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Use of irradiated pollen technique to recover haploids in bitter gourd

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ABSTRACT

Haploids play a crucial role in plant breeding as they contain only one set of chromosomes, facilitating the rapid development of homozygous lines. Parthenogenetic haploids have been successfully produced using the irradiated pollen technique. Embryo rescue facilitates the development of an underdeveloped or lethal embryo into a viable plant. Inbred development through embryo culture has yet to be utilized in bitter gourd (*Momordica charantia* L.). An investigation was undertaken in a bitter gourd to recover haploids through the ^{60}Co gamma-irradiated pollen technique, and an efficient protocol for embryo culture was standardized. Male flowers of bitter gourd genotype MC-139 irradiated at different gamma-ray doses were used for artificially pollinating female flowers. Intact seeds and excised embryos were cultured in E20A medium supplemented with 0.01 mg l^{-1} IAA, which was found as the most suitable medium for embryo induction and subsequent culture. The use of intact seeds extracted 15 days after pollinating with ^{60}Co gamma irradiated (90 Gy) pollen resulted in a mutant plant. A significant difference was observed in the size of the guard cell, pollen grain diameter and chloroplast number per guard cell for the plant under T_9 (90 Gy) compared to the plants under other treatments. The plant (T_9) produced only sterile pollen, and the seed set was not observed when self-pollinated.

Key words: *Momordica charantia* L.**,** Gamma rays, Mutagen, Embryo culture, ^{60}Co.

INTRODUCTION

The nutritional and medicinal properties of the cucurbitaceous vegetable crop, bitter gourd (*Momordica charantia* L.), are gaining attention worldwide. Among the various compounds, charatin, the hypoglycaemic principle of bitter gourd, can reduce blood sugar.

In Kerala, though bitter gourd is a popular vegetable, F_1 hybrid development is limping due to the lack of quality inbreds. Inbreeding and selection are the conventional methods of inbred development. Another approach is to induce haploids, which, following chromosomal doubling, can help in obtaining homozygous lines in a single step. The irradiated pollen technique has been used effectively to develop parthenogenetic haploids in several species (Dong *et al*., 7), but no inbred line has been developed in bitter gourd. As one of India's most important vegetable crops, it is essential to exploit all opportunities for developing superior inbred lines, which are prerequisites for F_1 hybrid development.

This study aimed to induce haploids in bitter gourd using irradiated pollen and embryo culture techniques, as the manipulation of these haploid cells has implications for plant breeding and genetic research in bitter gourd.

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MATERIALS AND METHODS

The experiment was performed with the bitter gourd genotype MC-139 raised in a polyhouse at the Department of Vegetable Science, College of Horticulture, Kerala Agricultural University, Thrissur, India, during the period 2018–2019. For pollen irradiation, the male flowers were treated with gamma rays (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy). The irradiated pollen was used to pollinate the female flowers during morning hours between 7 and 8 a.m. Observations were recorded on fruit set (%) and total No. of seeds and seeds with developed embryos per fruit after 15 days of pollination.

For optimization of the appropriate period for embryo culture, the fruits developed through normal pollen were harvested seven to seventeen days after pollination. Extracted seeds underwent surface-sterilization, and for media standardization, seeds and embryos from unirradiated pollen were inoculated and cultured on different media, E20A supplemented with 0.01 mg l^{-1} IAA and E20A supplemented with IAA (0.1 mg l^{-1}) and BAP (5.0 mg l^{-1}).

Two distinct techniques were used to culture the embryos, *i.e.*, either the embryos were taken out of the seeds and placed directly on the culture medium, or the embryos were left in the whole seeds and cultured with hilum facing the medium. Regenerated plantlets, after hardening, were transferred to the polyhouse. Since the plant recovery percentage was minimal, parameters

such as guard cell size, chloroplast numbers per guard cell, and pollen grain characteristics were considered for determining the plant ploidy level. The data obtained on various parameters were statistically analyzed using WASP 2.0.

RESULTS AND DISCUSSION

To optimize the optimal timeframe for embryo culture, fruits resulting from regular pollen were harvested between seven and seventeen days after pollination. Developed seeds were observed after 12 days of pollination. The number of seeds per fruit was found to be the maximum in fruit harvested 15 days after pollination, and there was no significant difference for this trait after 15 days of pollination (Table 1). Hence, all fruits developed through pollinating with irradiated pollen were harvested 15 days after pollination for embryo culture. The influence of irradiation dose on fruit set and number of seeds and seeds with developed embryo per fruit in bitter gourd genotype MC-139 was investigated. A hundred per cent fruit set was obtained for all the irradiation doses from 10 to 100 Gy (Table 2). Kurtar *et al*. (13) and Guler *et al*. (11) reported successful fruit set at all gamma-ray doses (25 to 200 Gy) in squash and bottle gourd (50 and 75 Gy), respectively. The mean number of seeds and seeds with developed embryos per fruit decreased significantly with an increase in irradiation dose. The 100 Gy irradiated pollen produced the lowest number of seeds per fruit and seeds lacked any embryo induction. Treatment T9 (90 Gy) had only 0.27 seeds with developed embryos per fruit. Results are in confirmation with previous findings where the number of embryos per fruit decreased as the irradiation level increased from 50 to 200 Gy in bottle gourd (Guler *et al*., 11) and 50 to 150 Gy in pumpkin (Berber *et al*., 3), respectively. The percentage of seed and embryo response to *in vitro* germinated plants developed through unirradiated pollen was significantly the highest in E20A supplemented with 0.01 mg l^{-1} IAA media compared to E20A supplemented with IAA (0.1 mg l^{-1}) and BAP (5 mg l^{-1}) (Tables 3 and 4). The percentage of abnormal seedlings (distorted fold-like cotyledons) was also the least in E20A + 0.01 mg l^{-1} IAA medium. Hence, the preferred media for embryo induction and subsequent culture in the experiment is E20A + 0.01 mg l^{-1} IAA. The most widely used medium for embryo rescue in cucurbits is the E20A medium, developed by Sauton and Dumas de Vaulx (17) for developing haploids in melon. Godbole and Murthy (8, 9) suggested E20A + 0.011 mg l^{-1} IAA medium for embryo induction in melon. The same media composition gave good results in bottle gourd (Guler *et al*., 11).

Embryos and seeds with developed embryos were extracted 15 days after pollination, cultured in E20A medium supplemented with 0.01 mg l^{-1} IAA, and incubated at 25°C under specific photoperiodic

Table 1. Identification of embryo excision period for embryo culture in bitter gourd.

Days after pollination	No. of developed seeds/fruit
7	0 (0.71)^{e}
8	0 (0.71)^{e}
9	0 (0.71)^{e}
10	0 (0.71)^{e}
11	0 (0.71)^{e}
12	5.40 (2.42)±0.10^{d}
13	14.60 (3.89)±1.45^{c}
14	21.66 (4.70)±2.19^{b}
15	29.13 (5.44)±2.53^{a}
16	29.06 (5.43)±2.60^{a}
17	30.13 (5.53)±1.96^{a}
CD at 5%	0.113
CV (%)	5.59

[a]Data are Mean ± Standard deviation, n = 15; values in parentheses are square root transformed.
Values in column followed by the same letter(s) are not significantly different.

Table 2. Effect of irradiation dose on fruit set, No. of seeds and seeds with developed embryo/fruit.

Irradiation dose (Gy)	Fruit set (%)	No. of seeds/ fruit	No. of seeds with developed embryo/ fruit
T0 (0-control)	100	29.13±2.53^{a}	29.13 (5.44)±2.53^{a}
T1(10)	100	25.13±1.77^{b}	5.80 (2.51)±0.56^{b}
T2 (20)	100	24.93±1.33^{bc}	4.13 (2.14)±0.83^{c}
T3 (30)	100	24.8±2.88^{bc}	3.67 (2.03)±0.72^{c}
T4 (40)	100	23.93±1.39^{bcd}	2.87 (1.83)±0.52^{d}
T5 (50)	100	23.33±2.02^{cde}	1.40 (1.36)±0.63^{e}
T6 (60)	100	22.8±2.27^{def}	0.93 (1.16)±0.70^{f}
T7 (70)	100	22.47±4.6^{def}	0.61 (1.00)±0.63^{fg}
T8 (80)	100	22.13±2.5^{ef}	0.53 (0.98)±0.52^{gh}
T9 (90)	100	21.33±2.09^{f}	0.27 (0.84)±0.46^{hi}
T10 (100)	100	18.67±1.84^{g}	0 (0.71)^{i}
CD at 5%		1.75	0.16
CV (%)		10.41	11.97

[a]Data are Mean ± Standard deviation, n = 15; values in parentheses are square root transformed.
Values in column followed by the same letter(s) are not significantly different.

Table 3. Response of seed in relation to media composition.

Treatment	Per cent of intact seeds forming		
	Normal plantlets/ seedlings	Abnormal seedlings (%)	Days to leaf emergence
TSM_1 (E20A+ IAA (0.01 mg l^{-1})	84.2**	15.8	43.33±5.23
TSM_2 (E20A + IAA (0.1 mg l^{-1}) + BAP (5 mg l^{-1})	22.97	77.03**	46.26±4.46

**Significant at 1% level (t test)

Table 4. Embryo response in relation to medium composition.

Treatment	Per cent of embryos forming		
	Normal plantlets/ seedlings	Abnormal seedlings (%)	Days to leaf emergence
TEM_1 (E20A + IAA (0.01 mg l^{-1})	68.67**	31.33	31.87±2.75
TEM_2 (E20A + IAA (0.1 mg l^{-1}) + BAP (5 mg l^{-1})	35.33	64.67**	37.27±2.84**

**Significant at 1% level (t test)

conditions. According to earlier reports, the best embryo rescue period was 21 to 35 days after pollination in cucumber (Claveria *et al*., 5) and melon (Gonzalo *et al*., 10). Baktemur *et al*. (2) observed 35 days after pollination as the best stage of embryo rescue in squash. A mutant plant was generated when artificial pollination was done using pollen that had received 90 Gy of radiation. In cucurbits, varying responses to pollen irradiation were observed by earlier workers. Dosage in the range of 100–300 Gy, particularly, is successful in cucumber for obtaining haploid plants (Chun *et al*., 4). A higher irradiation dose of 250-300 Gy induced haploid and chimeric plantlets in melon (Ari *et al*., 1). Since radiation resistance is linked to pollen sensitivity, irradiation should be customized to each species.

Different culture systems (intact seed or embryo removed) had varying levels of regeneration efficiency at different irradiation levels. Per cent of intact seeds forming normal plants ranged from 88.89 (control) to 22.22 (TS_9) (Table 5). Days for leaf emergence and visible root ranged from 43 (control) to 86.33 (TS_8) and 14.66 (control) to 35 days (TS_9). Excised embryos, developed after artificial pollination using pollen that had received 80 and 90 Gy irradiated doses, failed to produce normal plantlets. Only abnormal seedlings (distorted fold-like cotyledons) and callus growth were observed. Days for leaf emergence and visible root ranged from 31.67 (control) to 74.67 (TE_7) and 9.66 (control) to 15.83 days (TE_7) (Table 6). A significant reduction in the percentage of normal plants and an increase in the percentage of abnormal cultures were observed as the irradiation doses increased (Tables 5 and 6). Palenius *et al*. (14) reported the use of intact seed as a successful method for the regeneration of embryos of the male parental line of 'Galia' muskmelon (*Cucumis melo* L. var. *reticulatus* Ser.). *In vitro* rooting was obtained from E20A medium supplemented with 0.01 mg l^{-1} IAA and activated charcoal (3 gl^{-1}), which took 7.2 days for root initiation, and six roots were produced after three weeks of culture. Similar findings were previously reported in (Saha *et al*., 15) bitter gourd. The statement suggests two possible interpretations based on two facts: first, charcoal adsorbs inhibitory substances in the medium, and second, it establishes a darkened environment that results in the accumulation of photosensitive auxin or co-factors.

Table 5. Effect of irradiation treatment on intact seed response in E20A medium supplemented with 0.01 mg l^{-1} IAA.

Irradiation dose (Gy)	Percentage of intact seeds forming			
	Normal plants	Abnormal seedlings	Days to leaf emergence (No.)	Days to visible root (No.)
TS_0 (0-control)	88.89 (70.73)^{a}	11.11 (19.26)^{h}	43^{e}	14.66^{g}
TS_1 (10)	77.77 (61.93)^{b}	22.23 (28.07)^{g}	51.67^{d}	20.33^{f}
TS_2 (20)	68.89 (56.13)^{c}	31.11 (33.87)^{f}	52.33^{cd}	23.16^{e}
TS_3 (30)	66.67 (54.80)^{c}	33.33 (35.19)^{f}	56.33^{c}	25.5^{d}
TS_4 (40)	53.33 (46.92)^{d}	46.67 (43.07)^{e}	64.66^{b}	28^{c}
TS_5 (50)	42.22 (40.52)^{e}	57.78 (49.48)^{d}	66^{b}	28.33^{c}
TS_6 (60)	35.56 (36.59)^{ef}	64.44 (53.41)^{cd}	84^{a}	30.15^{bc}
TS_7 (70)	31.11 (33.87)^{fg}	68.89 (56.12)^{bc}	85.67^{a}	30.83^{b}
TS_8 (80)	26.67 (31.09)^{gh}	73.33 (58.91)^{ab}	86.33^{a}	31^{b}
TS_9 (90)	22.22 (28.07)^{h}	77.78 (61.93)^{a}	86^{a}	35^{a}
CD at 5%	4.84	4.84	4.38	2.18
CV	6.17	6.46	3.8	4.79

Values in parentheses are Arc Sine transformed
Means followed by the same letter(s) in the same column are not significantly different.

Table 6. Effect of irradiation treatment on embryo response in E20A medium supplemented with mg l^{-1} IAA.

Irradiation treatment (Gy)	Percentage of embryo forming				
	*Normal plants	*Abnormal seedlings	**Callus	Days for leaf emergence (No.)	Days for visible root (No.)
TE_0 (0- control)	80 (64.29)^{a}	20 (25.72)^{e}	0 (0.71)^{b}	31.67^{g}	9.66^{e}
TE_1 (10)	71.11 (57.70)^{ab}	28.89 (32.29)^{de}	0 (0.71)^{b}	39.50^{f}	12.16^{d}
TE_2 (20)	62.22 (52.19)^{bc}	37.78 (37.80)^{cd}	0 (0.71)^{b}	40.50^{f}	12.50^{cd}
TE_3 (30)	55.56 (48.19)^{c}	44.44 (41.80)^{c}	0 (0.71)^{b}	46.33^{e}	13.66^{bcd}
TE_4 (40)	40 (39.19)^{d}	60.00 (50.81)^{b}	0 (0.71)^{b}	53.83^{d}	14.00^{bc}
TE_5 (50)	35.56 (36.59)^{de}	64.44 (53.41)^{b}	0 (0.71)^{b}	59.50^{c}	14.83^{ab}
TE_6 (60)	26.67 (30.97)^{ef}	73.33 (59.03)^{b}	0 (0.71)^{b}	72.00^{b}	15.00^{ab}
TE_7 (70)	20 (26.56)^{f}	71.11 (57.52)^{b}	8.89 (3.02)^{a}	74.67^{a}	15.83^{a}
TE_8 (80)	0 (0.74)^{g}	86.67 (69.02)^{a}	13.33 (3.64)^{a}	-	-
TE_9 (90)	0 (0.74)^{g}	88.89 (70.73)^{a}	11.11 (3.37)^{a}	-	-
CD at 5%	7.8	8.74	0.678	2.58	1.58
CV (%)	12.83	10.3	26.56	2.86	6.78

*Values in parentheses are Arc Sine transformed
**Values in parentheses are square root transformed
Means followed by the same letter in the same column are not significantly different

As the irradiation level increased, a reduction in the percentage of plants successfully hardened was noticed (Table 7). A single bitter gourd mutant plant (T_9) survived. Different developmental stages of mutant and normal diploid plants are shown in Fig. 1. A significant reduction in the size of the guard cell and pollen grain and the number of chloroplasts per cell was noticed in the plant under T_9 (Table 8, Fig. 2). Studies conducted in winter squash (Kurtar and Balkaya, 12) and *Cucumis melo* var. *momordica* (Godbole and Murthy, 8) revealed larger stomatal size and higher number of chloroplasts in the guard cells of diploids compared to haploids. All other treatments except T_9 (90 Gy) produced

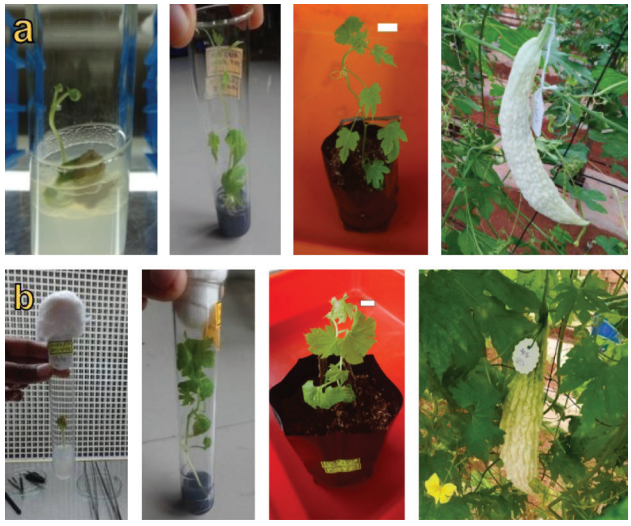

Fig. 1. Different developmental stages of mutant (a) and normal diploid (b) plants. Bars represent 1 cm for *in vitro* cultured plantlet in black polythene bag.

Table 7. Biometric parameters of hardened bittergourd plants.

Irradiation treatment (Gy)	Percentage of plants successfully hardened (after 20 days)	Days taken for 1st male flower emergence	Days taken for 1st female flower emergence
T_0 (0-control)	81.57	37.5±1.35^{c}	42±1.25^{c}
T_1 (10)	79.1	37.8±1.4^{c}	41.8±1.40^{c}
T_2 (20)	71.18	38.1±0.74^{c}	40.9±3.21^{c}
T_3 (30)	70.9	42.6±1.71^{b}	45.2±1.40^{b}
T_4 (40)	69.04	46.9±1.37^{a}	48.8±1.14^{a}
T_5 (50)	57.14	46.6±0.84^{a}	48.1±1.37^{a}
T_6 (60)	53.57	46.8±1.23^{a}	49±1.15^{a}
T_7 (70)	47.82	47.5±1.51^{a}	49±1.25^{a}
T_8 (80)	41.67	47.2±1.09^{a}	48.80±1.48^{a}
T_9 (90)	10.00	47.00	49.00
CD at 5%		1.63	2.08
CV (%)		2.99	3.62

*Based on 9 No. of treatments (except T_9)
Mean ± Standard deviation, n=10 except for treatment T_8 (n=5) and T_9 (n=1)
Mean ± Standard deviation followed by the same letter(s) in the same column are not significantly different

Table 8. Characteristics of leaf guard cell, pollen grain and number of chloroplasts per guard cell.

Irradiation treatment (Gy)	Leaf guard cell			Diameter of pollen grain (µ)	Stainable pollen (%)
	Length (µ)	Width (µ)	No. of chloroplasts/ cells		
T_0 (0-control)	19.62±1.33[a]	5.38±0.74[a]	12.17±1.19[a]	67.98±1.51[a]	100
T_1 (10)	19.5±0.79[a]	5.64±0.84[a]	12±1.21[a]	68.04±1.49[a]	100
T_2 (20)	19.52±1.17[a]	5.53±0.71[a]	11.75±0.97[a]	68.44±3.46[a]	100
T_3 (30)	20.14±0.8[a]	5.52±0.53[a]	11.66±1.55[a]	67.76±4.65[a]	100
T_4 (40)	20.2±1.51[a]	5.55±0.2[a]	12.17±1.7[a]	69.62±3.52[a]	100
T_5 (50)	19.82±1.51[a]	5.71±0.68[a]	11.58±1[a]	68.36±1.63[a]	100
T_6 (60)	19.65±1.08[a]	5.63±0.49[a]	11.92±0.67[a]	68.39±1.85[a]	100
T_7 (70)	20.19±0.48[a]	5.73±0.62[a]	12.33±0.49[a]	68.15±1.82[a]	100
T_8 (80)	19.94±0.49[a]	5.68±0.47[a]	12.42±0.51[a]	67.41±1.93[a]	100
T_9 (90)	16.23±0.48[b]	4.66±0.2[b]	6.83±0.58[b]	37.34±4.42[b]	0
CD at 5%	0.83	0.48	0.85	2.31	
CV (%)	5.34	10.86	9.29	4.42	

Mean ± Standard deviation, n = 12
Mean ± Standard deviation followed by the same letter in the column are not significantly different

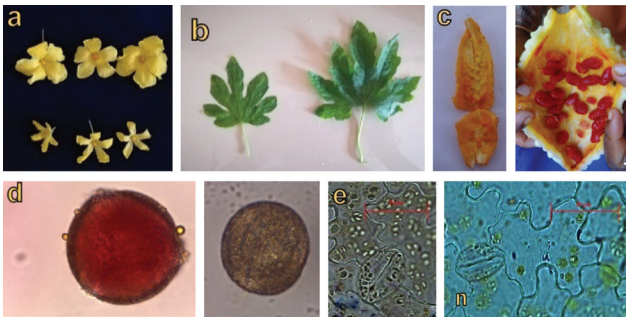

Fig. 2. Comparison of normal diploid and mutant plants.

a. Male flower: diploid (above), mutant (below); b. Leaf: diploid (right), mutant (left); c. Ripe fruit developed through self-pollination: diploid (right), mutant (left); d. Pollen grain: diploid (left), mutant (right); e. Leaf guard cell: diploid (left), mutant (right) (c) bar = 1cm, (d, e) bar = 20 µm, Magnification -100 X

stainable pollen, indicating plants' normal diploid nature. Plants developed through pollination with irradiated pollen of 90 Gy (T_9) produced only sterile pollen. Moreover, the seed set was not observed when it was self-pollinated, which can be attributed to abnormal ploidy levels in the plant. It was less vigorous and had small-sized leaves and flowers. *In-vitro* generated plants under treatment T0 to T8 exhibited normal characteristics (Fig. 2). According to previous reports, haploid plants exhibited similar morphological characteristics in watermelon (Sari *et al*., 16) and melon (Dal *et al*., 6).

E20A medium supplemented with 0.01 mg l^{-1} IAA and the use of intact seeds extracted 15 days after pollinating with ^{60}Co gamma irradiated (90 Gy) resulted in a mutant plant. Since the characteristics of leaf guard cells, pollen grains, and morphological analysis of flowers and leaves do not exactly corroborate the ploidy level of plants, confirmation of ploidy through karyotype analysis or flow cytometry is desirable (Table 8).

AUTHORS' CONTRIBUTION

Conceptualization of research (PT, RPK, SMR); Designing of the experiments (PT, RPK, KS); Contribution of experimental materials (SP, VK); Execution of field/lab experiments and data collection (RPK, VK); Analysis of data and interpretation (RPK, PT); Preparation of the manuscript (RPK, PT).

DECLARATION

The authors declare that they do not have any conflict of interest.

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