British Biotechnology Journal
8(1): 1-10, 2015, Article no.BBJ.14073
ISSN: 2231–2927

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Gamma Radiation: Physical Elicitor for *In vitro* Culture Techniques of *Cajanus cajan*

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Authors' contributions

This work was carried out in collaboration between all authors. Author DN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors TT, SAH, MZ and SS managed the analyses of the study, managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ BBJ/2015/14073
Editor(s):
(1) Mahalingam Govindaraj, ICRISAT, Patancheru, India.
Reviewers:
(1) Simone Aquino, Health Department, Universidade Nove de Julho, Brazil.
(2) Anonymous, University of Maroua, Cameroon.
(3) Nii Korley Kortei, Department of Nuclear Agriculture and Radiation Processing, University of Ghana, Ghana.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1168&id=11&aid=9530

Original Research Article

Received 17th September 2014
Accepted 25th November 2014
Published 1st June 2015

ABSTRACT

Gamma rays are very important in mutation breeding and in *in vitro* mutagenesis in order to develop required features of plants and increase the genetic variability. *Cajanus cajan* when subjected to absorbed doses 30 Gy, 50 Gy, 100 Gy, 150 Gy and 200 Gy showed a direct co-relation between callus induction, regeneracy frequency and absorbed doses of gamma radiation as compared to control. Gamma irradiation resulted in the induction of autonomous growth in callus, which led to the formation of callus tumors resembling the shape of crown gall tumors. Gamma irradiation in the present study proved to be an important tool in increasing the breeding efficiency, and regeneration frequency, especially that of the recalcitrant varieties.

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Keywords: Callus tumors; regeneracy; crown gall; in-vitro; gamma rays.

Key Message: *Gamma irradiation with optimum absorbed doses under in-vitro conditions proves as a physical elicitor for callus initiation and plantlet regeneration.*

ABRREVIATIONS

INMAS: Indian Institute of Nuclear Medicine and Applied Sciences; 2,4-D: 2,4-Dichlorophenoxyacetic acid; NAA: 1-Naphthaleneacetic acid; IAA: indole-3-acetic acid; IBA: Indole-3-butyric acid; BAP: 6-Benzylaminopurine; KGh^{-1}: Kilo Gray per hour; γ- rays: gamma rays; Gy: Gray.

1. INTRODUCTION

In vitro mutagenesis is a combination of *in vitro* culture and mutation induction which provides the opportunity to increase variability of an economically important cultivar is used on plants in developing varieties that are agriculturally and have high productivity potential. Utilization of *in vitro* plant tissue culture techniques increased the benefiting of agriculture and agricultural industries from biotechnological applications. Therefore, the rapid development of biotechnology in recent years accelerated the studies on development of modernized improvement methods and increased the genetic variability [1]. Among the factors that play a role in increasing the plant variability, hybridization, recombination, spontaneous or induced mutations are used most frequently. The rate of spontaneous mutations is too low to be considered for practical purposes. Therefore, physical and chemical mutagens might be used together with *in vivo* or *in vitro* techniques to increase the mutation frequency [1,2].

A low cost alternative for the breeding program is the induction of mutagenesis by radiation (different types of radiation) which has been used in plant breeding, which increases the genetic variability and allows desirable genotypes to be obtained [3-5]. Gamma rays are also very important in mutation breeding and in *in vitro* mutagenesis in order to develop required features of plants and increase the genetic variability. The many mutant varieties, which are resistant to diseases, cold, salt and with high quality, have been developed [1].

The irradiation of *in vitro* explants presents some advantages, such as the ease of handling the material, the fast obtainment of subsequent generations and, in some cases, *in vitro* evaluation. This technique has already been used successfully in ornamental plants [6] and several fruit plants, such as: citrus [7], banana fruit [8] and rootstock of (Gravesac) grape [9].

The combined use of gamma radiation and *in vitro* cultivation was also tried by Novak et al. [10]; Sandford et al. [11]; Gavazi et al. [12]. Atak and Alikamanolu [13] reported that in meristem cultures from irradiated explants of 3 different types of soybean plants, gamma irradiation affected regeneration capabilities.

The present study focuses on the callus induction and regeneracy frequency of the experimental plant under *in vitro* conditions after irradiation with various doses of gamma radiation.

2. MATERIALS AND METHODS

2.1 Plant Material

Cajanus cajan L. was selected for the present study. *Cajanus cajan* L. which ranks sixth in the area of production in comparison of other grain legumes is one of the most valuable legumes grown in semi-arid and sub-tropical areas of the world. It is used in the treatment of kidney ailments, hepatitis, measles, sickle cell anemia, abdominal tumors, diabetes and traumatism. It is also used as an anti-inflammatory and antibiotic effects as well as a sedative drug.

2.2 Irradiation of Materials

The seeds were irradiated with gamma radiation of absorbed doses 30 Gy, 50 Gy, 100 Gy, 150 Gy and 200 Gy. The device used was Gamma Cell GC-5000 BRIT–BOMBAY. The source of gamma radiation was Cobalt-60; with a dose rate 2.08 Kilo Gray per hour (2.08 KGh^{-1}) at Indian Institute of Nuclear Medicine and Applied Sciences (INMAS) New Delhi.

2.3 Growth Media Preperation

Murashige and Skoog [14] basal nutrient medium was selected and used in the course of the present investigation (Table 1). The pH of the medium was adjusted to 5.6- 5.8 with a few

drops of 0.1 (N) NaoH or 0.1 (N) HCl before autoclaving. In order to solidify the medium, 8 gm of agar agar was mixed with one litre of medium.

Table 1. Composition of the MS Medium

Constituents	Amount (mg/l)
Macronutrients	
Ammonium nitrate	1650
Potassium nitrate	1900
Magnesium sulphate	370
Potassium dihydrogen phospate	170
Calcium chloride	440
Micronuutrients	
Boric acid	6.2
Mangenese sulphate	22.3
Zinc sulphate	8.6
Sodium molybdate	0.25
Copper sulphate	0.025
Cobalt chloride	0.025
Potassium iodide	0.83
Iron source	
Ferrous sulphtae	27.8
EDTA (Disodium salt)	37.3
Myo-inositol	100
Organics	
Glycine	2.0
Thiamine –HCL	0.1
Pyrodoxine – HCL	0.5
Nicotinic acid	0.5
Carbon source	
Sucrose	3000

The medium was then boiled to dissolve the agar powder and poured into clean and dry culture tubes (6"×1", corning) each containing 20 ml of culture medium. Finally, the culture tubes were plugged with non- absorbent cotton. Media were sterilized by autoclaving at 15 lbs for 15 minutes at 121°C. Several plant growth regulators both natural and artificial e.g. 2,4-D, NAA, IAA, IBA, CPA, Kinetin, BAP etc. were used in different concentrations and combinations for the growth and differentiation of tissues.

2.4 Statistical Analysis

Each experiment was performed three times and all the determinations obtained from three replicates (N=3). The data values were submitted for analysis of variance for each factor (dose and developmental stages) and their interaction. One-way analysis-ANNOVA (HSD, P≤0.05) test (GraphPad Prism 5, 2003 Analytical Software) was used.

3. RESULTS AND DISCUSSION

3.1 Results

Different explants from leaf, inter-node, hypo-cotyl and embryonic axes (Fig. 1, Fig. 2) (in–vitro grown seedlings) after proper sterilization were individually cultured on callus induction medium using different (BAP, 2, 4-D and Kinetin of concentrations [Tables 2, 3]. BAP, in general, was observed to be more efficient for callus induction, while as 2, 4-D and Kinetin were effective only at higher concentrations. A significant variation in growth was observed when non-regenerating callus, regenerating callus, and micro-shoots attained from gamma irradiated cultures were compared with respect to control (without irradiation). With an increase in absorbed doses of gamma radiation, there was a significant (P<0.05) increase in callus induction (Table 4). Gamma irradiation resulted in the induction of autonomous growth in callus, which led to the formation of callus tumors resembling the shape of crown gall tumors (Fig. 3). The growth response of directly regenerated plantlets

Table 2. Callus induction from various explants on MS medium supplemented with various (BAP mg/l) concentrations. (Data scored after 2 weeks)

MS	Inter-node cuttings		Leaf		Hypocotyl	
(PGR mg/l)	Response	Remarks	Response	Remarks	Response	Remarks
BAP (0.50)	+1	Initial swellings at two cut ends. Swelling followed by callusing , gradually engulfing other parts. Callus compact, white in colour.	-1	Intiation of the callus from the margins of leaf disc. Callus was brown and friable, Growth rate was compertively less.	+1	Initial swellings at two cut ends. Swelling followed by callusing but in less frequency. Growth rate was compertively less.
BAP (1.00)	+1		-1		+1	
BAP (2.00)	+3		+1		+2	

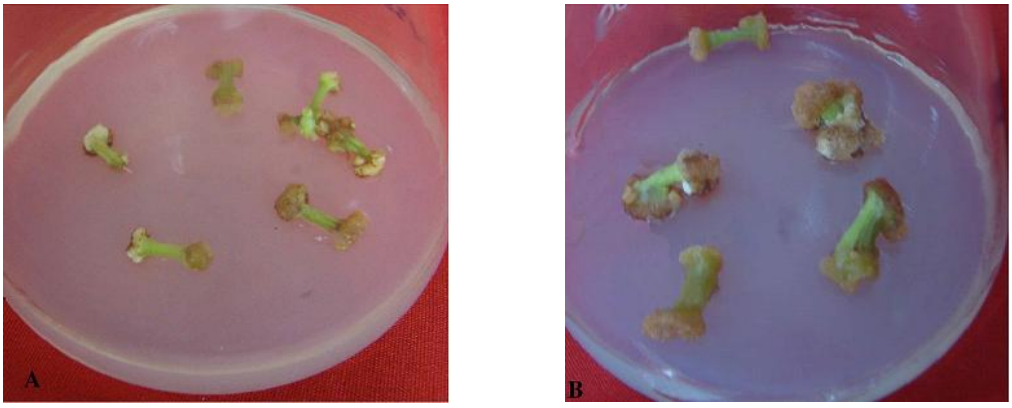

Fig. 1. Callus induction from explants on MS medium supplemnted with 2.0 mg/l BAP
A: Callus intiation from hypocotyl B: Callus intiation from inter- node cuttings

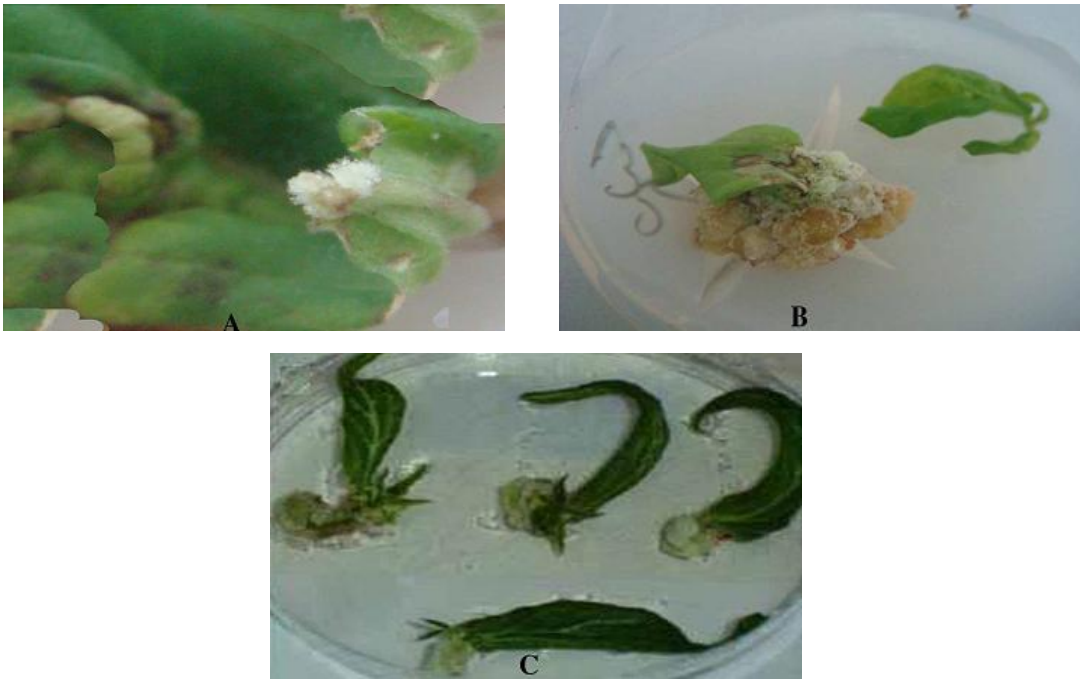

Fig. 2. Regeneration from leaf
A, B: Initiation and formation of callus from leaf (MS media with BAP 2 mg/l and 0.50 mg/l 2,4-D).C: Induction of shoots (MS media with BAP 5.0 mg/l and 1.0 mgl 2,4-D)

showed a significant increase in number of shoots up to 4 weeks with increasing absorbed doses of gamma radiation (Fig. 4). However, after 4 weeks due to necrosis, plantlets at absorbed dose 200 Gy were not able to withstand irradiation effects, leading to decrease in the number of shoots. Gamma irradiation significantly affected the number of shoots in callus mediated regenerants (Table 5). A direct co-relation was found between callus induction and absorbed doses of gamma radiation as compared to control (Fig. 5). Callus mediated shoot regeneration at 200 Gy dose was not achievable due to inability of plantlets to withstand irradiation effects because of necrosis in the callus.

3.2 Discussion

Among the tested concentrations, 2.0 mg/l BAP was the most effective for callus induction, produced friable and white callus from inter-node cuttings, hypocotyl and compact and yellow callus from the leaf which is in accordance with

Geetha et al. [15]. Autonomous growth has been shown in tissues transformed by bacteria [16], viruses [17,18], chemicals [19,20] and genetic factors [21,22]. In the present study, there was a significant increase in callus induction with increase in doses of gamma radiation, which resulted in induction of autonomous growth in callus resulting in formation of callus tumors resembling the shape of crown gall tumors, showing a major shift in metabolic pathways. The change in γ-irradiated tissues may be due to acquisition of the capacity to synthesize hormones. Our results are in accordance with the findings of K. N. Pandey, 1982 [23] who showed crown gall formation in *Haworthia* callus. The ability of crown gall tissues to synthesize hormones is believed responsible for their capacity to grow autonomously [24-28]. Studies also showed that callus exposed to γ -irradiation acquired new capacities for the synthesis of cell division factors [29]. These studies established some degree of similarity between γ-irradiated *Cajanus cajan* callus and crown gall tumor with respect to autonomous growth and the occurrence of cell division factors. Moreover, gamma rays application at low doses, have been reported to the biosynthesis of endogenous phytohormones (e.g. indole acetic acid, gibberellic acid, cytokinins) and nitrogenous compounds [30]. Another study [31] has also demonstrated that a critical endogenous level of growth regulators has to be attained before cell division and organogenesis could occur.

It is reasonably conjectured that gamma ray could stimulate callus regeneration by some mechanism such as activation of retrotransposome [32]. These observations demonstrated the probable mutations that have taken place in the *Cajanus cajan* tissues due to the gamma irradiation. Apart from the dose effects, the responses were controlled by a number of parameters, including the genotype, the type of explant, the orientation of the explant on the culture medium, and the origin of the explant from the mother plant [33]

Fig. 3. Induction of crown gall formation in 8-weeks' old callus after treatment with absorbed dose of 100 Gy of gamma radiation

Fig. 4. Induction of multiple shoots from irradiated callus tumors at 8-weeks of growth stage at absorbed dose of 150Gy

Table 3. Variation in morphogenetic responses of various explants when MS medium supplemented with various (BAP mg/l) concentrations (Data scored after 4 weeks)

	MS+PGR (mg/l)	Explant		
	BAP + 2,4-D	Leaf	Inter-node cuttings	Hypocotyl
Callus percentage	**0.50±0.25**	15 .11±1.19	33.10±1.56	25.19±1.55
	1.00±0.50	15.56±1.11	38.56±1.16	27.87±1.15
	2.00±0.50	16.52±1.13	44.50±1.03	28.18±1.19
Shoot regeneration percentage	**0.50 +0.25**	7.71±1.09	21.09±1.03	13.80±1.00
	1.00 +0.50	8.18±1.03	25.57±1.11	15.57±1.11
	2.00 + 1.00	8.62±1.03	31.80±1.00	16.57±1.11
	5.00 +1.00	8.62±1.03	35.17±2.00	16.59±2.00
Mean no. of Shoots per plant	**0.50 + 0.25**	0.55±1.11	5.55±1.11	2.01±1.00
	1.00 + 0.50	1.60±1.12	6.03±1.09	2.11±1.33
	2.00 + 1.00	2.00±1.11	7.00±1.33	3.39±1.09
	5.00 + 1.00	2.03±1.09	10.00±1.05	4.01±1.33

Table 4. Variation in callus growth supplemented with optimised auxin and cytokinin concentration at different doses of gamma radiation

	Treatments	MS+ 1 mg/l (2-4,D)	MS +0.5 mg/l (N.A.A)	MS+2.0 mg/l (BAP)
4 weeks old	Control	1.00±0.03 (0.00)	0.80±0.02 (0.00)	1.57±0.04 (0.00)
	30Gy	1.04±0.02 (4.00)	0.84±0.02 (5.00)	1.76±0.05 (12.10)
	50Gy	1.10±0.03 (10.00)	0.99±0.02 (23.75)	1.84±0.05 (17.20)
	100Gy	1.21±0.03 (21.00)	1.04±0.03 (30.00)	1.92±0.05 (22.30)
	150Gy	1.50±0.05 (50.00)	1.19±0.03 (48.75)	194±0.05 (24.01)
	200Gy	1.52±0.03 (50.11)	1.21±0.03 (50.0)	195±0.05 (24.20)
8 weeks old	Control	1.11±0.03 (0.00)	0.87±0.02 (0.00)	1.65±0.05 (0.00)
	30Gy	1.17±0.03 (5.40)	0.90±0.02 (3.40)	1.80±0.05 (9.09)
	50Gy	1.37 ± 0.03 (24.00)	1.06±0.03 (21.84)	2.19±0.06 (33.00)
	100Gy	1.40±0.04 (26.13)	1.13±0.03 (29.88)	2.42±0.06 (46.66)
	150Gy	1.69±0.05 (52.25)	1.30±0.03 (49.42)	2.43±0.06 (47.27)
	200Gy	1.73±0.05 (55.83)	1.32±0.03 (50.48)	2.93±0.08 (30.41)
12 weeks old	Control	1.19±0.03 (0.00)	0.93±0.02 (0.00)	1.96±0.05 (0.00)
	30Gy	1.39±0.03 (16.80)	0.98±0.03 (5.37)	2.37±0.07 (20.90)
	50Gy	1.46±0.04 (23.00)	1.10±0.03 (18.30)	2.40±0.07 (36.36)
	100Gy	1.51±0.03 (26.89)	1.24±0.03 (33.33)	3.52±0.07 (79.59)
	150Gy	1.52±0.03 (27.01)	1.40±0.04 (51.00)	3.56±0.10 (81.63)
	200Gy	1.87±0.04 (57.14)	1.46±0.03 (32.00)	3.59±0.10 (83.16)

**P≤ 0.05, The values represent Mean±SE (n=3), CD at 5%, Treatments: 0.0237*, Developmental Stages: 0.030* Treatment × Developmental stages: 0.052*, Parenthesis shows percent variation*

Under *in vitro* conditions, regeneracy frequency in the present study, showed a direct co-relation with increase in doses of gamma radiation as compared to control. The maximum enhancement was observed with an absorbed dose of 150 Gy. However, a dose dependent reduction was observed with an absorbed dose of 200 Gy. The callus growth due to necrosis stopped altogether with 200 Gy after 8 and 12 weeks stage. The better responses observed for the 150 Gy could suggest that the type of chromosomal alterations that took place eventually produced a change in the morphology. The higher gamma-ray doses may have produced other modifications that caused the tissues and calli to become necrosed. These results are consistent with the findings of Kulkarni et al. [34]; Kharkwal, [35] which showed an increase in shoot multiplication of *Musa* sp. due to γ-irradiation. Similarly, γ-radiation stimulated plant regeneration in cultures of *Eleusine coranna* [36] and improved shoot organogenesis [37]. All of the stress factors induce a common reaction of somatic cells manifested by their de- and re- differentiation. Stress factors can stimulate morphogenic process under *in vitro* conditions. A stimulatory

effect was reported in somatic tissues cultures by Maluszynski et al. [38].

Some treatments enhanced callus growth and regeneration, some enhanced callus growth only, while some of them inhibited both callus growth and regeneration. The increasing γ-radiation doses induced decrease in callus fresh weight and regeneration percentage. Identified factors controlling stress specific response genes can help to elucidate the stress response in plants [39]. The reduction on plantlet development at specific doses of irradiation is considered normal. According to Mohan Jain [40], it is related to the fact that cells which underwent irradiation and suffered some type of physiologic or chromosome damage present lower mitotic capacity, in contrast with cells that did not suffer irradiation, justifying the observations found in the present study. The detrimental effect of gamma irradiation on plants appears to be chromosomal or non-chromosomal and the most important effect is the retardation in growth and finally, death [41].

Table 5. Variation in number of *in vitro* raised microshoots (MS -2mg/l BAP) at different doses of gamma radiation

	Treatments	4- weeks	8- weeks	12 – weeks
No. of Shoots/plant	**Control**	4.66±2.08 (0.00)	4.96±0.57 (0.00)	5.33±2.08 0.00
	30Gy	3.63±1.15 (22.10)	4.33±0.57 (12.70)	5.0±1.00 (6.19)
	50Gy	4.43±3.05 (4.94)	5.09±1.52 (2.62)	6.66±2.51 (81.24)
	100Gy	4.66±2.08 (0.00)	6.31±2.10 (27.22)	6.90±2.03 (32.69)
	150Gy	6.00±1.00 (28.76)	7.33±2.00 (62.90)	8.08±1.15 (37.52)
	200Gy	7.33±1.52 (57.30)	5.98±1.52 (2.42)	5.50±2.03 (10.69)
Average shoot length (cm)	**Control**	5.66±0.71 (0.00)	7.40±0.96 (0.00)	7.83±0.76 (0.00)
	30Gy	5.50±1.50 (41.25)	5.76±1.49 (49.36)	6.00±0.50 (53.83)
	50Gy	5.83±1.04 (23.89)	6.08±0.78 (45.96)	7.66±0.73 (20.04)
	100Gy	6.06±0.76 (13.05)	7.36±0.72 (21.70)	8.33±1.04 (23.08)
	150Gy	6.66±0.78 (20.89)	8.16±2.75 (13.19)	9.09±1.09 (53.00)
	200Gy	3.16±0.76 (58.75)	4.46±1.38 (52.55)	4.50±0.50 (58.45)
No. of leaves/plant	**Control**	5.31±1.54 (0.00)	6.24±2.51 (0.00)	7.21±1.52 (0.00)
	30Gy	5.12±0.89 (40.42)	5.33±3.05 (14.30)	6.32±1.40 (5.29)
	50Gy	6.33±1.40 (23.83)	7.52±2.05 (33.38)	8.00±2.00 (7.41)
	100Gy	7.66±1.58 (7.82)	7.66±1.55 (42.15)	8.88±0.52 (34.66)
	150Gy	8.00±1.73 (3.73)	8.53±1.05 (46.90)	9.01±1.52 (40.41)
	200Gy	6.93±1.40 (16.61)	7.01±0.01 (62.16)	7.05±1.03 (53.37)

**P≤ 0.05, The values represent Mean±SE (n=3), CD at 5%, Treatments: 0.103*, Developmental Stages: 0.133*, Treatment × Developmental stages: 0.230*, Parenthesis shows percent variations*

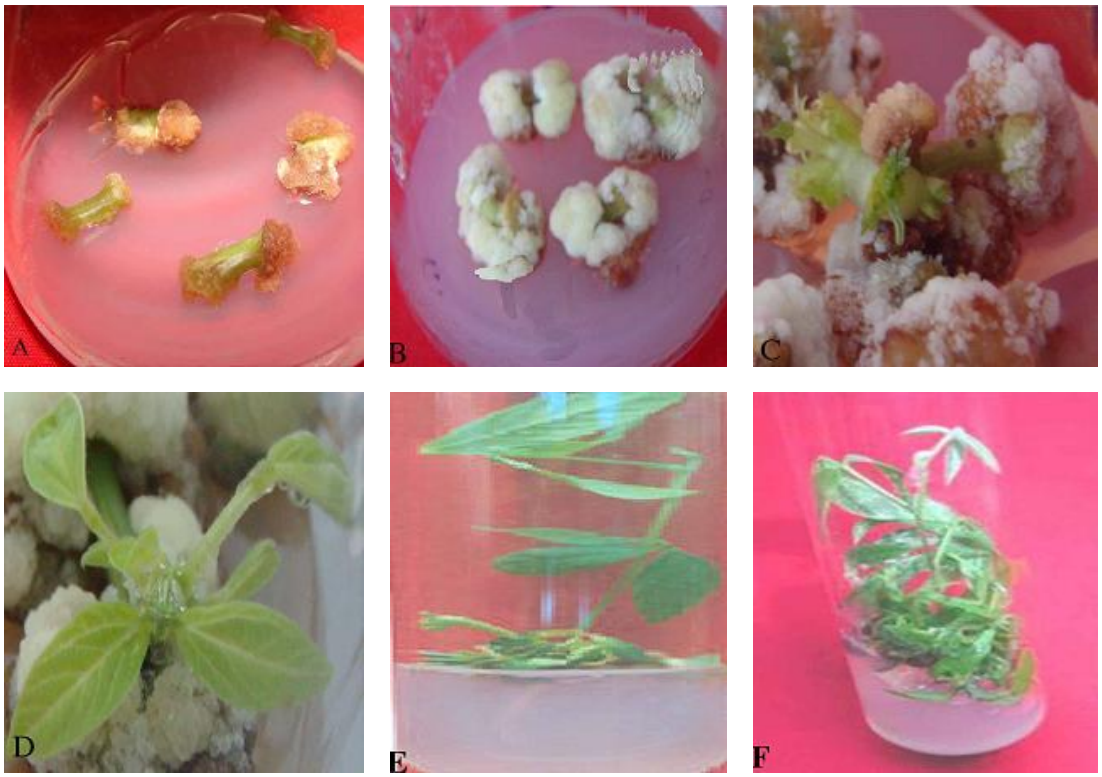

Fig. 5. Increase in callus formation/regeneration frequency after irradiation with different doses of gamma radiation at 2 weeks, 4 weeks and 8 weeks of growth. A: Callus growth in controlled cultures. B: Callus growth in irradiated cultures. C: Induction of shoots in controlled cultures. D: Induction of shoots in irradiated cultures. E: Induction of multiple shoots in controlled cultures. F: Induction of multiple shoots in irradiated cultures.

4. CONCLUSION

Gamma rays prove to be an important tool in increasing the breeding efficiency, and regeneration frequency, especially that of the recalcitrant varieties. Molecular marker assistant selection can also be introduced with such irradiation. *In vitro* irradiation is a powerful method for *Cajanus cajan* improvement as suitable doses of gamma rays besides inducing various physiological and biochemical alterations stimulate callus initiation and plantlet regeneration. Under *in vitro* conditions, results hold 150 Gy as threshold dose for increasing plant growth, plant vigour and development. In this context, further work is required where the response of plant to different doses of gamma radiation can be elaborated.

ACKNOWLEDGEMENTS

I acknowledge with gratitude the help rendered by faculty of Science, Jamia Hamdard, for providing me the necessary lab facilities to effectively carry out this work. My thanks are due to INMAS, New Delhi for providing me Gamma irradiation facility. The financial support from UGC New Delhi is highly acknowledged.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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