper envelopes and stored in silica gel for one week before DNA isolation. Once completely dehydrated, the tissues were ground using a standard vortex and metal beads to obtain a powder. After grinding, DNA was extracted from all 38 mutant cowpea lines using a low-cost genomic DNA extraction protocol [18].

### 2.3. Quality Control of DNA by Gel Electrophoresis

To control the quality of the DNA, quantification was performed using gel electrophoresis. Genomic DNA was tested on a 2% agarose gel in electrophoresis with 0.5 µl of TBE buffer and 4.5 µl of ethidium bromide (fig. 4). Quantification was carried out by evaluating the intensity of ethidium staining compared to different concentrations of the standard lambda DNA ladder (3 ng; 4.5 ng; 6 ng; 10 ng; 15 ng; 20 ng; 34 ng; 54 ng; and 75 ng). After quantification, the samples were diluted to 3 ng and 0.075 ng. The latter concentration is the optimal concentration for PCR.

### 2.4. Primer Design

The primers used for PCR amplification were designed due to the unavailability of the cowpea genomic sequence for all loci. Therefore, mRNA was used as the cowpea sequence, and the sequence was compared via BLAST to the genome of Phaseolus vulgaris, the closest relative of cowpea with a fully sequenced and annotated genome found on NCBI. This allowed for the estimation of intron locations. Regions were chosen where the total genomic sequence is ≤ 1800 bp after the BLAST sequence against the Phaseolus genome on phytozome.net. Primer design was carried out using primer3(http://biotools.umassmed.edu/bioapps/primer3_www.cgi)
(http://biotools.umassmed.edu/bioapps/primer3_www.cgi) with parameters for TILLING (Tm 67-73°C and optimal length of 24 with a range of 18 to 27). Sixteen EST primer combinations were designed and used for PCR amplification. Primers containing sequences complementary to the target region, along with a DNA polymerase, which gives the method its name, are key components for enabling selective and repeated amplification. Once the PCR products were obtained, the primers were tested by electrophoresis on a 1.5% agarose gel.

### 2.5. DNA Amplification by PCR

Polymerase Chain Reaction (PCR) is a molecular biology technology used to amplify a single copy or a few copies of a DNA fragment to generate, over several orders of magnitude, thousands or even millions of copies of a particular DNA sequence. This method relies on thermal cycling, consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication. Primers (short DNA fragments) containing sequences complementary to the target region, along with a DNA polymerase, which gives the method its name, are key components for enabling selective and repeated amplification. As PCR progresses, the generated DNA is itself used as a template for replication, triggering a chain reaction in which the DNA template is exponentially amplified.

In this study, several components and reagents were used, including:

- Genomic DNA of cowpea
- Taq Buffer
- dNTP Mix
- 16 primers complementary to the 3' ends (three primers) of each of the sense and antisense strands
- Taq Polymerase
- Thermocycler using the PCRTM70 program

The obtained PCR products were tested on agarose gel (300 ml of 1.5% agarose with 6 µl of ethidium bromide) to detect the presence of amplification (Fig 1).

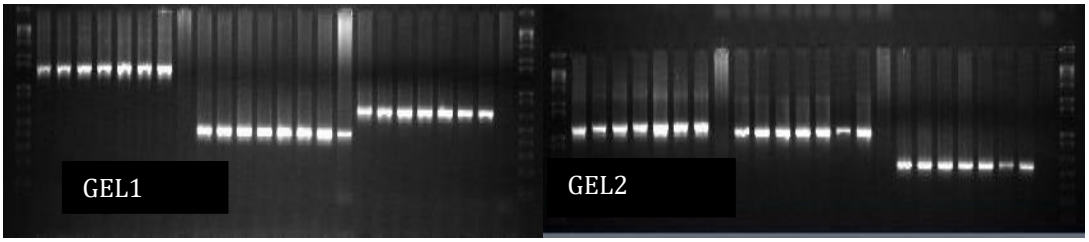


**Figure 1** Primer Test on Agarose Gel

Gel1: Primer 2R-2F; 4F-4R; 5F-6R; Gel2: Primer11F-11R; 15F-15R; 9F-9R

### 2.6. Extraction and Purification of CEL I

Once the PCR was performed, the products were denatured to generate heteroduplexes between wild-type and mutant DNA strands. Mismatch cleavage was achieved by incubation with a nuclease, and the products were visualized using denaturing polyacrylamide gel electrophoresis and a gel reading platform such as the Li-Cor DNA analyzer. Although several nucleases have been identified for mismatch cleavage [19, 20, 21, 22], CEL I nuclease from celery is commonly used. CEL I is a single-strand-specific nuclease related to S1 nuclease, and CEL I, S1, and mung bean nucleases have all been shown to be useful for mutation discovery using standard TILLING methods [23].

CEL I is a single-strand-specific nuclease related to S1 nuclease, and CEL I, S1, and mung bean nucleases have all been shown to be useful for mutation discovery using standardized TILLING methods [23].

In this section, we performed the extraction and purification of CEL I. Celery was first pressed, and a buffer was added to the juice to maintain the pH. The juice particles were removed by microcentrifugation and gravity filtration. Ammonium sulfate was then added to the juice until 25% saturation. The single-strand-specific nuclease remained in solution at this saturation, but other proteins were precipitated and removed by microcentrifugation. The supernatant was collected, and ammonium sulfate was added until 85% saturation, where the desired single-strand-specific nucleases precipitated. The pellets were collected by microcentrifugation and resuspended in a buffer. A total of four buffer washes were performed to remove the salt and further concentrate the enzyme. The purified enzyme was then collected, and its activity was tested.

### 2.7. Digestion of Genomic DNA with CEL I and Evaluation on Agarose Gel

After PCR amplification, the digestion of genomic DNA was performed with CEL I. The CEL I mixture was prepared with PCR water (2400 µl), CEL I buffer (420 µl), CEL I (10 µl), and 20 µl of the mixture were added to each PCR product. The mixture was incubated at 45°C for 15 minutes in a thermocycler. To stop the reaction, 10 µl of 0.25 M EDTA per sample were added, mixed, and centrifuged. For the evaluation of all samples, a 1.5% agarose gel was prepared, and all digested products were detected using LI-COR denaturing polyacrylamide gels.

## 3. Results

DNA extraction was successfully performed from all lines (figure). The sixteen EST primer combinations tested on cowpea produced amplification, except one which was excluded from the 5F-5R analysis (Fig 1). The cleaved products were detected using IRD 700 and 800 channels. Four EST primers showed polymorphism between the varieties (Figure 2 to 6). The product sizes ranged from 400 to 3000 bp.

A total of 33 mutations were found: 12 in A, 13 in B, 3 in C, 3 in D, and 2 in E2. Two individual lines, one from population A and the other from population B, had more than one base change detected in an amplicon.

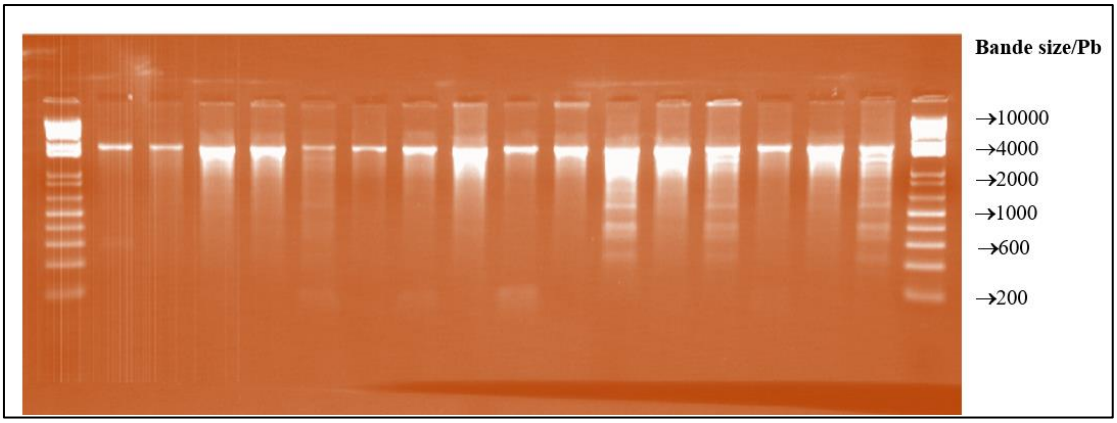


**Figure 2** Gel image for primer 2F-2R of mutation TILLING discovery using crude celery juice for enzymatic mismatch cleavage

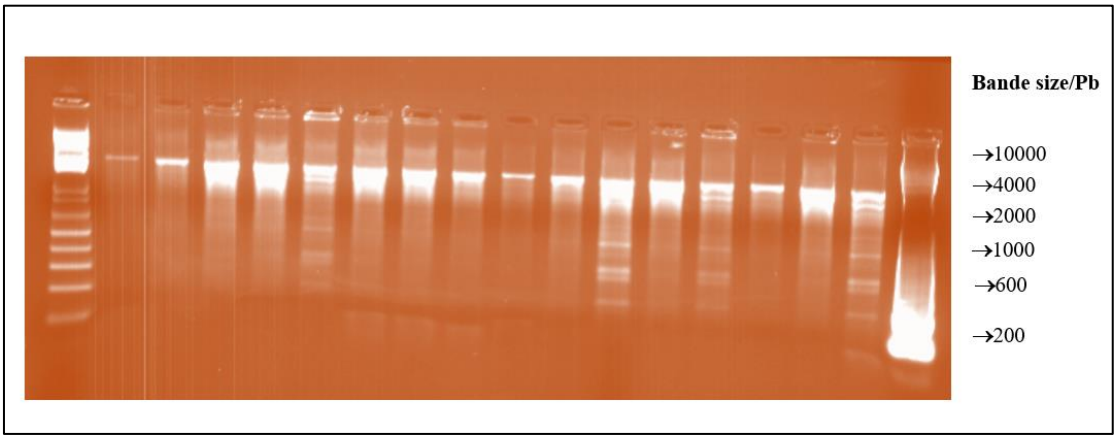


**Figure 3** Gel image for primer 1F-1R of mutation TILLING discovery using crude celery juice for enzymatic mismatch cleavage

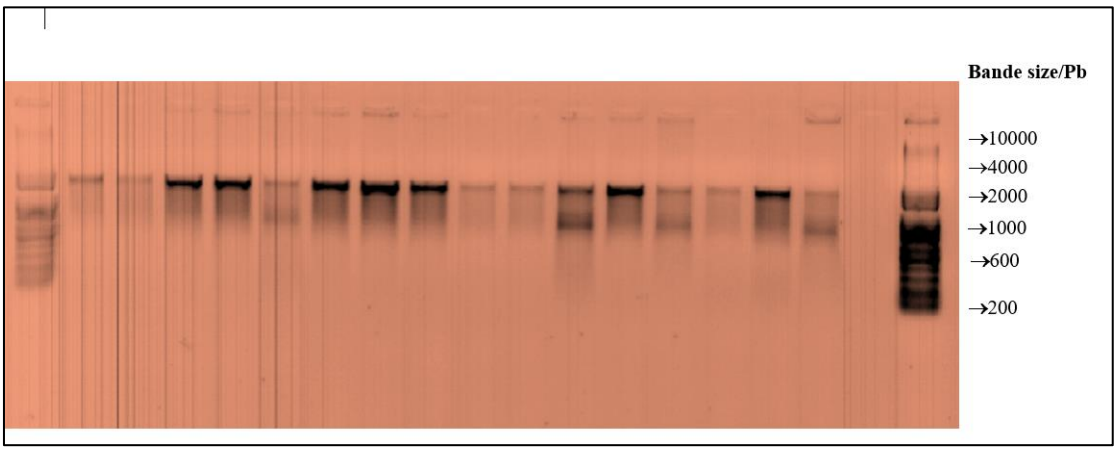


**Figure 4** Gel image for primer 3F-3R of mutation TILLING discovery using crude celery juice for enzymatic mismatch cleavage

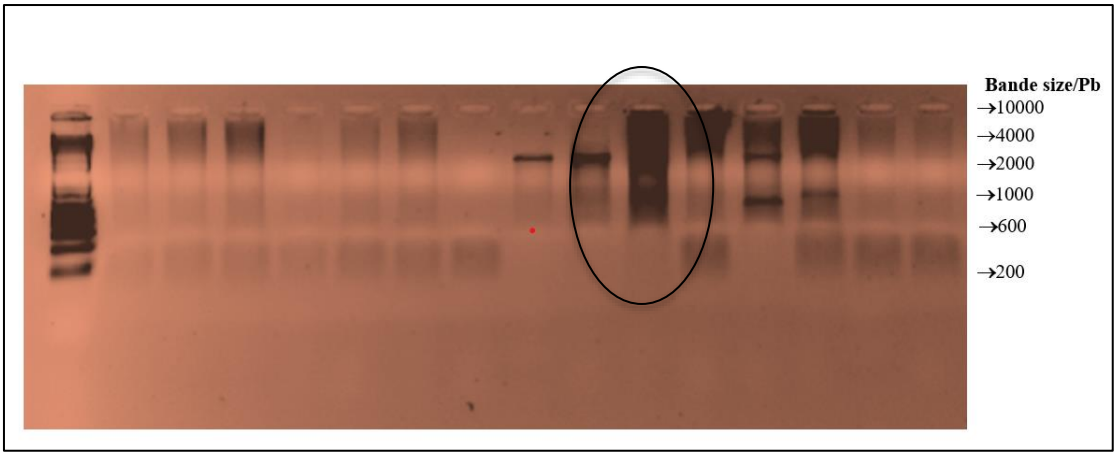


**Figure 5** Gel image for primer 4F-4R of mutation TILLING discovery using crude celery juice for enzymatic mismatch cleavage

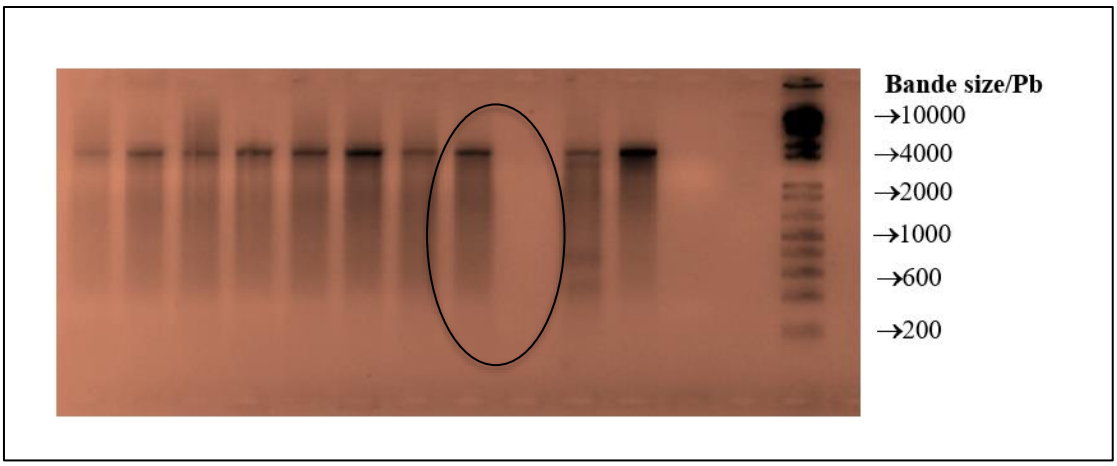


**Figure 6** Gel image for primer 2F-2R of mutation Eco-TILLING discovery using crude celery juice for enzymatic mismatch cleavage

The mutation density was estimated as the total number of mutations divided by the total number of base pairs examined (amplicon size × individuals examined). For each target, 200 bp were subtracted from the amplicon size to adjust for the 100 bp regions at the top and bottom of the TILLING gel images that are difficult to analyze. Populations A and C showed similar mutation densities (~1/160 kb for both).

The mutation density in population A was approximately 1/550 kb and about 1/400 kb in population B. Populations C and D had the same mutation distribution with 4% truncation mutations, 48-51% missense mutations, and 66-59% silent mutations. The distribution in population B was 12% truncation mutations, 43% missense mutations, and 68% silent mutations. Population A significantly deviated from these mutation distributions as no truncations were found, with 56% missense mutations and 44% silent mutations (pairwise comparison of mutation distribution in A to the distribution in each population: B $\chi 2 = 23.5$, $p < 0.001$; C $\chi 2 = 14.62$, $p < 0.05$; D $\chi 2 = 6.05$, $p < 0.05$).

However, none of the mutation distributions were significantly different from the expected distribution calculated from the irradiation-induced changes in the targets (5, 33% truncations, 56% missense mutations, and 51% silent mutations).

## 4. Discussion

### 4.1. Effects of Gamma Rays on Cowpea Mutations

Unlike point mutations induced by EMS, which are primarily G/C to A/T transitions, gamma rays cause deeper DNA damage. As ionizing radiation, they induce double-strand breaks, leading to chromosomal rearrangements, deletions, translocations, and inversions [24]. These effects can generate a broader genetic diversity than point mutations, which is beneficial for varietal selection. The effect of gamma rays on the cowpea genome structure is reminiscent of observations made in soybeans, where studies have shown that some mutations could be amplified due to genetic redundancy [25]. However, in cowpea, which is considered diploid, it is possible that the effects of double-strand breaks can be managed without significantly affecting plant viability. In contrast to diploid species, polyploids such as soybeans present challenges for mutation detection due to the presence of homologous gene copies that can mask or complicate genetic variation [26].

To evaluate the effectiveness of cowpea as a model for high-throughput mutation discovery via TILLING, we examined 36 targets in three varieties of mutagenic populations and identified a total of 33 induced mutations. Our observations indicate that induced mutagenesis in cowpea produces populations with mutation frequencies compatible with high-throughput TILLING operations. Indeed, the results show that populations A and D have the highest mutation frequencies, followed by populations C and B. The distribution of mutations observed in these populations is consistent with expectations. However, the mutation frequencies in these cowpea populations were not higher than those reported for barley and maize [22, 27].

Although population B was irradiated with a lower dose than population A, the resulting mutation frequency was lower. It is possible that the genetic background affects the efficiency of irradiation, as observed in other species such as wheat, barley, and rice [28, 29]. However, differences due to other environmental or experimental conditions cannot be excluded. Populations B and C share the same genetic background, but population B was irradiated with a lower dose of gamma rays than population C, resulting in a mutation density approximately twice as high as that of population C. We noted that the irradiation treatment of cowpea seeds with different doses can vary in mutation frequency from one experiment to another, probably due to the effect of environmental conditions on the plant's response. Therefore, it is expected that mutagenesis experiments conducted in different locations with different irradiation doses may result in very different mutation frequencies. This intraspecific variability highlights the importance of genetic and environmental factors in the effectiveness of mutagenesis [30].

However, although visible mutations were observed more frequently when the irradiation dose was increased to 300 Gy, the proportion of irradiated seeds that germinated and grew was halved as the dose was raised to 340 Gy. Therefore, more severe mutation protocols can increase the mutation frequency [31], but they also significantly reduce the recovery of viable seeds. Additionally, we found that by digesting the template DNA to remove a parasitic amplicon, it was possible to improve mutation detection.

### 4.2. Optimization of Mutation Detection

Complex mutations caused by gamma rays require a rigorous methodological approach for their identification. In the case of soybeans, it has been demonstrated that nonspecific amplification of homologous genes can hinder the effective detection of mutations via TILLING [25]. A similar strategy could be adopted for cowpea, by integrating targeted restriction enzymes or pre-testing primers to refine the detection of gamma-ray-induced mutations.

TILLING used for maize and soybeans has successfully overcome these obstacles by pre-testing primers before amplification and optimizing PCR conditions [32]. For cowpea, this approach could enable better identification of mutations, particularly those involved in abiotic stress resistance and the improvement of agronomic yield.

### 4.3. Agronomic Applications and Perspectives

Our results confirm that cowpea has significant potential for high-throughput TILLING. By integrating advanced mutation detection strategies and leveraging the effects of gamma rays in a controlled manner, it would be possible to improve the precision and efficiency of genetic selection for this species.

The use of gamma rays to induce mutations in cowpea opens promising prospects for varietal selection. Similar to soybeans, where allelic series are combined through crossbreeding to optimize specific traits, it would be feasible to develop mutagenic cowpea lines with improvements in drought tolerance, disease resistance, and nitrogen fixation optimization.

However, the variability observed in mutation frequencies according to irradiation doses suggests that further studies are needed to refine mutagenesis protocols. It would be pertinent to test different exposure doses and analyze their impact on the genomic stability of cowpea to maximize benefits without compromising seed viability.

## 5. Conclusion

In conclusion, this study highlighted the effectiveness of TILLING and Eco-TILLING methods for detecting mutations in cowpea varieties subjected to water stress. The results show that mutagenic populations exhibit mutation frequencies compatible with high-throughput TILLING operations, with mutation densities varying according to irradiation doses and environmental conditions. Gamma-ray-induced mutations generated significant genetic diversity, which is beneficial for varietal selection. However, further studies are needed to optimize mutagenesis protocols and maximize benefits without compromising seed viability. By integrating advanced mutation detection strategies, it is possible to improve cowpea's tolerance to abiotic stresses and optimize its agronomic yield.

In our future improvement perspectives, we plan to conduct additional studies on the sequencing protocols of identified genes to see their involvement in mechanisms of resistance to salinity or water stress, in order to create more efficient varieties without compromising seed viability.

## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declared no conflict of interest.

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