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Agronomic and Physicochemical Characterization of Gamma Irradiation on M4 Cowpea (*Vigna unguiculata* [L.] Walp.) Mutants

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Abstract Gamma irradiation has been successfully used as a powerful mutagen for inducing various legume crops. This study evaluated the effect of gamma irradiation-induced variability on the agronomic, physicochemical and yield attributes of cowpea plants. The study was conducted in a screenhouse in a complete randomized block design with 8 replications. The M4 mutant lines were derived from gamma irradiation with a Tswana background. Tswana is a late-maturing local commercial variety exhibiting a prostrate growth habit with a yield potential of 1-2 ton^{-1}ha. The mutant lines include Tswana-300Gy-202, Tswana-300Gy-214, Tswana-400Gy-49, Tswana-400Gy-49, Tswana-500Gy-31, and Tswana-500Gy-53. Analysis of variance for agronomic traits and nutritional factors revealed induced genetic variability between the mutant and their Tswana background. A significant variation of a 1 or 2-day prolonged period to emergence in mutant lines compared to the Tswana variety suggested a novel-induced variation. The maximum and the lowest Chl-a content was observed for the Tswana-500Gy-53 line (0.510 µg ml^{-1}) and Tswana (0.276 µg ml^{-1}) respectively. However, an induced consistent suppressed Chl-a was observed for Tswana-300Gy-202 and Tswana-300Gy-214 relative to other mutant lines. The carotenoid levels were highest for Tswana-500Gy-53 (0.220 µg ml^{-1}), while Tswana-300Gy-202 (0.126 µg ml^{-1}) had the lowest carotenoid content. The maximum NPQt and PhiNPQ were observed for Tswana-500Gy-53 (5.070; 0.651) and Tswana-500Gy-31 (4.122; 0.594), resulting in lower Fv/Fm and Phi2 compared to Tswana control (3.741). Tswana-400Gy-85 had the highest crude fibre concentration (6.190g/kg) compared to the control, while the lowest concentration was observed for Tswana-400Gy-49 (5.047g/kg). Tswana-500Gy-31 recorded the lowest tannin content (0.097), followed by Tswana-500Gy-53 (0.098), while Tswana-400Gy-85 had the highest tannin level (0.260) compared to the control and other mutant lines. The yield-related attributes were not significantly affected by gamma-irradiated mutagenesis except the 100 SW. Tswana-300Gy-214 (18.125 g) and Tswana-400Gy-49 (18.250 g) had the highest 100SW compared to the Tswana control (16.500 g). They also had higher 100 SW than Tswana-400Gy-85 (16.750 g) and Tswana-500Gy-53 (16.875 g) respectively. Further studies could consider the antinutritional and genetic analysis of the point of mutation and the existence of silent mutations where the DNA did not have a noticeable effect.

Keywords Gamma Irradiation, Mutant,

Photosynthetic Fluorescence, Chlorophyll, Carotenoids, Yield

1. Introduction

In the face of climate change, which is threatening the productivity of most major crops, the need to increase the feeding capacity of the world's growing population is becoming a global concern. Crops such as cowpeas (*Vigna unguiculata* L. Walp) have been recognized as strategic crops to increase food security [1]. Cowpea is a leguminous crop with agronomic, environmental, and economic benefits, which helps to improve the diets and income status of individuals in underserved communities across Africa, Asia, and South America [1], [2]. Due to its ability to thrive in various environmental conditions, it is an important Sub-Saharan African crop and an important component of crop production and the economy of the region [3]. Cowpea plants contribute to long-term environmental improvement through their ability to fix biological nitrogen, which has positive effects on the soil by increasing microbial diversity [4]. One of the adaptive mechanisms of plants to abiotic stresses is associated with biochemical and transcriptional regulation of nitrogen sources [5]. As such cowpea is considered a resilient plant to climate change and to the endangering agricultural production [6]. In Botswana, cowpea production has been reported to be low despite widespread cultivation, with most farmers classified as smallholders [7]. The resulting low productivity may be exacerbated by using cultivars with inherent low-yield potential and a lack of improved and high-yielding cultivars.

With the aim of increasing cowpea growth, development, yield and quality, several breeding methods, such as inbreeding, backcrossing or introgression breeding, mutation breeding, hybrid breeding, molecular marker-assisted selection, genetic engineering, and gene editing, have been implemented to improve the productivity of many crops including cowpea [8]. Among these techniques, induced mutation breeding has been used in crop improvement in recent years due to its low cost and rapid and robust results [4]. Compared with other breeding methods, this approach has shown the ability to generate variation for genetic enhancement in many crops as well as for breeding in a relatively shorter time [6], [7]. This approach is critical for the development of superior crop varieties, which have a significant economic impact on agriculture, food production, and, ultimately, food security. Gamma irradiation, among other induced mutation approaches, has been proven to be one of the most effective and successful methods for generating improved cultivars in terms of yield, quality, and resilience to biotic and abiotic limitations [8]. It has a long-term history as a main tool of environmental mutagenesis for the purpose of plant breeding (more than 60 years). During this time, several mutant lines from various plant species and cultivars with better traits emerged in many places throughout the world [9]. Meanwhile, carbon metabolism through photosynthesis is a critical activity for plants to perform their routine and primary cellular processes during growth and development [10]. In that regard, using chlorophyll fluorescence characteristics to examine photosynthesis is a beneficial tool that can serve as an indicator of thylakoid membrane efficiency and functional changes in the photosynthetic apparatus [11]. Chlorophyll fluorescence is considered a quick and effective probe for physiological changes associated with plant photosynthesis efficiency [12] and has been accurately used for evaluating the effects of various stress factors, such as high and low temperature [13] radiation quality and intensity [14] water stress [15], salinity [16]. For most plants including cowpea, several chlorophyll fluorescence parameters such as the maximum quantum efficiency of photosystem II photochemistry (Fv/Fm), the quantum yield of Photosystem II (Phi2), the quantum yield of non-regulated dissipation process (PhiNO), Non-photochemical quenching (NPQ), the quantum yield of non-photochemical quenching (PhiNPQ), Photosystem I Open Centres (PSIOC), Photosystem I Active Centres (PSIAC) can directly reflect the changes in photosynthetic characteristics of plants subjected to different conditions.

However, the gamma irradiation process could destroy the growth and photosynthetic pigments with a concomitant loss of photosynthetic capacity. Several studies on the effects of gamma irradiation on the agronomic and physicochemical aspects of various crop species including cowpeas [17], chickpeas [18], mungbean [19] and peas [20] have been conducted. Research has reported stimulatory effects of low gamma irradiation (<150 GY), such as an increased survival rate, an increased germination%, a reduced number of days to 50% emergence, 50% flowering and 50% maturity on a variety of legumes, including cowpea [21], [22]. However, an irradiation dose of 200 GY or above can lead to undesirable alterations such as a decreased survival rate, increased days to 50% emergence, 50% flowering, 50% maturity, and reduced chlorophyll content [19], [20], [23]. Despite the high nutritional and functional value, cowpea grains contain some primary and secondary compounds such as starch, crude fibre and tannins. Excess of these compounds is regarded as anti-nutritive as they interfere with the bioavailability and absorption of proteins and minerals [24], [25]. Gamma radiation has also been proven beneficial in increasing overall nutritional qualities, including some desired modifications in the functional properties of seed flours in Faba bean and red kidney beans [26]. Given the impact of gamma irradiation on crop improvement, this study aimed to investigate the effect of gamma irradiation on the agronomic, physicochemical characteristics and yield attributes of cowpeas.

2. Materials and Methods

2.1. Plant Materials

Seeds of the Tswana cowpea (control) and the six-gamma-irradiated mutant lines with a Tswana background (Table 1) were obtained from the breeding program of the National Agricultural Research Development Institute (NARDI), Botswana. The mutant lines were derived from 300, 400, and 500 gamma rays at the International Atomic Energy Agency (IAEA), Agriculture and Biotechnology Laboratory in Vienna, Austria, using the CO60 source Gammacell Model. Progenutant selection for morphological and agronomic characteristics was carried out on M2-M4 plants at the Department of Agricultural Research breeding program and the results demonstrated a high survival rate. Therefore, this study investigated cowpea mutant lines below based on morphological, physiological and yield aspects.

Table 1. Cowpea M4 mutant lines

Entries number	Cowpea Mutant lines
1	Tswana -0Gy
2	Tswana-300Gy-202
3	Tswana-300Gy-214
4	Tswana-400Gy-49
5	Tswana-400Gy-85
6	Tswana-500Gy-31
7	Tswana-500Gy-53

M4: 4th progeny lines; Gy: Gray derived unit of ionizing irradiation dose; 0 presents the control; "300Gy, 400Gy, 500Gy" presents gamma irradiation doses; "202, 214, 49, 85, 31 and 53" presents codes for the M4 progeny lines.

2.2. Experimental Site and Design

The experiment was conducted in a screenhouse using polythene bags, at the National Agricultural Research Development Institute (NARDI) during the 2022/2023 cropping season. The experiment was subjected to a randomized complete block design (RCBD) with 8 replicates. The seeds were sown in polythene bags filled with mixed soil at a depth of approximately 2-3 cm. The plants were watered to field capacity and agronomic management was carried out throughout their growing season.

2.3. Agronomic Traits and Yield Parameters

Agronomic traits were measured on the number of days to 50% emergence, 50% flowering and days to 50% maturity. For yield parameters, all the pods from the 8 replications for each variety were harvested and used to analyse the following yield traits; the number of pods per plant, pod length, pod weight (g), number of seeds per pod and 100-seed weight (g).

2.4. Photosynthetic Pigments

Plant leaves were collected from three randomly selected biological replications 5 weeks from the date of planting. The third developed leaves were detached from the plants in the late morning, where they were snap-frozen in liquid nitrogen and immediately stored at -80 ℃ until the extraction and determination of chlorophyll and carotenoid contents. Chlorophyll and carotenoid contents were extracted using methods described previously by Asimovic et al. [27] and Kamble [28]. A total leaf sample of 1 g was mixed using an Automill Tkken. Afterward, the powder was blended with 80% acetone and 0.5 g of magnesium carbonate ($MgCO_3$) powder. The sample was then gently ground, refrigerated at 4 ℃ for 4 hours, and centrifuged for 5 minutes at 500 rpm. The supernatant was collected, and a spectrophotometer was used to quantify the total chlorophyll, Chl-a, Chl-b, and carotenoid contents. The absorbance of the chlorophylls was measured at 645 nm and 663 nm, while the absorbance of the carotenoids was measured at 480 nm and 510 nm. As a control, 80% acetone was used. The existing concentrations of chlorophylls and carotenoids in the leaf extracts were determined using the following equations [29].

$$\boldsymbol{Chlorophyll\ a}\ \ (\boldsymbol{mg.g^{-1}FW}) = \{12.7(\mathrm{OD}_{663}) - 2.69(\mathrm{OD}^{645})\} * \frac{\mathrm{V}}{1000*\mathrm{W}} \quad (1)$$

$$\boldsymbol{Chorophyll\ b}\ \ (\boldsymbol{mg.g^{-1}FW}) = \{22.9(\mathrm{OD}^{645}) - 4.68(\mathrm{OD}^{663})\} * \frac{\mathrm{V}}{1000*\mathrm{W}} \quad (2)$$

$$\boldsymbol{Total\ Chlorophyll}\ \ (\boldsymbol{mg.g^{-1}FW}) = \{20.2(\mathrm{OD}_{645}) + 8.02(\mathrm{OD}_{663})\} * \frac{\mathrm{V}}{1000*\mathrm{W}} \quad (3)$$

$$\boldsymbol{Carotenoids}\ \ (\boldsymbol{mg.g^{-1}FW}) = \frac{7.6(\mathrm{OD}_{480}) - 1.49(\mathrm{OD}_{510}}{\mathrm{d}*1000*\mathrm{W}} * \mathrm{V} \quad (4)$$

Where:
OD_{663}, OD_{645}, OD_{480}, OD_{510} = Optical densities at a specific wavelength
V = Volume of an extract
W = Fresh weight of extracted tissue
d = Length of light path (d = 1.4cm)

2.5. Determination of Chlorophyll Fluorescence Parameters

A handheld MultiSpeQ device v1.0 (Michigan State University, USA) connected to the PhotosynQ Platform (http://www.photosynq.org) was used to collect data on chlorophyll fluorescence-based photosynthetic traits from three randomly selected replicates. Five leaves were selected from each plant, and the MultiSpeQ device was gently pressed open and then clamped onto the leaf. Measurements were taken 3-5 minutes after the device was clamped onto the leaf. The photosynthetic traits at the top of the leaf canopy were measured, including the maximum quantum efficiency of PSII photosystem (Fv/Fm), the quantum yield of photosystem II (Phi2), the quantum yield of the non-regulated dissipation process (PhiNO), quantum yield of non-photochemical quenching (PhiNPQ), non-photochemical quenching (NPQt), PSI open centres, and PSI active centres.

2.6. Starch Determination

The 5 g sample of seeds was ground using a grinder and then sieved through a 0.5 mm sieve to obtain powdered samples. Only 1.25 g of the powdered samples was weighed into a 25 ml conical flask and then homogenized with 12.5 ml of a 1% hydrochloric acid solution. The mixture was placed in a shaking water bath at 95-100 ℃ for 15 minutes. Subsequently, 7.5 ml of distilled water was added to cool the mixture. Then, 2.5 ml of 4% phosphor-wolframic acid was added to the mixture to precipitate the nitrogenous matter. The solution was then diluted to 25 ml and filtered through Whatman Grade 2 filter paper to obtain a clear solution. Thereafter, 5 ml of the filtrate was transferred into a 2 dm^3 polarization tube, and the polarization value was measured using a polarimeter. To calculate the total starch in the samples, the formula provided by ISO 9001: 2008 was used as follows:

$$\%\boldsymbol{Starch} = [\frac{\boldsymbol{Vts}*a_0}{\boldsymbol{\alpha^{20}_D}*\mathbf{L_P}*\mathbf{Ws}} * \mathbf{100} \quad (5)$$

Where:

Vts: Total volume of sample

$\mathbf{a_0}$: Polarization value

L_P: Length of polarization tube

α^{20}_D: Conversion factor (specific rotation) of Ewers method for starch

Ws: weight of sample

2.7. Extraction and Determination of Crude Fibre

Extraction and determination of crude fibre were performed according to the AOAC 2000 procedure. The seed samples were ground to a particle size of 1mm using a grinder. Exactly 1 g of celite and 3 g of the ground sample (W1) were weighed with an accuracy of ±1 mg and placed into a crucible. 60 ml of cold Scharrer reagent was added, and the mixture was boiled for precisely 30 minutes from the onset of boiling. A vacuum was then used to drain the acid from the samples, followed by washing with 400 ml of deionized water using a water spray device. The addition of 60 ml of cold Scharrer reagent and the subsequent boiling, draining and washing procedure were done twice. Thereafter, the samples were washed three times with 25 ml of acetone and two times with 25 ml of diethyl ether. After the final wash, the crucible was placed in an incineration dish and dried with its contents for at least 90 minutes in a drying oven set at 130 ℃. The crucible and the incineration dish were then allowed to cool in a desiccator until they reached a constant weight. This weight represented the crucible containing crude fibre and ashes (W2). Later, the filter crucible and the incineration dish were placed in a muffle furnace for ashing at a temperature of (500 ± 25) ℃ for three hours and then reweighed after cooling in a desiccator. This weight represented the crucible containing ashes (W3).

$$\boldsymbol{Crude\ fiber\ content}\left(\frac{g}{kg}\right) = \frac{(m^2 - m^3)}{m_1} * \mathbf{100} \quad (6)$$

Where;

m_1 is the mass, in milligrams of the ground sample.

m_2 is the mass, in milligrams, of the incineration dish with the filter crucible with the residue obtained after drying at 130 ℃.

m_3 is the mass, in milligrams, of the incineration dish with the filter crucible with the residue obtained after ashing at (500 ± 25) ℃.

2.8. Extraction and Determination of Condensed Tannins

Extraction and determination of condensed tannins were done following a method by FAO/IAEA [30]. A 200 mg finely ground seed sample was placed in a conical flask with 50% aqueous methanol. The samples were then put in a shaking water bath at 30 ℃ for 2 hours. After this, the contents were centrifuged at 4 ℃ for 20 minutes at 3,000 xg. The supernatant was collected and kept on ice for use in determining the tannins. 1 ml of the extracted tannins from each sample was pipetted into 100*12 mm test tubes. Then 6.0 ml of the butanol-HCl reagent and 0.2 ml of the ferric reagent were added to each of the test tubes. The tubes were swirled, covered with glass marbles, and then heated on a heating block at 97-100 ℃ for 60 minutes. After heating, the tubes were allowed to cool, and the absorbance was read at 550 nm. The blank sample was prepared from an unheated mixture consisting of 1.0 ml of extract, 6 ml of butanol, and 0.2 ml of ferric reagent.

$$\boldsymbol{Condensed\ Tannins}\ (\%\ \boldsymbol{in\ DM}) = \frac{\boldsymbol{A550nm}*\mathbf{78.26}*\boldsymbol{Dilution\ Factor}}{\%\ \boldsymbol{Dry\ mater}} \quad (7)$$

Where:

A550nm= absorbance at 550nm

78.26- conversion factor

Dilution factor- 1

2.9. Statistical Analysis

The analysis of variance and means comparison were statistically evaluated using SAS statistical software (version SAS Institute 2004) where significant means were compared using the least significant difference (LSD) test at a risk level of 5% ($p<0.05$).

3. Results

3.1. Agronomic Traits in Response to Mutation

Statistical analysis showed that the number of days to 50% emergence varied from 7 to 9 days. The Tswana control had the lowest number of days (7 days) to 50% emergence. However, gamma irradiation significantly prolonged the number of days to 50% emergence. The mutant lines Tswana-300Gy-202, Tswana-400Gy-85 and Tswana-500Gy-53 took a significant 2 days longer to reach 50% emergence than the Tswana control. In addition, Tswana-300Gy-214, Tswana-400Gy-49, and Tswana-500Gy-31 mutant lines took an additional day to reach 50% emergence relative to the Tswana control. Nonetheless, the number of days to 50% flowering and the number of days to 50% maturity were not significantly different ($p<0.05$) between Tswana and the mutant lines (Table 2). Although the difference was insignificant, the Tswana-500Gy-31 and Tswana-500Gy-53 took 65 and 66 days respectively to reach 50% flowering, shorter than the 68 days for the Tswana variety. However, they reached maturity within 110 and 111 days respectively compared to the 108 for the Tswana variety.

3.2. Physiological Response of Mutant Lines to Gamma Irradiation

3.2.1. Chlorophylls Content of Mutant Lines

Analysis of variance showed that gamma irradiation affected chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and the total chlorophyll content. The Chl-a content varied from 0.276 to 0.510 μg ml^{-1}. The maximum Chl-a concentration was observed for the Tswana-500Gy-53 line (0.510 μg ml^{-1}), while the Tswana control had the lowest Chl-a content (0.276 μg ml^{-1}). Gamma-irradiated mutants, namely, Tswana-400Gy-49, Tswana-400Gy-85, Tswana-500Gy-31, and Tswana-500Gy-53 had a significantly higher chlorophyll-a content (0.445; 0.475; 0.443; 0.510 μg ml^{-1}) than the Tswana control (0.276 μg ml^{-1}). Similarly, these mutant lines, except Tswana-500Gy-31 showed a significantly high content of Chl-b relative to the Tswana control. However, the highest Chl-b content was observed in the Tswana-400Gy-85 (0.633 μg/ml), while the lowest was recorded for the Tswana-300Gy-202 (0.341 μg ml^{-1}). The Tswana-400Gy-49 showed the highest total chlorophyll concentration of 1.107 μg ml^{-1}), while the lowest total chlorophyll concentration of 0.333 μg ml^{-1} was observed in the Tswana-500Gy-31 line. In addition to increased levels of chlorophyll-b, mutant lines Tswana-400Gy-49 and Tswana-400Gy-85 further demonstrated a significant increase in total chlorophyll content of 1.062 and 1.107 μg ml^{-1} respectively compared to 0.661 μg ml^{-1} of Tswana control. In contrast, significantly lower total chlorophyll contents amounting to 0.330 and 0.381 μg ml^{-1} were observed for Tswana-500Gy-31 and Tswana-500Gy-53, respectively, compared to the Tswana control (Table 3).

Further comparisons between mutant lines showed a clear trend of a significantly suppressed chlorophyll-a for Tswana-300Gy-202 and Tswana-300Gy-214 in comparison to other mutant lines. Despite no difference in Chlorophyll-a amongst mutant lines, Tswana-500Gy-53 had the highest levels of chlorophyll-a. In terms of total Chlorophyll, variations exist between the mutant lines. The mutant Tswana-400Gy-49 and Tswana-400Gy-85 lines exhibited significantly higher total chlorophyll content, followed by Tswana-300Gy-202 and Tswana-300Gy-214. The Tswana-500Gy-31 and Tswana-500Gy-53 mutants exhibited the lowest total chlorophyll compared to other mutants (Table 3).

3.2.2. Carotenoids Contents

A significantly higher carotenoid concentration was observed in the Tswana-500Gy-53 (0.220 μg ml^{-1}) line, while the lowest carotenoid content was observed in the Tswana-300Gy-202 (0.126 μg ml^{-1}) line. Only mutant lines Tswana-400Gy-85 and Tswana-500Gy-53 had significantly higher concentrations of carotenoids 0.210 and 0.220 μg ml^{-1} respectively in comparison to the Tswana control (0.150 μg ml^{-1}). On the other hand, Tswana-300Gy-202 and Tswana-300Gy-214 showed a significantly lower carotenoid content than other mutant lines. The Tswana-300Gy-214 showed a significant 0.072 μg ml^{-1}, 0.081 μg ml^{-1} and 0.091 μg ml^{-1} lower carotenoid content compared to the Tswana-400Gy-49 Tswana-400Gy-85, Tswana-500Gy-31 and Tswana-500Gy-53 lines respectively (Table 3).

Table 2. Agronomic traits in response to mutation in M4 cowpea mutant lines

Variety	Days to Emergence	Days to flowering	Days to maturity
Tswana-0	7.000 ±0.463^{c}	68.000 ±5.625	108.000 ±8.123
Tswana-300Gy-202	9.000 ±1.302^{ab}	69.000 ±4.764	112.000 ±7.726
Tswana-300Gy-214	8.000 ±0.707^{b}	71.000 ±5.890	113.000 ±5.792
Tswana-400Gy-49	8.000 ±0.641^{b}	70.000 ±4.518	113.000 ±4.970
Tswana-400Gy-85	9.000 ±1.408^{a}	71.000 ±3.623	114.000 ±5.780
Tswana-500Gy-31	8.000 ±1.246^{b}	65.000 ±4.689	110.000 ±4.301
Tswana-500Gy-53	9.000 ±1.302^{ab}	66.000 ±5.743	111.000 ±1.309
Significance	***	Ns	Ns
SEM	1.15051	25.36224	33.93622
P –value	0.0009	0.170	0.423
CV %	13.02964	7.340481	5.227158
LSD	1.0778	5.0602	5.8534

Means followed by the same letters in the same column are not significantly different at p<0.05. Standard errors of the means (n=8), Significance codes 0.001'***', 0.01 '**', 0.05 '*', Ns = not significant. A paired t-test was used; a significant difference was defined as p<0.05.

Table 3. Chlorophyll a, b, total chlorophyll, and carotenoid contents in response to gamma irradiation in the mutant lines

Variety	Chlorophyll *a* (µg ml^{-1})	Chlorophyll *b* (µg ml^{-1})	Total chlorophyll (µg ml^{-1})	Total carotenoids (µg ml^{-1})
Tswana-0	0.276 ±0.097^{b}	0.385 ±0.153^{b}	0.661 ±0.219^{b}	0.150 ±0.061^{bc}
Tswana-300Gy-202	0.334 ±0.023^{b}	0.341 ±0.053^{b}	0.675 ±0.074^{b}	0.126 ±0.034^{c}
Tswana-300Gy-214	0.318 ±0.071^{b}	0.553 ±0.141^{b}	0.670 ±0.212^{b}	0.129 ±0.038^{c}
Tswana-400Gy-49	0.445 ±0.031^{a}	0.617 ±0.044^{a}	1.062 ±0.074^{a}	0.201 ±0.013^{ab}
Tswana-400Gy-85	0.475 ±0.055^{a}	0.633 ±0.074^{a}	1.107 ±0.116^{a}	0.210 ±0.010^{a}
Tswana-500Gy-31	0.443 ±0.045^{a}	0.489 ±0.055^{ab}	0.330 ±0.034^{c}	0.201 ±0.023^{ab}
Tswana-500Gy-53	0.510 ±0.05^{a}	0.566 ±0.020^{a}	0.381 ±0.034^{c}	0.220 ±0.024^{a}
Significance	**	**	***	**
SEM	0.0334	0.0516	0.0755	0.019
P- value	0.001	0.003	0.0001	0.010
CV %	14.487	18.482	18.740	18.654
LSD	0.102	0.157	0.229	0.058

Means followed by the same letters denote nonsignificant differences, while those followed by different letters denote significant differences. Standard errors of the means (n=3), Significance codes 0.001 '***', 0.01 '**', 0.05 '*', Ns = not significant. A paired t-test was used; a significant difference was set at p<0.05.

3.2.3. Chlorophyll Fluorescence-related Photosynthetic Parameters of the Mutant Lines

Induced significant variation was observed for the non-photochemical quenching (NPQt), the quantum yield of non-photochemical quenching (PhiNPQ), the maximum quantum yield of photosystem II photochemistry (Fm/Fv), the quantum yield of photosystem II (Phi2) and the quantum yield of non-regulated dissipation process (PhiNO) between Tswana-control and the mutant lines. However, no significant variation was observed for the Photosystem II open centres and Photosystem II active centres (Table 4). The NPQt mean values range from 0.912 to 5.070. Tswana-300Gy-214 (0.912) exhibited significantly lower NPQt mean values compared to the control (3.741) and other mutant lines, whereas Tswana-500Gy-53 (5.070) had significantly higher NPQt mean values, followed by Tswana-500Gy-31 (4.122). The Tswana-300Gy-202 (0.412), Tswana-300Gy-214 (0.203) and Tswana-400Gy-85 (0.372), Tswana-500Gy-53 (0651) and Tswana-500Gy-31 (0.594) had significantly higher PhiNPQ mean values relative to the Tswana-control (0.388). On the other hand, Tswana-300Gy-214 (0.203) had significantly lower PhiNPQ mean values compared to the control (0.388) and other mutant lines. In terms of the Fv/Fm, Tswana-500gy-53 (0.456) showed significantly lower mean values

compared to the Tswana control (0.601.

Despite the insignificance between the Tswana-control (0.601), Tswana-300Gy-202 (0.598), Tswana-300Gy-214 (0.698), Tswana-400Gy-49 (0.603) and Tswan-400 Gy-85 (0.626), an increased mean value of Fv/Fm was observed. Further comparison of the mutant lines and the control showed significantly lower Phi2 mean values for the Tswana-500Gy-31 (0.257) and Tswana-500Gy-53 (0.213) compared to the control (0.423), Tswana-300Gy-202 (0.436), Tswana-300Gy-214 (0.567), and Tswana-400Gy-85 (0.431). The Tswana-300Gy-214 (0.567) recorded significantly higher Phi2 value in comparison to Tswana-400Gy-49 (0.381), Tswana-500Gy-31 (0.257) and Tswana-500Gy-53 (0.213). A similar trend was observed for PhiNO as Tswana-300Gy-214 (0.230) recorded significantly higher PhiNO value compared to the control (0.162), Tswana-300Gy-202 (0.153), Tswana-400Gy-49 (0.189), Tswana-500Gy-31 (0.149) and Tswana-500Gy-53 (0.136). Whereas significantly lower PhiNO values were recorded for the Tswana-300Gy-202 (0.153), Tswana-500Gy-31 (0.149) and Tswana-500Gy-53 (0.136) compared to Tswana-300Gy-214 (0.230).

3.3. Yield Trait Variation in M4 Cowpea Mutants

The number of pods per plant, pod weight, pod length, number of seeds per pod and the number of branches per plant did not differ between the Tswana and mutant lines. However, the 100 seed weight (100 SW) varied significantly and it ranged from 16.500 g and 18.250 g. The lowest 100 SW was observed for the Tswana variety, while the highest was shown for Tswana-400Gy-49. The significant increases in 100 SW of 18.125 g and 18.250 g were shown in Tswana-300Gy-214 and Tswana-400Gy-49 respectively compared to the Tswana control (16.500 g). Similarly, Tswana-300Gy-214 and Tswana-400Gy-49 have a significantly higher 100 SW than Tswana-400Gy-85 (16.750 g) and Tswana-500Gy-53 (16.875 g) respectively. Thus, a significant 1.500 g and 1.375 g higher 100 SW respectively (Table 5).

3.4. Quantification of Total % Starch, Crude Fibre and Tannins

Tswana control (5.097) had significantly lower crude fibre content compared to Tswana-300Gy-202 (5.366) and Tswana-300Gy-214 (5.290). The highest crude fibre was observed for Tswana-400gy-85 (6.190). The concentrations of condensed tannins ranged from 0.098 to 0.260. The significant highest concentration of condensed tannins was observed for Tswana-400Gy-85 (0.260). Despite the non-significance observed for % total starch, the starch concentration was reduced in mutant lines compared to the control (Table 6).

Table 4. Chlorophyll fluorescence of cowpea mutants in respond to gamma irradiation

Variety	NPQt	PhiNPQ	Fv/Fm	Phi2	PhiNO	PS1AC	PS1OC
Tswana-Control	3.741 ±0.141^{b}	0.388 ±0.179^{c}	0.601 ±0.110^{ab}	0.423 ±0.043^{ab}	0.162 ±0.037^{bc}	2.498 ±0.567	0.798 ±0.281
Tswana-300GY-202	2.869 ±0.745^{bc}	0.412 ±0.066^{c}	0.598 ±0.063^{ab}	0.436 ±0.054^{ab}	0.153 ±0.012^{c}	2.596 ±0.353	0.787 ±0.263
Tswana-300GY-214	0.912 ±0.325^{d}	0.203 ±0.055^{d}	0.698 ±0.030^{a}	0.567 ±0.041^{a}	0.230 ±0.019^{a}	2.691 ±0.237	0.840 ±0.030
Tswana-400GY-49	2.442 ±0.997^{c}	0.430 ±0.121^{bc}	0.603 ±0.074^{ab}	0.381 ±0.097^{bc}	0.189 ±0.025^{b}	2.720 ±0.164	0.767 ±0.034
Tswana-400GY-85	2.021 ±0.574^{cd}	0.372 ±0.079^{cd}	0.626 ±0.043^{a}	0.431 ±0.065^{ab}	0.196 ±0.015^{ab}	2.657 ±0.387	0.742 ±0.061
Tswana-500GY-31	4.122 ±0.668^{a}	0.594 ±0.063^{ab}	0.494 ±0.035^{bc}	0.257 ±0.011^{cd}	0.149 ±0.011^{c}	3.098 ±0.046	0.569 ±0.212
Tswana-500GY-53	5.070 ±1.094^{ab}	0.651 ±0.075^{a}	0.456 ±0.048^{c}	0.213 ±0.016^{d}	0.136 ±0.016^{c}	2.226 ±0.634	0.508 ±0.125
Significance	***	**	**	**	***	Ns	Ns
SEM	0.417	0.058	0.037	0.051	0.012	0.248	0.101
P-Value	0.000	0.001	0.006	0.004	0.0001	0.401	0.231
CV %	23.857	2.145	10.869	22.797	11.989	16.268	24.427
LSD (0.05)	1.264	0.176	0.111	0.154	0.036	0.752	0.306

Standard errors of the means (n=5), Means separated using Least Significant Difference (LSD) at P<0.05. ***, **, * Significance at P< 0.05. 0.01, 0.001, Ns indicate non-significance at p=0.05. Where NPQ=non-chemical quenching, PhiNQ= quantum yield of Non photochemical quenching, Fv/Fm= maximum quantum efficiency of photosystem II, Phi2= Photosystem II quantum yield, PhiN0= quantum yield of non-regulated dissipation process, PSIOC = Photosystem I Open Centres, PSIAC= Photosystem I Active Centres,

Table 5. Yield component response to gamma irradiation in M4 cowpea mutant lines

Variety	Number of pods per plant	Pod weight (g)	Pod length (g)	Number of seeds per pod	100 seed weight (g)	Number of branches
Tswana-0	17.000 ±4.440	42.500 ±9.971	16.625 ±1.188	13.000 ±1.847	16.500 ±1.773^{b}	6.000 ±0.991
Tswana-300Gy-202	17.000 ±3.357	45.875 ±8.114	16.750 ±0.707	12.000 ±0536	17.652 ±1.188^{ab}	6.000 ±1.035
Tswana-300Gy-214	19.000 ±7.453	50.375 ±20.410	16.875 ±1.356	12.000 ±1.126	18.125 ±1.356^{a}	7.000 ±1.309
Tswana-400Gy-49	16.000 ±4.324	41.375 ±7.963	16.750 ±1.389	12.000 ±1.309	18.250 ±0.707^{a}	6.000 ±1.035
Tswana-400Gy-85	17.000 ±7.126	42.500 ±20.043	16.500 ±0.926	12.000 ±1.126	16.750 ±1.488^{b}	6.000 ±0.756
Tswana-500Gy-31	20.000 ±2.815	47.375 ±9.486	15.500 ±1.604	11.000 ±1.598	17.500 ±0.756^{ab}	7.000 ±1.512
Tswana-500Gy-53	18.000 ±4.899	42.750 ±9.736	15.875 ±1.260	12.000 ±1.061	16.875 ±0.835^{b}	7.000 ±1.598
Significance	Ns	Ns	Ns	Ns	*	Ns
SEM	26.849	175.959	1.482	1.661	1.482	1.464
P –value	0.735	0.809	0.215	0.63	0.033	0.413
LSD	5.206	13.328	1.2233	1.295	1.223	1.216

Means followed by the same letters denote non-significant differences, while those followed by different letters denote significant differences. Standard error of the mean (n=8). Significance codes; 0001 '***', 0.01 '**', '*', Ns = not significant. A paired t-test was used; a significant difference was set at p<0.05.

Table 6. Induced effect of gamma irradiation on total starch, crude fibre and tannins of cowpea mutant lines

Variety	Total % Starch	Crude Fibre	Condensed Tannins
Tswana-control	39.284 ±0.133	5.097 ±0.061^{c}	0.120 ±0.015^{b}
Tswana-300Gy-202	38.999 ±0.064	5.366 ±0.083^{b}	0.105 ±0.004^{b}
Tswana-300Gy-214	38.230 ±0.958	5.290 ±0.056^{b}	0.115 ±0.038^{b}
Tswana-400Gy-49	38.933 ±0.687	5.047 ±0.060^{c}	0.104 ±0.012^{b}
Tswana-400Gy-85	37.220 ±0.825	6.190 ±0.130^{a}	0.260 ±0.052^{a}
Tswana-500Gy-31	37.880 ±0.785	5.127 ±0.042^{c}	0.097 ±0.010^{b}
Tswana-500GY-53	37.811 ±1.317	5.110 ±0.060^{c}	0.098 ±0.027^{b}
Significance	Ns	***	***
SEM	0.514	0.043	0.016
P-Value	0.110	0.0001	0.0004
CV%	2.323	1.415	21.732
LSD (0.050)	1.560	0.132	0.049

Means separated using Least Significant Difference (LSD) at P<0.05. ***, **, * Significance at P<0.05. 0.01, 0.001, NS indicate non-significance. Standard error of the mean (n=3).

4. Discussion

Inducing cowpea mutations could facilitate the development of new sources of genetic variation with desirable mutations while minimizing the adverse effects on seedling survival and healthy plant growth in the subsequent generations. The distinct morphophysiological variation between Tswana control and mutant lines was restricted to the number of days to 50% seedling emergence. Relative to the Tswana control, the mutant lines had a prolonged number of days to 50% seedling emergence in Tswana-300Gy-202, Tswana-300Gy-214, Tswana-400Gy-49, Tswana-400Gy-85, Tswana-500Gy-31, and Tswana-500Gy-53 mutant lines. This observation suggests that the Tswana variety is stable and adaptable, and that had shown to be a useful variety in arid and semi-conditions [31], [32]. However, the prolonged period for seed emergence in mutant lines could be attributed to the induced physiological alterations, which include suppression of cell division in the meristematic zone of growing seedlings and a reduction in plant cell DNA repair mechanisms [33], [34]. This also resulted in the interference with protein synthesis, essential metabolic enzyme activity, and hormonal balance [35], [36], leading to disorders and inhibition of

germination hence an extended number of days to 50% emergence in mutant lines. These findings support the previous reports which indicated that gamma dosage intensity above 100 Gy is detrimental as opposed to low radiation doses which stimulate early emergence, increased percentage germination, and field survival with healthy and vigorous seedlings [32], [37]. Despite the induced variations in terms of days to emergence between Tswana and mutant lines, the number of days to flowering and the number of days to maturity were not affected. On the contrary, previous studies reported a significant decrease in the number of days to 50% flowering and 50% maturity in gamma-irradiated plants [29], [38], [39]. In other studies, on peas, soybeans, and cowpeas, the number of days to 50% flowering was increased [33], [40]. However, our study suggested the induced prolonged novel genetic variation in terms of germination while maintaining the background desirable traits such as flowering and maturity.

The process of exposing seeds to gamma radiation has been found to have a positive effect on the pigment system, which in turn enhances the process of photosynthesis and the subsequent plant growth and development processes [31], [41]. The effective light-harvesting complex, excess light energy quenching, and the scavenging of free radicals via chlorophylls and carotenoids' respective mechanisms play an essential role in improving the photosynthetic capacity [42], [43]. The mutation did not affect the Chl-a, Chl-b, total chlorophyll content, and carotenoid levels in the Tswana-300Gy-202 and Tswana-300Gy-214 mutant lines. This might result in lower light harvesting and photosynthetic capacities in those varieties. This will ultimately reduce the distribution of photoasimilates during photosynthesis. The reduced chlorophylls and carotenoids further correlated with reduced NPQt, with a concomitant unaffected quantum efficiency of the Photosystem II (PSII) light-harvesting antenna (LHCII). This altogether suggested a compromised mechanism that involves defending the saturated reaction centres from over-excitation and photoinhibition [44], [45]. On the contrary, a promoted synthesis of Chl-a and Chl-b was observed in Tswana-400Gy-49, Tswana-400Gy-85, Tswana-500Gy-31, and Tswana-500Gy-53 mutant lines. However, a markedly reduced total chlorophyll in Tswana-500Gy-31 and Tswana-500Gy-53 was observed. This might have resulted into the generation of extremely reactive free radicals that destroy or alter the photosynthetic pigments, thylakoid membranes, the antioxidant system, and important cell constituents, resulting in the overall reduction of the photosynthesis process [46]–[48].

The increased levels of chlorophyll-a and chlorophyll-b in the Tswana-400Gy-49 and Tswana-400Gy-85 were accompanied by maintained levels of quantum efficiency (Fv/Fm), suggesting greater light absorption at different wavelengths and its conversion into light energy. This resulted in improved photosynthetic capability and overall plant growth [46], [49], [50]. Despite the increased chlorophyll levels in Tswana-500GY-53 compared to Tswana control, the Fv/Fm and reduction in Phi2 were inhibited compared to the Tswana control. The decrease in Fv/Fm has been reported to be linked with impaired photosynthesis and eventual adverse effects on plants including the decline in the rate of cellular respiration, poor growth and development and eventually decrease in grain yield [51], [52]. Similarly, the reduction in Phi2 has been reported to induce photoinhibition and stress leading to the inactivation of PSII reaction centres [53]. Furthermore, Kuhlgert et al [54] reported that lower Phi2 is an indication of a reduced fraction of excited electrons bound to photosystem II, which converts light energy into carbohydrates. Similar results of decreased Fv/Fm, Phi2 and PhiNO have been reported in different studies of cowpea, rice [55]–[57]. However, the increase in chlorophylls and NPQt levels, along with the unchanged 100 seed weight, may be due to an efficient flow of electrons at the acceptor side of PSII and a feedback de-excitation mechanism to dissipate excess energy [58], [59]. These factors could potentially contribute to crop productivity.

An inverse correlation was observed between all mutant lines and the Tswana control for the number of branches, number of pods per plant, pod weight, pod length, and number of seeds per pod. Despite such no effect, the 100 SW was markedly increased in Tswana-300Gy-214 and Tswana-400Gy-49 compared to the Tswana. Despite the increase in 100 SW, there were no variations among other yield-related attributes such as the number of pods per plant, pod length, pod weight, the number of seeds per pod, and the number of branches per plant. This suggested the existence of silent mutations where the DNA did not have a noticeable effect [60]. Meanwhile, cowpea improvement also involves incorporating nutritional considerations that will expand the scope and aims of agriculture and food production, contributing to an integrated concept of food and nutrition security [61]. Despite no significance in starch contents, the mutant lines had lower starch content than the control. Shahat et al. [62] reported that the irradiation degrades the starch granules of cowpea and induces structure changes in starch molecules. Increment in the crude fibre content was observed for Tswana-400Gy-85, Tswana-300Gy-202 and Tswana-300Gy-214. Abdallah et al [63] reported that diets that have little fibre have been associated with difficulty in easing bowels and diseases of the colon like piles, appendicitis, and cancer. In that respect, the increased crude fibre in Tswana-400Gy-85, Tswana-300Gy-202 and Tswana-300Gy-214 offers them as potential excellent sources of dietary fibre and might be very useful in adding bulk to food to relieve constipation [64]. In addition to increased crude fibre, the Tswana-400Gy-85 mutant line also showed decreased levels of condensed tannins. This further suggested that the ingestion of Tswana-400Gy-85 grains with reduced levels of tannins is important for human health due to its high digestibility and possible increased bioavailability of minerals and essential amino acids as well as antioxidants

properties [65].

5. Conclusions

Gamma irradiation is one of the breeding strategies that plays a role in improving cowpea crops in terms of agronomic and nutritional value. Evaluation of the M4 mutant lines revealed that despite the prolonged days to emergence, both days to flowering and maturity were unaffected. The prolonged 50% days to emergence for Tswana-300Gy-202,
Tswana-300Gy-214, Tswana-400Gy-49 Tswana-400Gy-85, Tswana-500Gy-31, and Tswana-500Gy-53 could be attributed to the induced prolonged germination novel genetic variation while maintaining the background desirable traits such as flowering and maturity. The reduced NPQt level and the nonaffected Chl-a, Chl-b, total chlorophyll, carotenoid and Fv/Fm of the Photosystem II (PSII) light-harvesting antenna (LHCII) in Tswana-300Gy-202 and Tswana-300Gy-214 suggested a compromised mechanism that involves defending the saturated reaction centres from over-excitation and photoinhibition. Regarding the nutritional value, the increased crude fibre content in Tswana-400Gy-85, Tswana-300Gy-202 and Tswana-300Gy-214 offers them as potential excellent sources of dietary fibre and tannins, the latter commonly in Tswana-400Gy-85. These are essential for improving digestibility, bioavailability of minerals and essential amino acids, as well as antioxidant properties. Further studies could consider evaluating these mutant lines under field experiments and nutritional studies to elucidate their agronomic performance, genetic variations, genetic point of mutation and their nutritional value.

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