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Effect of gamma ray irradiated pollen technique on seed development pattern in *Citrus*

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Abstract

In modern fruit breeding, to reduce the breeding cycle, induction of haploid plants through irradiated pollen technique is of paramount importance. However, the major drawback is the sensitivity of pollen to higher irradiation doses as it induces abnormality in double fertilization process. Hence, optimization of irradiation doses for maximum recovery of healthy seed is very important. Present work analyzed the seed developmental pattern of *Citrus grandis*, pollinated with gamma irradiated pollen (100-500 Gy) of *C. limon, C. limetta*, and *C. sinensis*. Not a single fruit was retained on the tree till maturity in *C. grandis* × *C. limon* crosses; however, in other two cross combinations, fruits were harvested at maturity only up to 300 Gy irradiation doses. Among the harvested fruit, normal seed decreased gradually with increasing irradiation doses and less than 5 normal seeds per fruit were obtained at 300 Gy with maximum in *C. grandis* × *C. limetta* cross. However, abnormal and empty number no. of seeds has increased significantly with increasing irradiation doses. Developmental pattern of embryo and endosperm within normal seeds was also influenced significantly by irradiation treatment. At higher doses, seeds number with both embryo and endosperm was decreased gradually and at 300 Gy, it was reduced to 59.47% as compared to control.

Key words: Abnormal seed, embryo, empty seed, endosperm, irradiation

Introduction

In *Citrus* as well as in other woody species, long reproductive cycle, heterozygosity, large canopy size and self-incompatibility pose major hindrances for breeding and genetic research (Germanà and Chiancone 2001). However, the problems of long reproductive cycle, large canopy size and self-incompatibility can be overcome by using different plant bio-regulators, training and pruning operation and by using different cross compatible pollen parents, respectively. But it is very difficult to overcome the problem of heterozygosity which ultimately hinders the varietal improvement programme because for the development of new varieties, we need homozygous parental lines to make crosses. In fruit crops like citrus, development of homozygous inbred line by selfing each parental population is very difficult because it will produce different hybrids in every selfing due to their heterozygous nature. To overcome this problem, production of haploid progenies is of utmost importance as chromosome doubling of these haploid progenies will make them into complete homozygous diploid plants. It means, homozygosity in heterozygous population can easily be obtained in single step through haplodization. In *Citrus*, haploids have been produced by anther culture (Germanà et al. 1994; Germanà and Chiancone 2003) and interploid hybridization (Germanà and Chiancone 2001). However, these methods have not been effective because these haploids are very weak and grow more slowly than diploid plants (Germanà 1997). This suggests an alternative method to develop to produce haploids in this recalcitrant genotype. Irradiated pollen technique (UV, gamma rays and X-rays) is currently used successfully to induce *in situ* haploid plants (Kundu et al. 2017). Irradiated pollens are genetically inert, physiologically active and can easily be germinated on the stigma, but are not able to fertilize the egg cell and the polar nuclei. Hence, these pollens might be used to stimulate parthenogenesis including gynogenic haploid production. Due to its simple application, good penetrability, reproducibility, high mutation frequency

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and lower number of disposal problems, gamma rays are commonly used in haploidy programs (Chahal and Gosal 2002). But the major constraint of using gamma irradiation technique is the sensitivity of *Citrus* pollen to dehydration hence, they may lose their viability at higher irradiation doses (Kundu et al. 2016). This may results into abnormality in the seed developmental process by disturbing double fertilization and subsequently the development of embryo and endosperm, in a dose-related manner. Sometimes, embryos obtained at higher irradiation doses, get degenerated an early stage of fruit growth resulting no healthy seed recovery at the maturity of the fruit. However, seeds having well developed embryo is the key component for any breeding programme as without recovering these haploid embryos from pseudo-fertilized seed, it is impossible to carry forward the haploidy programme. Hence, study on seed development pattern as affected by gamma irradiation treatment of pollen grains is very essential before starting the breeding programme. No studies have been encountered till date in citrus concerning optimization of gamma ray doses for maximum recovery of normal seeds with well developed embryo and endosperm. Therefore, the present study aimed at examining the effect of gamma rays irradiation on seed developmental pattern in *Citrus*.

Materials and methods

The field experiment for pollination was carried out at the main orchard of Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute (IARI), New Delhi during 2013-14. Three different *Citrus* species, namely, sweet orange (*Citrus sinensis*) cv. Mosambi, lemon (*C. limon*) cv. Kagzi Kalan and sweet lemon (*C. limetta*) were selected as pollen sources for the experiment based on the previous year's study on pollen viability. Pummelo (*C. grandis*), a monoembryonic diploid *Citrus* species was taken as seed parent to test the seed developmental pattern after pollinating with irradiated and non-irradiated (control) pollen grains of selected pollen parents.

Twenty unopened flowers approaching anthesis were separately collected from mature plants of each of these 3 pollen parents in the morning (09:00 to 09:30 AM). After removal of petals and stigma, the anthers with filament were put in glass petri dishes and kept under sunlight for proper dehiscence. Irradiation treatment was performed on these anthers in a gamma chamber by Cobalt 60 gamma rays (Nuclear Research Laboratory, IARI, New Delhi, India) at 100, 300, and 500 Gray (Gy). One petri dish with flower of each of these 3 pollen parents was kept in clean and dry place for further use as non-irradiated pollen (control). To evaluate the effect of gamma ray irradiated pollens on seed developmental pattern, flowers of *C. grandis* were emasculated on the same day, before anthesis (09:00 to 10:30 AM; maximum 5 flowers per cluster) and bagged with white muslin cloth bags (40×40 cm) to prevent uncontrolled pollination. After removing the bags, hand pollination of these emasculated flowers were carried out on the same day (10:30 AM to 01:00 PM) by brushing the stigmas with both irradiated and non-irradiated pollen grains (control) and re-bagging was done thereafter. After pollination, green shed net was used to make the boundary surrounding the pummelo plants for isolating them from adjoining field to ensure environmental safety. The bags were removed 10 days after pollination (DAP).

At the maturity, fruits were harvested by cutting the fruit stalk from the base of the fruit with a sharp knife. Thereafter, these harvested fruits were brought to the laboratory of Division of Fruits and Horticultural Technology and cut into two halves for the collection of seeds from each fruit. After extraction of the seeds from the fruits, total number of seeds in each fruit were counted and they were classified into three groups *viz*., normal (fully developed seeds), abnormal (poorly developed and shrivelled seeds) and empty seeds (seeds with only bony seed coat but without endosperm). Length and width of normal and abnormal seeds were measured by using digital vernier calliper while weight of normal, abnormal and empty seeds was measured by using digital weighing balance. Thereafter, bony seed coat was removed from each normal seed to get the embryo and endosperm present inside. The length and weight of embryo + endosperm was measured by the methods similar to length and weight of normal and abnormal seed. Normal seeds with both embryo and endosperm and with only endosperm were counted by splitting the endosperm in to two halves.

The experiment was laid out on completely randomized design with 5 replications. Statistical analysis was performed using Microsoft Excel (2007) and statistical analysis software (SAS 9.3; SAS Institute, Cary, NC, USA) and the mean values were compared using Tukey's Honest Significant Difference (THSD) Test.

Results and discussion

Number of seeds per fruit

During maturity, we did not get any fruits in *C. grandis* × *C. limon* cross at any exposure doses along with control (non-irradiation). At the same time, not a single fruit was retained on the tree till maturity at 500 Gy treatment in other two cross combinations also, although there was a satisfactory fruit setting at 40 days after pollination in all the cross combination at 500 Gy. This observation might be due to the lethal effect of 500 Gy irradiation dose on *C. sinensis* and *C. limetta* pollen resulting in no fruit retention at maturity in *C. grandis* × *C. sinensis* and *C. grandis* × *C. limetta* crosses at 500 Gy. However, *C. limon* pollen might be highly sensitive to even lower irradiation doses or cross incompatible with *C. grandis*. However, perusal of data (Table 1) pertaining to number of seeds/fruit indicated that seed development followed a typical pattern in each and every fruit harvested at maturity. Total number of seeds of 3 different categories (normal, abnormal and empty), formed inside the fruit (Fig. 1) differed significantly due to pollen parent and amount of irradiation doses. This variation in number of seeds per fruit following pollination with irradiated pollen might be due to the differences in pollen parent as the sensitivity of pollen grains to the concentration of irradiation doses varying with the genotypes (Kundu et al. 2014; Ali et al. 1998). Regardless of irradiation doses, significantly higher number of normal (46.33 seeds/fruit) and empty seeds (66.27 seeds/fruit) per fruit was formed in *C. grandis* × *C. sinensis* crosses while abnormal seeds per fruit were counted at significantly higher amount in *C. grandis* × *C. limetta* crosses (40.13). Irrespective of pollen parent, number of normal seeds decreased rapidly with the increased doses of irradiation (Fig. 2) and at higher dose (300 Gy), the rate of reduction of normal seed formation was recorded at 96.91% as compared to non-irradiated pollen (control). Gamma irradiation in general and higher doses in particular reduced pollen viability and germinabilty which may lead chromosomes impairing hence, restrict double fertilization resulting in to ovule degeneration and formation of less number of normal seeds (Bermejo et al. 2011). Earlier, Zhang and Lespinasse (1991) in apple and Ollitrault et al. (1996) in Clementine also found that pollination with irradiated pollen leads to the formation of parthenocarpic fruits (without seeds). Further, they reported that higher the irradiation doses, more pronounced the tendency of parthenocarpic fruit formation. In the present study, only 3.5 numbers of normal seeds were obtained at 300 Gy while it was 4.8 at 100 Gy doses. These findings are in corroboration with those reported by Froelicher et al. (2007) in mandarin orange; Yahata et al. (2010) in Banpeiyu pummelo. However, a reverse pattern was followed for abnormal and empty seed formation at higher irradiation doses. At 300 Gy, an increase in abnormal seed formation by 131.98% and empty seeds by 893.41% per fruit was recorded as compared to respective controls. Poor seed set and formation of abnormal seeds following pollination with irradiated pollen is a common phenomenon which reflects the

Table 1. Effect of pollen parent and gamma ray irradiation doses on total number of seeds per fruit in *Citrus grandis*

Treatment	Total number of seeds per fruit		
	Normal	Abnormal	Empty
Pollen parent (P)			
C. sinensis	46.33^{a}	25.00^{b}	66.27^{a}
C. limetta	34.80^{b}	40.13^{a}	45.87^{b}
SEm (+)	0.41	0.46	0.51
LSD (< 0.05)	1.20	1.36	1.50
Irradiation dose (I)			
Control	113.40^{a}	19.70^{c}	9.10^{c}
100 Gy	4.80^{b}	32.30^{b}	68.70^{b}
300 Gy	3.50^{b}	45.70^{a}	90.40^{a}
500 Gy	-	-	-
SEm (+)	0.50	0.56	0.62
LSD (< 0.05)	1.78	2.01	2.23
P × I Interaction			
C. sinensis			
Control	133.00^{a}± 2.55	17.80^{e}± 1.48	12.00^{e}± 1.58
100 Gy	3.60^{cd}± 0.89	21.20^{de}± 1.79	77.40^{b}± 2.07
300 Gy	2.40^{d}± 0.55	36.00^{c}± 1.22	109.40^{a}± 2.51
500 Gy	-	-	-
C. limetta			
Control	93.80^{b}± 2.86	21.60^{d}± 1.52	6.20^{f}± 1.30
100 Gy	6.00^{c}± 1.00	43.40^{b}± 1.82	60.00^{d}± 2.35
300 Gy	4.60^{cd}± 0.89	55.40^{a}± 2.70	71.40^{c}± 1.95
500 Gy	-	-	-
SEm (+)	0.70	0.80	0.88
LSD (< 0.05)	3.13	3.54	3.91

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P < 0.05 (Tukey's Honest Significant Difference Test); DAP = days after pollination

(a) (b) (c)

Fig. 1. Different types of seeds in pummelo (*Citrus grandis*) after crossing with irradiated pollen of sweet lemon (*C. limetta*). A: Normal seeds; B: Abnormal seeds; C: Empty seeds

failure of fertilization and subsequent events rather than an earlier rejection of irradiated pollen by stigma. Similar findings were also reported earlier in kiwi fruit (Chalak and Legave 1997; Musial and Przywara 1998). Data pertaining to the interaction between pollen parent and irradiation doses also showed significant variation in seed development pattern in *Citrus*. Minimum number of normal seeds per fruit was formed in *C. grandis* × *C. sinensis* at 300 Gy (2.40 seeds/fruit) which was statistically at par with *C. grandis* × *C. limetta* at same irradiation dose (4.60 seeds/fruit). It is pertinent to mention that in both the cross combinations, less than 6 normal seeds/fruit was formed at 100 and 300 Gy irradiation treatment (Table 1) which may be considered as seedless fruit. However, as compared to respective control, the rate of reduction of normal seed per fruit at 300 Gy treatments was higher in *C. grandis* × *C. sinensis* crosses (98.20%) as compared to *C. grandis* × *C. limetta* crosses (95.10%). On the other hand, maximum number of abnormal and empty seeds per fruit was obtained maximum in *C. grandis* × *C. limetta* (55.40 seeds) and *C. grandis* × *C. sinensis* crosses (109.40 seeds/fruit) at 300 Gy, respectively. However, it was counted minimum when *C. grandis* flowers were pollinated with non-irradiated pollens of *C. sinensis* (17.80 seeds/fruit) and *C. limetta* (6.20 seeds/fruit), respectively. The effect of irradiation on seed development pattern is reported to be male genotype dependent (Chalak and Legave 1997) and higher resistance of *C. limetta* pollen to higher exposure rate than *C. sinensis* pollen (Kundu et al. 2014) might be one of the important reasons for higher proportion of normal seed set in *C. grandis* × *C. limetta* crosses at higher irradiation doses.

Seed weight and size

A significant variation has been noted on the weight and size of 3 different types of seed, formed inside the harvested fruits due to pollen parent, irradiation

Fig. 2. Size of different category of seeds, formed in pummelo (*Citrus grandis*), after crossing with irradiated pollen of sweet lemon (*C. limetta*). A: Normal seeds; B: Abnormal seeds; C: Empty seeds

doses and their interactions (Tables 2 and 3). Regardless of irradiation doses, weight of normal seed was recorded significantly higher in *C. grandis* × *C. limetta* crosses (138.63 mg) while abnormal and empty seed weight were recorded maximum in *C. grandis* × *C. sinensis* crosses (42.13 and 8.71 mg). Similar trend was also recorded for seed size (Fig. 3) with maximum width of normal seed (6.90 mm) in *C. grandis* × *C.*

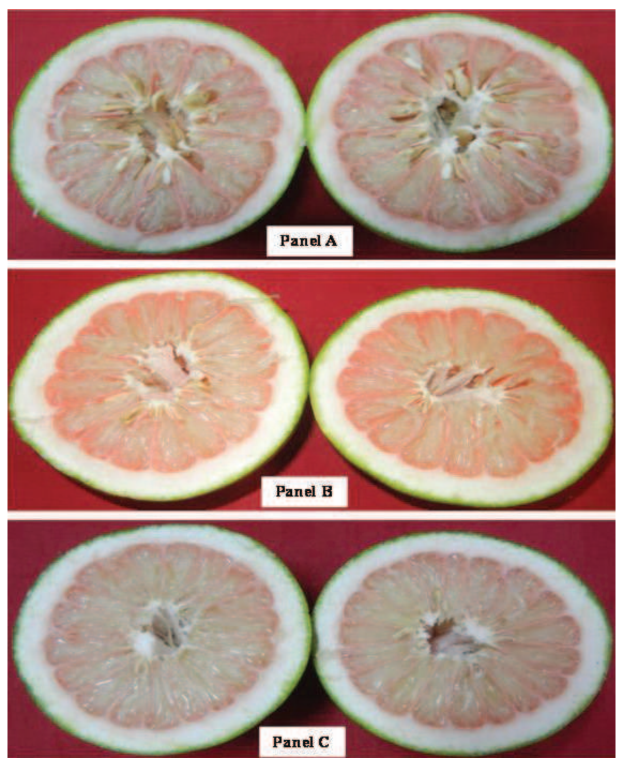

Fig. 3. Pattern of normal seeds, formed within pummelo (*Citrus grandis*) fruit, after pollinated with sweet lemon (*C. limetta*) pollen, irradiated at various doses of gamma ray exposure *viz*., Non-irradiation (Control) (Panel A); 100 Gy (Panel B); 300 Gy (Panel C)

Table 2. Effect of pollen parent and gamma ray irradiation doses on seed weight of *Citrus grandis*

Treatment	Seed weight (mg)		
	Normal	Abnormal	Empty
Pollen parent (P)			
C. sinensis	125.22^{b}	42.13^{a}	8.71^{a}
C. limetta	138.63^{a}	30.76^{b}	5.40^{b}
SEm (+)	0.53	0.44	0.14
LSD (< 0.05)	1.58	1.30	0.42
Irradiation dose (I)			
Control	194.63^{a}	46.07^{a}	4.40^{c}
100 Gy	107.78^{b}	31.37^{b}	7.50^{b}
300 Gy	93.36^{c}	31.90^{b}	9.27^{a}
500 Gy	-	-	-
SEm (+)	0.66	0.54	0.17
LSD (< 0.05)	2.34	1.92	0.63
P × I Interaction			
C. sinensis			
Control	193.00^{a}± 2.25	46.33^{a}+ 1.78	4.60^{d}+ 0.20
100 Gy	95.43^{c}± 1.43	42.27^{b}+ 1.80	10.13^{b}+ 0.49
300 Gy	87.23^{d}± 1.87	37.80^{c}+ 1.46	11.40^{a}+ 0.96
500 Gy	-	-	-
C. limetta			
Control	196.27^{a}± 2.96	45.80^{a}± 2.17	4.20^{d}± 0.28
100 Gy	120.13^{b}± 1.54	20.47^{e}± 2.13	4.86^{d}± 0.28
300 Gy	99.50^{c}± 2.50	26.00^{d}± 1.34	7.14^{c}± 0.61
500 Gy	-	-	-
SEm (+)	0.93	0.76	0.25
LSD (< 0.05)	4.12	3.38	1.10

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P < 0.05 (Tukey's Honest Significant Difference Test); DAP = days after pollination

limetta crosses however, in abnormal seed, it was found maximum in *C. grandis* × *C. sinensis* crosses (5.93 mm). This higher weight and size of normal seeds in *C. grandis* × *C. limetta* crosses as compared to *C. grandis* × *C. sinensis* crosses might be due to less sensitivity of *C. limetta* pollen to higher exposure rate than *C. sinensis* pollen (Kundu et al. 2014) which may cause better fertilization of egg cells of *C. grandis* resulting in to better seed development in this cross combination. On the other hand, due to higher sensitivity of *C. sinensis* pollen to increased irradiation doses, pollen tube of *C. sinensis* either degenerated within the style of *C. grandis* before reaching to the ovarian wall or causes pseudo-fertilization, resulting in to poor development of normal seeds and increased tendency of seed abortion at early as well as advanced stages of seed development in *C. grandis* × *C. sinensis* crosses as compared to *C. grandis* × *C. limetta* crosses, resulting into higher size and weight of abnormal and empty seeds in *C. grandis* × *C. sinensis* crosses. These findings are in agreement to those reported by Pandey et al. (1990), Zhang and Lespinasse (1991) and Froelicher et al. (2007) in different crops. Irrespective of pollen parent, length and width as well as the weight of normal and abnormal seed decreased gradually with the increasing concentration of irradiation doses while reverse pattern was observed for empty seed weight. Slow rate of embryo and endosperm development within the seed as well as less synthesis and deposition of storage products in the endosperm cells at higher irradiation doses might reduce the weight of normal and abnormal seeds (Musial and Przywara 1998). The maximum decrease in length of normal (18.34%) and abnormal seed (15.20%); width of normal (13.51%) and abnormal seed (0.18%) as compared to respective controls, were observed at 300 Gy. A perusal of interaction between pollen parent and irradiation doses clearly indicate a significant variation in seed size and weight. The weight of normal seed was recorded maximum in *C. grandis* × *C. limetta* crosses at control (196.27 mg) which was statistically at par with *C. grandis* × *C. sinensis* crosses at control (193.00 mg); however, the maximum length of normal seed (14.69 mm) was recorded in *C. grandis* × *C. sinensis* crosses at control with minimum at 300 Gy in the same cross combination (11.80 mm). Further, the rate of reduction of weight of normal seed at 100 and 300 Gy as compared to respective control was higher in *C. grandis* × *C. sinensis* (50.55 and 54.80%, respectively) than *C. grandis* × *C. limetta* (38.79 and 49.30%, respectively) crosses. Similar pattern of reduction was also observed in length and width of normal and abnormal seed with minimum reduction in *C. grandis* × *C. limetta* crosses which might be due to genetic differences in different species (Kundu et al. 2014).

Embryo and endosperm development

Developmental pattern of embryo and endosperm after pollination with irradiated pollen was significantly affected by the pollen parent, amount of irradiation doses and their interactions (Table 4). The mean effect

Table 3. Effect of pollen parent and gamma ray irradiation doses on seed size of *Citrus grandis*

Treatment	Seed length (mm)		Seed width (mm)	
	Normal	Abnormal	Normal	Abnormal
Pollen parent (P)				
C. sinensis	12.88^{a}	12.16^{a}	6.46^{b}	5.93^{a}
C. limetta	13.21^{a}	11.64^{b}	6.90^{a}	5.23^{b}
SEm (+)	0.13	0.09	0.13	0.08
LSD (< 0.05)	NS (0.37)	0.27	0.40	0.24
Irradiation dose (I)				
Control	14.50^{a}	13.03^{a}	7.33^{a}	5.49^{a}
100 Gy	12.80^{b}	11.62^{b}	6.35^{b}	5.78^{a}
300 Gy	11.84^{c}	11.05^{c}	6.34^{b}	5.48^{a}
500 Gy	-	-	-	-
SEm (+)	0.16	0.11	0.16	0.10
LSD (< 0.05)	0.56	0.40	0.59	NS (0.35)
P × I Interaction				
C. sinensis				
Control	$14.69^{a} \pm 0.49$	$13.18^{a} \pm 0.41$	$7.07^{ab} \pm 0.21$	$5.19^{cd} \pm 0.09$
100 Gy	$12.15^{c} \pm 0.60$	$11.84^{b} \pm 0.29$	$6.10^{b} \pm 0.16$	$6.46^{a} \pm 0.15$
300 Gy	$11.80^{c} \pm 0.29$	$11.46^{b} \pm 0.27$	$6.19^{b} \pm 0.98$	$6.14^{ab} \pm 0.16$
500 Gy	-	-	-	
C. limetta				
Control	$14.32^{ab} \pm 0.67$	$12.87^{a} \pm 0.46$	$7.60^{a} \pm 0.33$	$5.78^{bc} \pm 0.50$
100 Gy	$13.45^{b} \pm 0.64$	$11.39^{b} \pm 0.47$	$6.61^{ab} \pm 0.34$	$5.10^{d} \pm 0.47$
300 Gy	$11.87^{c} \pm 0.35$	$10.65^{c} \pm 0.34$	$6.50^{b} \pm 0.38$	$4.82^{d} \pm 0.20$
500 Gy	-	-	-	-
SEm (+)	0.22	0.16	0.23	0.14
LSD (< 0.05)	0.98	0.70	1.03	0.62

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P < 0.05 (Tukey's Honest Significant Difference Test); DAP = days after pollination

of irradiation treatment obviously indicates that the length and weight of embryo + endosperm within normal seed decreased gradually with the increasing concentration of irradiation doses. The maximum length (9.01 mm) and weight (108.78 mg) of embryo + endosperm was obtained at control. However, the decrease in length (30.74%) and weight (18.62%) as compared to respective controls was recorded maximum at 300 Gy. This might be due to the formation of this embryo and endosperm tissue from the unfertilized nucleus of the central cell. Jensen et al. (1977) observed that in unfertilized cotton ovules, the ultrastructure and the amount of starch and lipid in the diploid cellular endosperm was similar to those of the central cell. Also in maize, endosperm formed after double fertilization contained numerous starch grains, whereas in autonomous endosperm starch was not observed (Laikova 1976) resulting into increased size and weight of fertilized endosperm than unfertilized one. Actually pollination with irradiated pollen triggered same physiological process in ovules as like normal pollen gains, however, the induced proembryos were usually distinctly smaller and their development deprived as compared to controls, resulting development of smaller size of embryo and endosperm by irradiated pollen (Musial and Przywara 1998). The present findings are in agreement to the earlier reports of Nicoll et al. (1987) in apple; Sniezko and Visser (1987) in pear. Musial and Przywara (1998) reported that irradiated endosperms always contained very low amounts of storage products resulting into formation of smaller endosperms as compared to control. Regardless of irradiation doses, significantly higher length (7.99 mm) and weight of embryo + endosperm (111.98 mg) were measured in *C. grandis* × *C. limetta* and *C. grandis* × *C. sinensis* crosses, respectively. A perusal of interactions evidently showed variation among different treatments. The maximum length of embryo + endosperm was recorded in fruits obtained from either of the cross combination with non-irradiated pollens, having statistical similarity; however, the rate of reduction of length of embryo + endosperm at higher irradiation doses as compared to control was significantly higher in *C. grandis* × *C. sinensis* (41.59%) than *C. grandis* × *C. limetta* crosses (19.61%). On the other hand, irrespective of pollen parent, significant reduction of normal seeds with both embryo and

Table 4. Effect of pollen parent and gamma ray irradiation doses on embryo and endosperm development in normal seed of *Citrus grandis*

Treatment	Length of embryo + endosperm (mm)	Weight of embryo + endosperm (mg)	Seeds with embryo + endosperm (%)	Seeds with only endosperm (%)
Pollen parent (P)				
C. sinensis	7.05^{b}	111.98^{a}	75.44a	24.56^{a}
C. limetta	7.99^{a}	84.62^{b}	79.57^{a}	20.45^{a}
SEm (+)	0.06	5.19	1.70	1.70
LSD (< 0.05)	0.18	15.3	NS (5.00)	NS (5.01)
Irradiation dose (I)				
Control	9.01^{a}	108.78^{a}	98.37^{a}	1.63^{c}
100 Gy	7.30^{b}	97.60^{a}	75.65^{b}	24.35^{b}
300 Gy	6.24^{c}	88.53^{a}	58.50^{c}	41.53^{a}
500 Gy	-	-	-	-
SEm (+)	0.08	6.35	2.08	2.08
LSD (< 0.05)	0.27	NS (22.73)	7.44	7.44
P × I Interaction				
C. sinensis				
Control	$9.21^{a} \pm 0.12$	$120.22^{a} \pm 2.12$	$98.65^{a} \pm 0.62$	$1.35^{d} \pm 0.62$
100 Gy	$6.56^{d} \pm 0.20$	$114.87^{ab} \pm 2.25$	$71.00^{bc} \pm 6.19$	$29.00^{bc} \pm 6.19$
300 Gy	$5.38^{e} \pm 0.03$	$100.87^{ab} \pm 2.51$	$56.67^{d} \pm 9.13$	$43.33^{a} \pm 9.13$
500 Gy	-	-	-	-
C. limetta				
Control	$8.82^{a} \pm 0.32$	$97.33^{ab} \pm 1.58$	$98.08^{a} \pm 0.87$	$1.92^{d} \pm 0.87$
100 Gy	$8.04^{b} \pm 0.21$	$80.33^{ab} \pm 2.71$	$80.29^{b} \pm 5.68$	$19.71^{c} \pm 5.68$
300 Gy	$7.09^{c} \pm 0.31$	$76.20^{b} \pm 1.11$	$60.33^{cd} \pm 10.83$	$39.73^{ab} \pm 10.78$
500 Gy	-	-	-	-
SEm (+)	0.11	8.98	2.94	2.94
LSD (< 0.05)	0.48	39.93	13.07	13.07

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P < 0.05 (Tukey's Honest Significant Difference Test); DAP = days after pollination

endosperm was observed with increasing concentration of irradiation, resulting into only 40.53% seeds recovery with both embryo and endosperm at highest level of irradiation (300 Gy) as compared to control. However, a positive relation was found between irradiation doses and seeds with only endosperm and 25.48 times higher recovery of seeds with only endosperm at 300 Gy as compared to non-irradiated pollen (control). It might be due to embryo abortion after pollination with irradiated pollen which has been reported earlier by Zhang and Lespinasse (1991) in apple, Pandey et al. (1990) in kiwi fruit, Froelicher et al. (2007) in mandarin and Yahata et al. (2010) in citrus. Musial and Przywara (1998) reported this gradual reduction of seeds with both embryo and endosperm with increased irradiation doses as 'Hertwig effect'. This 'Hertwig effect' was also reported previously in apple (James et al. 1985), kiwi fruit (Chalak and Legave 1997), muskmelon (Cuny et al. 1993) and in rose (Meynet et al. 1994). Irrespective of irradiation doses, the maximum recovery (79.57%) of seeds with both embryo and endosperm was recorded with *C. limetta* pollen parent. A peep into interaction clearly showed that maximum per cent of seeds with both embryo and endosperm were formed in *C. grandis* × *C. sinensis* crosses at control (98.65%) which was statistically at par with *C. grandis* × *C. limetta* crosses at control; however, at higher irradiation doses (300 Gy) it was obtained maximum (60.33%) in *C. grandis* × *C. limetta* crosses with low rate of reduction as compared to control (38.49%) than *C. grandis* × *C. sinensis* crosses (42.55%). On the other hand, the rate of increase of seeds with only endosperm at 300 Gy as compared to respective control was recorded much higher in *C. grandis* × *C. sinensis* crosses (32.10 times) than *grandis* × *C. limetta* crosses (20.69 times). This variation might be due to the higher sensitivity of *C. sinensis* pollen to 300 Gy irradiation dose as compared to *C. limetta* pollen (Kundu et al. 2014).

The present investigation revealed that at the higher irradiation dose of 300 Gy, the pollen of *Citrus limetta* showed higher resistance to gamma ray irradiation than *C. sinensis* as evidenced from the data of seed developmental pattern. However, pollen grains of *C. limon* either highly sensitive to even lower dose of irradiation or cross incompatible with *C. grandis*, resultied into no fruit retention at maturity in *C. grandis* × *C. limon* crosses. Hence, *C. limetta* can be employed as more reliable pollen parent in haploidy programme of *Citrus*. Apart from this, irradiation dose of 500 Gy was found lethal for all the *Citrus* genotypes evaluated under the study as pollen parent resulted into no fruit retention till maturity and no seed recovery at 500 Gy in either of the cross combinations. Hence, irradiation doses below 500 Gy should be used for haploidy programme of *Citrus*. But from the findings of different fruit crops advocated that the frequency of haploid induction is much higher at higher irradiation doses. In present study, as the seeds were degenerated at higher irradiation doses, showing, early recovery of the ovule following pollination with irradiated pollen at higher doses but well before the start of their degeneration. Therefore, *in vitro* culture of these ovules might be the viable step for maximum regeneration of haploid plants in near future. Since a large amount of variability exists in *Citrus* the response of other important *Citrus* genotypes to irradiation doses should be studied in detail in future. Moreover, from the present experiment it was also observed that the irradiation doses of 100 Gy and above are highly effective for the production of seedless fruit of citrus which has high market demand. Hence, beside haploidy programme, this gamma irradiated pollen technique can also be emerged as a potential tool for parthenocarpic fruit production.

Authors' contribution

Conceptualization of research (AD); Designing of the experiments (AD); Contribution of experimental materials (MK); Execution of field/lab experiments and data collection (MK); Analysis of data and interpretation (MK); Preparation of manuscript (AD, MK).

Declaration

The authors declare no conflict of interest.

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