Gamma rays induced morphological, flowering and palynological modifications in horse gram (*Macrotyloma uniflorum*)

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Abstract

Aim: The quest was framed to analyse the effect of high dose of gamma rays on morphological, flowering, and palynological traits in horse gram (*Macrotyloma uniflorum*).

Methodology: Two horse gram varieties PAIYUR 2 and CRIDA 1-18 R were mutated using 32 mutagenic combinations. Sterile plants produced by gamma rays:400 Gy were utilized to study the alterations in morphological, flowering characters in 20 randomly selected plants, and palynological traits using scanning electron microscope.

Results: Gamma rays (GR):400 Gy produced sterile plants with altered ideotype and reproductive traits. It reduced expression of yield attributing traits, delayed first flowering, modified anther, and pollen size. The palynological changes included genotype dependant variation in pili number, size and exine ornamentation.

Interpretation: High gamma ray dose is lethal to horse gram which was evidenced through grave modifications in morphological and palynological traits. These alterations resulted in sterility. Sterile plants tried to repair the irradiation induced damages and hence failed to perform routine reproductive functions.

Key words: Gamma rays, Horse gram, Mutagenesis, Palynological traits, Sterility

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Introduction

World's food demand is catered by only three major cereal crops viz. rice, wheat and maize despite great plant diversity (Cheng *et al.*, 2017). This narrow dependency poses threat to food production (Khouri *et al.*, 2014). India is not an exception. To sustain food and nutritional security, diversification of rural cropping pattern is important (Mabhaudhi *et al.*, 2017). The gradual yield loss in these major cereal crops and emerging stresses also necessitate diversification (Chivenge *et al.*, 2015). The cultivation pattern of traditional Indian rural agriculture comprises of many underutilized legume crops. Exploitation of such underutilized but potential legumes is important under Indian dry land agriculture (Bhartiya *et al.*, 2015) as rainfed farming accounts for 67% of total cultivable land in the country, which contribute to 44% of food grain production (CRIDA, 1997).

Horse gram (*Macrotyloma uniflorum* (Lam.) Verdc) is an underutilized legume crop. It is grown in 3.26 lakh ha (Fuller and Murphy, 2018) during *Rabi* season under dry and marginal land conditions by resource poor farmers in India. It has emerged as a potential commercial crop owing to its inherent ability for drought tolerance (Sharma *et al.*, 2015), suitability for inter and mixed cropping (Krishna, 2010) and diversified uses e.g. as dal for a subsidiary source of protein (Kadam *et al.*, 1985), nutrient loaded (Prasad and Singh, 2015) fodder for livestock and green manuring to add fertility status of the soil (Bhardwaj *et al.*, 2013).

The area, production and productivity of horse gram are declining (Aditya *et al.*, 2019). This decline is due to horse gram (i) is sown after northeast monsoon and largely depends on residual moisture, (ii) has fairly long duration (90-120 days), (iii) produce flowers till harvest because of indeterminate growth habit, and (iv) experience terminal drought.

Evolution of horse gram genotypes with short to medium duration and determinate growth habit could sustain the yield. Application of classical hybridization techniques in horse gram is limited due to small sized flowers and significant flower dropping. Induced mutagenesis offers scope for inducing variability in horse gram (Priyanka *et al.*, 2019). Mutation induced alterations in plant types, fertility status and seed yield have been reported in many plants including pulses (Koti *et al.*, 2004). Earlier Savaskan (2002) and Singh and Singh (2001) reported induction of pollen sterility at higher mutagenic doses. An insight into the mutation induced variations in pollen function provides knowledge for linking these changes with plant reproductive performance. The alterations in reproductive biology of plants can be efficiently studied using scanning electron microscope (Durate-Silva *et al.*, 2010; Rick, 1942). reported significant variations in pollen shape and size under irradiated treatments. Sari-Gorla *et al.* (1996) reported that the occurrence of mutations during or immediately after the first pollen mitosis had resulted in formation of non-functional pollen grains. Koti *et al.* (2004) reported varied reproductive efficiency in UV-irradiated population of soybean. Therefore, in the present study, attempts were made to understand the gamma rays-induced morphological and

flowering modifications as well as alterations in the pollen and anther morphology of horse gram using SEM.

Materials and Methods

Healthy, well filled, and genetically pure uniform sized seeds of two popular and well adapted horse gram varieties "PAIYUR 2" and "CRIDA 1-18 R" were subjected to mutagenesis with the following combinations. GR: 100 to 400 Gy; EB: 100 to 400 Gy; G100 Gy+EB 100 Gy; G 200 Gy+EB 200 Gy, G300 Gy+EB 300 Gy; G400 Gy+EB 400 Gy; GR+EMS: 100Gy+0.3% EMS, 200 Gy+0.3% EMS, 300 Gy+0.3% EMS and 400 Gy+0.3% EMS and two controls. For combination treatment of GR and EB, the seeds were first irradiated with GR followed by GR at designated doses. For GR and EMS combination treatment, the seeds were first irradiated with GR then chemically mutagenized with EMS. The EMS concentration of 0.3% was fixed based on the findings of Bolbhat *et al.* (2012). A total of 500 seeds were mutagenized per treatment. Gamma rays and Electron Beam (EB) irradiations treatments were performed at Bhabha Atomic Research Centre, Mumbai. For EMS (Sigma, USA) treatment, the presoaked seeds (6 hours) were immersed in EMS solution prepared in sodium phosphate buffer (pH of 7.0) for 5 hrs with intermittent shaking. The volume of mutagenic solution was maintained ten times proportion to that of seed volume. Treated seeds were thoroughly washed in running tap water for half an hour to eliminate the residual effect of mutagen. Excess moisture in the seed coat was removed with folds of blotting paper.

Mutagen treated seeds along with control were sown immediately at 30 x 15 cm spacing with 4m row length in the research blocks attached to the Department of Pulses, Centre for Plant Breeding and Genetics, TNAU, Coimbatore, Tamil Nadu during *Rabi* season 2017. All the critical cultivation practices were adopted *in toto* including need-based irrigations. Since GR: 400 Gy, caused drastic effect on flowering behavior and floral structures, data were recorded critically from such mutagenic treatment and compared with 20 randomly selected plants in controls for morphological, flowering and palynological investigations. While, for palynological study, the individual complete sterile plants from GR: 400 Gy were utilized for SEM studies.

Plant height was measured from ground level to the tip of the main axis at maturity and expressed in centimeter. The leaf area was calculated by multiplying the length and breadth of the widest region of third fully opened leaf in the main stem at peak flowering (Kanagaraj and Satish, 2017). Same third leaf was utilized to measure chlorophyll fluorescence using chlorophyll fluorometer OS1P(OPTI-SCIENCES) at bright sun shine hours. Total number of days taken from the date of sowing to production of first flower was documented as days to first flowering. The total number of flowers in all clusters was counted at peak flowering, and the average was calculated. The days taken from first flowering to cessation of pod formation in the main stem was calculated as economic flowering duration. Total number of primary branches on main stem was counted at prematurity stage

as per Mahajan *et al.* (2007). Pollen grains from freshly dehiscent anthers were stained with 1% potassium iodide solution, and examined under light microscope. The ratio between total number of well stained pollens and total pollen grains was calculated as pollen fertility percentage. Three microscopic plates of pollen were considered for each inflorescence and mean values were estimated. The percentage of well filled seeds was worked out for each plant at harvest and the mean seed fertility was calculated.

For SEM analysis, the anther samples were fixed in different concentrations of ethanol (30% to 90%) and dehydrated. The time of fixation in each concentration was two minutes. The organic solvent was replaced with liquid carbon-di-oxide by critical point drying. The samples were coated with low-vacuum sputter coating by EMITECH (SC7620) super cool sputter coater, and mounted rigidly on a 12 mm diameter specimen stub using double sided adhesive carbon conducting tape. The instrument FEI QUANTA 250 was used to characterize the specimens. Pollen size was measured in polar (P) and equatorial (E) axis and pollen shape index (P/E) was calculated. The data were statistically analyzed by SPSS software (Version 10.0).

Results and Discussion

Two horse gram varieties were subjected to mutagenesis by applying single doses of gamma rays, EB and combinations of gamma rays with EB and EMS in order to induce variability for agro-economic traits. Both EB and GR at 400 Gy modified the plant ideotype. However, the treatment GR: 400 Gy produced remarkable and drastic changes in plant height, number of branches, leaf area, days to first flowering, economic flowering duration, number of flowers per cluster, and palynological traits. It also produced complete sterile plants in both varieties.

Gamma rays reduced plant height to a greater extent in sterile plants in both varieties (Fig. 1a). The reduction percent was 26 and 19.7 in PAIYUR 2 and CRIDA 1-18 R, respectively. Kumar and Srivastava (2011) reported reduction in plant height under irradiated condition. This is due to alterations in the meristematic cell division followed by hormonal imbalance and changes in the functionality of important metabolic enzymes (Kumar and Dwivedi, 2014). The decline in number of branches was pronounced in CRIDA 1-18 R variety (Fig. 1b). Sterile plants possessed light green coloured leaves with less leaf area. The leaf area of sterile plants of PAIYUR 2 and CRIDA 1-18 R were 22.55 and 19.42 cm², respectively (Fig. 1c), which differed significantly compared to their respective parents. The light coloured leaves produced abridged chlorophyll a/b ratio (Fig. 1d) due to mutagenic stress. Parida and Das (2005) and Gicquel *et al.* (2011) reported altered photosynthesis and down regulation of chloroplast gene. Stress induced changes in major metabolic pathways including photosynthesis have been reported in horse gram (Desingh and Kanagaraj, 2007). Gamma rays irradiation delayed flowering for 5 and 4 days in PAIYUR 2 and CRIDA 1-18 R, respectively (Fig. 1e), and dwindled the economic flowering duration in both varieties (Fig. 1f). It was more pronounced in the cultivar PAIYUR 2. Significant reduction was observed in average

number of flowers per cluster in sterile plants (Table 1) and it was 51.56% and 48.46% in PAIYUR 2 and CRIDA 1-18 R, respectively.

Horse gram has a typical papilionaceous petal orientation under normal growing conditions. However, irradiation in this study produced small sized floral buds with distorted and twisted petal orientation. The twisted petals were dark green and anthers in such flowers were fully dehiscent without having fertile pollen. In contrast, the fertile flowers possessed light yellowish petals and the fertility percentage were 93.48 and 90.21 in control treatments of PAIYUR 2 and CRIDA 1-18 R, respectively (Table 1). Under high dose of GR irradiation (400 Gy), the plants divert more photo-synthates and energy for repairing the irradiation induced damages that result in reduced flower size, number, and pollen count (Kakani *et al.*, 2003). Kumar and Srivastava (2011) and Nurmansyah *et al.* (2017) reported the production of smaller sized flowers with few tiny pollen grains, altered pollen morphology and germination percentage under irradiated condition. The negative effect of GR on pollen fertility is due to the collective effects of different meiotic aberrations and disturbances in normal karyokinensis and/or cytokinensis. Priyanka *et al.* (2020) reported a reduction in pollen fertility in M₁ generation of horse gram treated with gamma rays and EMS.

The alterations in reproductive biology of plants can be efficiently studied using scanning electron microscope. An insight into the mutation induced variations in pollen function will provide knowledge for linking these changes with plant reproductive performance. It was evident that the gamma irradiation had caused significant changes in the anther and pollen morphology and the alteration was genotype dependent. Gamma rays treatment reduced anther length (Table 1). The average anther length in sterile plants of PAIYUR 2 was 306.85 µm, while it was 279.4 µm in CRIDA 1-18 R. The anther width was increased (340 µm) in PAIYUR 2 but reduced in CRIDA 1-18 R. Plants with reduced anther size consequently produced small sized pollens (both in polar and equatorial axis) and changed pollen shape index (P/E). The average P/E index of sterile plants were 0.76 and 0.86 in PAIYUR 2 and CRIDA 1-18 R, respectively, while their respective controls possessed 0.80 and 0.93 (Table 1) that caused condensation in anther wall fibre matrix (Fig. 2a, 3a).

In the variety PAIYUR 2, irradiation produced shrunken pollen, crowded pili, and pili length variation (Fig. 2 b-d) which might have caused a blockade in pollen germination. The protruding pili are important for precise pollen flight and pollen tube development (Mackenzie *et al.*, 2014). Pili of exine origin act as an exit pathway for germinating pollen tube and helps in recognition of protein signals that initiate the development of a pollen tube. The presence of crowded apertures in sterile plants is the indication of physiological pollen damages. Further, it is hypothesized that irradiated plants thrive to repair the pollen damages by increasing the synthesis of exine borne components like sporopollenin rather than performing reproductive functions. Sporopollenin imparts resilience to pollens under stress (Brooks and Shaw, 1978). Kumar and Priyanka (2006) reported gamma

Fig. 1 (a-f) : Effect of Gamma rays radiation on (a) plant height; (b) number of primary branches in main stem; (c) leaf area (cm²); (d) chlorophyll (a/b) ratio; (e) days to first flowering and (f) economic flowering duration in horse gram.

Fig. 2 (a-d) : SEM micrographs of GR:400 Gy sterile pollen of the horse gram cultivar PAIYUR 2; (a) anther wall with condensed fibre matrix (5 µM, X c. 10000); (b) collapsed pollen (10 µM, X c. 5000); (c) pollen surface with crowded pili (5 µM, X c. 10000) and (d) pollen surface with reduced pili size (5 µM, X c. 30000).

Fig. 3 (a-d) : SEM micrographs of GR:400 Gy sterile pollen of the horse gram cultivar CRIDA 1-18R; (a) Anther wall with condensed fibre matrix (5 μ M, X c. 10000); (b) Characteristic spheroidal pollen with altered size (10 μ M, X c. 5000); (c) Pollen surface with no brochi and absence of muri and lamina (5 μ M, X c. 10000) and (d) pollen surface with modified pili size (5 μ M, X c. 30000).

Table 1 : Changes in palynological characteristics in horse gram cultivars after higher dose of (400 Gy) gamma rays

Characteristics	PAIYUR 2		CRIDA 1-18 R	
	Control	G - 400 Gy	Control	G - 400 Gy
Average no. of flowers per clusters	6.4 $\pm$ 0.33	3.10 $\pm$ 0.46	5.2 $\pm$ 0.28	2.68 $\pm$ 0.19
Pollen fertility (%)	93.48 $\pm$ 0.89	0.00	90.21 $\pm$ 0.76	0.00
Anther length (μ M)	367.65 $\pm$ 1.20	306.85 $\pm$ 0.98	421.25 $\pm$ 1.21	279.4 $\pm$ 0.64
Anther width (μ M)	285.00 $\pm$ 0.84	340.00 $\pm$ 1.20	421.00 $\pm$ 1.14	122.0 $\pm$ 0.91
Mean pollen size in polar axis (μ M) (P)	46.68 $\pm$ 0.78	33.28 $\pm$ 0.61	47.79 $\pm$ 0.49	46.13 $\pm$ 0.66
Mean pollen size in equatorial axis (μ M) (E)	51.93 $\pm$ 0.60	43.54 $\pm$ 0.88	51.11 $\pm$ 0.52	53.42 $\pm$ 0.84
Index of shape (P/E)	0.80	0.76	0.93	0.86
Pollen shape (P/E= 1:spheroidal; 0.7 to 0.97 oblate)	Oblate	Oblate, collapsed	Oblate	Oblate
Exine pattern	Reticulate	Reticulate	Reticulate	Reticulate but in patches
Pili number per 200 μ M	9.3 $\pm$ 0.23	21 $\pm$ 0.46	11.0 $\pm$ 0.34	7.25 $\pm$ 0.18
Average pili length (μ M)	2.73 $\pm$ 0.12	2.38 $\pm$ 0.14	2.24 $\pm$ 0.22	2.19 $\pm$ 0.19
Average pili width (μ M)	1.02 $\pm$ 0.11	1.03 $\pm$ 0.19	0.94 $\pm$ 0.13	1.46 $\pm$ 0.12
Pollen germination (%)	89.42 $\pm$ 1.2	0.0	90.26 $\pm$ 0.9	0.0
Seed fertility (%)	94.89 $\pm$ 0.55	0.00	90.43 $\pm$ 0.64	0.00

Data are presented as mean $\pm$ SE

rays induced altered pollen morphology, reduced pollen viability, pollen tube growth, and fertilization failure. These changes are due to GR induced abnormal microsporogenesis (Kuamr and Priyanka, 2006), variation in aperture number (Heslop - Harrison, 1971), and modification in signal receiving capacity of pollen proteins (Pfahler, 1981). In this study, variation in pollen morphology led to changes in aperture number and size (Fig. 2b-d and Table 1) which might be the reason for altered pollen function. Absence of functional pollen drastically affected seed set (Table 1).

A pronounced modification in the exine structure and ornamentation was witnessed in sterile pollens of CRIDA 1-18 R (Fig. 3b-d). In non-treated plants, the pollen sculpture was reticulate and polybrochate, but in sterile pollens only few brochi were present in a discontinuous pattern. The characteristic walls (muri) and spaces (lumina) were absent making the surface smoother compared to pollen structure in control. The deviations in exine ornamentation with reduced columellae head are

indicative of abnormal tapetal function. This atypical tapetal function is fatal for reproduction perspective as normal pollen (gametophyte) development depends on a close interaction with the tapetal (sporophytic) tissue. Tapetum supplies necessary nutrients and enzymes for pollen development (Lei and Liu, 2020). Defective tapetum is linked with non-functional male meiosis, failure of meiocyte maturation (Cui *et al.*, 2018) and reduced fertility (Chen *et al.*, 2018). Tapetum provide precursors for exine formation (Shivanna and Johri, 1985). Tapetum layer is also involved in biosynthesis of sporopollenin (Ariizumi and Toriyama, 2011). It is speculated that a kind of tapetal changes might have reduced sporopollenin content and thereby altered the exine ornamentation. Further, increased pili width in sterile pollen is an indication of photo-synthate diversion for repair *in lieu* of reproductive role which was evidenced through reduced pollen fertility and seed set (Table 1). Reduced pollen fertility under irradiated condition was reported due to modified cell division and decreased mitotic index (Savaskan and Toker, 1991); resulting in

reduced pollen tube growth (Bonhomme *et al.* 1998 a and b); variation in the ability of pollen tubes in fertilization (Grini *et al.*, 1999); and disturbance in zygote formation and embryo development (Denward, 2011). A positive correlation between pollen fertility with pollen germination and seed set was witnessed (Table 1). Similar trend was reported by Priyanka *et al.* (2020) and Choi *et al.* (2021). Further, Lasa and Bosemark (1993) opined that pollen sterility-based male sterility is the most successful, stable, and proven method in tapping the potential of heterosis in many crops including self-pollinated species. In the pollen based male sterility system the sterility is induced by abnormal microsporogenesis and therefore the chance for production of functional pollens is very rare. Therefore, a separate hybridization attempt involving gamma rays induced sterile plants (as female) and normal fertile parent (as male) was developed during the study period. It resulted in seed set (data is to be published) which indicated normal ovary function in sterile plants. Hence, from the findings of this study it can be concluded that GR:400 Gy modified the expression of morphological, flowering and phenological traits in horse gram. The changes in phonological traits resulted in male sterility without affecting the ovary function. In a plant breeding perspective, these results can be considered for development of pollen sterility based male sterility in horse gram. As the classical hybridization in horse gram involves manual emasculation and pollination. Small flower size reduces the efficiency and flower drop after pollination poses threat to the seed set percentage.

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Add-on Information

Author's contribution : **S. Priyanka:** Execution, data compilation, analysis and manuscript preparation; **R. Sudhagar:** Conceptualization, methodology, execution, data analysis and supervision; **C. Vanniarajan:** Data execution, revise, editing and fund acquisition; **K. Ganesamurthy:** Supervision, editing and resources; **J. Souframanien:** Methodology, revise, editing and

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