

Efficient induction of parthenogenetic haploids in African marigold (*Tagetes erecta* L.) using Co60 gamma-irradiated pollen of French Marigold (*Tagetes patula* L.)

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Abstract

Haploids and doubled haploids play a crucial role in crop improvement programs, including hybridisation, mutation breeding, and genetic and genomic studies. Induction of parthenogenesis using gamma-irradiated pollens is an efficient approach for inducing haploids and developing homozygous lines in horticultural crops. However, no research has been conducted using the induced parthenogenesis approach for haploid induction in the genus *Tagetes*. This study aims to assess the effectiveness of gamma irradiation at doses of 100, 200, 300, 400, and 500 Gy, using a Co60 (Cobalt-60) source, for haploid induction. The irradiated pollen of a distant tetraploid species, *Tagetes patula* genotype 'Pusa Deep', was used to induce haploids in the diploid marigold species, *T. erecta* genotypes 'DAMH-24' and 'DAMH-55'. The pseudo-fertilized ovaries were cultured on modified MS medium supplemented with 0.75 mg/l TDZ, 0.2 mg/l NAA, and 0.5 mg/l GA3. To better understand the effect of γ irradiation doses on haploid induction rate, a correlation analysis was performed. Out of 50 regenerants, 20 were selected to determine ploidy levels using morphological traits, chloroplast count, chromosomal count, and flow cytometry analysis. A plant with 3-7 chloroplasts in stomatal guard cells, $2n=x=12$ chromosomes, and 1C DNA (~54K mean Pi) was identified as haploid; the plant with 10-14 chloroplasts, $2n=2x=24$ chromosomes, and 2C DNA (~108K mean Pi) was diploid, while the plant with 16-20 chloroplasts, $2n=2x=24$ chromosomes, and 3C DNA (~154K mean Pi) was triploid. For efficient pollen inactivation and high-throughput haploid induction in *Tagetes erecta*, the 400 Gy treatment was found to be the most effective.

Key message

This study presents the first successful induction of haploids in *Tagetes erecta* via induced parthenogenesis using γ -irradiated pollen from the tetraploid species *Tagetes patula*.

Introduction

A haploid is defined as a plant that has a gametic number of chromosomes (n). The haploids are highly valuable for genetic and evolutionary studies and play a crucial role in crop improvement programmes like mutation, hybridisation, and transgenic development. The diploidisation of haploids leads to the complete fixation homozygosity in a single step, unlike traditional breeding approaches, and results in the production of doubled haploids (DHs). The DH, being considered an immortal population, is crucial for hybrid breeding (Pradeepkumara et al. 2023) and can be utilised for various genetic and genomic research (Schaart et al. 2021), QTLs analysis (Dash and Mishra 2024), and linkage mapping studies and genome sequencing (Scott et al. 2020).

Marigold (*Tagetes sp.*), a member of the Asteraceae family, is the leading flower crop grown in India and holds great significance in the livelihood security of small and marginal farmers. The genus *Tagetes*, a native to Mexico, comprises around 33 species, out of which only two species, namely, *T. erecta* L. (African marigold; $2n = 2x = 24$) and *T. patula* L. (French marigold; $2n = 4x = 48$) are commercially

cultivated all over the world. In India, marigold rank first both in area (66,130 ha) and production (603180 T), occupying 21.7% of the total area and 19.7% of the total production under flower crops (Anonymous 2020). Besides its aesthetic importance, *Tagetes erecta* L. is a high-value industrial crop for the extraction of carotene, especially lutein, which is used as an eye tonic for healthy vision. The marigold petals are also valued as poultry feed for intensifying the colour of egg yolk and broiler skin (Valdez-Baro et al. 2024). The marigold crop is considered a viable option in sustainable agriculture owing to its role in controlling the nematode population. The allelopathic effect of chemical alpha-terthienyl, present in marigold, keeps the population of root-knot nematode under control when it is planted as an intercrop (Liu et al. 2020).

Owing to the industrial importance of marigold, the demand for F_1 hybrids is immensely rising throughout the world. The F_1 hybrids in marigold possess novel traits, show tolerance to biotic and abiotic stresses and give higher returns to the farmers. In India, the indigenous F_1 hybrids are of meagre occurrence, and a significant amount of revenue is spent every year on the import of hybrid seeds. Recently, in India, institutes have taken up the systematic hybrid breeding program of marigold to meet the ever-increasing demands of farmers for hybrid seeds. Homozygous parental lines are crucial for hybrid seed production in any crop. Conventional breeding approaches require 6–8 generations of selfing to develop a completely homozygous line in this highly cross-pollinated crop. The several agricultural homozygosity can be fixed in a single generation within a tenure of ~ 1 year using the modern biotechnological approach based on doubled haploid. The induction of haploids via *in vitro* androgenesis (*via*. microspore or anther culture) or gynogenesis (*via*. unpollinated ovule/ovary culture) or induced parthenogenesis (*via*. culture of ovaries after pollination) and their subsequent diploidisation to create doubled haploids (DHs) offer a viable option for the rapid development of homozygous lines in marigold. *In vitro androgenesis* is a well-established technique in marigold for the development of DHs in marigold (Kumar et al. 2019). However, in the petaloid male sterile marigold hybrids that completely lack male reproductive organs, i.e. anthers (Sukwiwat et al. 2023), the maternal haploid induction through culture of female gametophyte is the only viable alternative. Using female gametophytes, the haploids can be induced through unpollinated ovary culture (*in vitro* gynogenesis; Kumar et al. 2020) and pollinated ovary culture approach (induced parthenogenesis).

In pollinated ovary culture technique, the maternal haploid can be induced by either pollination with irradiated/inactive pollen or pollination with the pollen of distant-related species. The irradiated pollen mostly contains inactivated sperm cells; however, they retain the ability to produce standard pollen tubes that can penetrate ovules and eventually result in parthenocarpic seed development and haploid embryo induction (Tran et al. 2023). For successful haploid induction through induced parthenogenesis, ionising irradiations (X-rays and gamma rays) can be used. However, the application of gamma rays is more common because of their greater tissue penetration, less lethality, high mutation rate, and convenience (Kurtar and Balkaya 2010). The generative nuclei inside pollen degenerate due to high energy of irradiations and are unable to undergo mitosis-II to produce two sperm nuclei and thus inhibit double fertilisation. This leads to *pseudo-fertilization* as male gametes are eliminated from participating in the

fertilisation process and stimulate haploid embryo induction through induced parthenogenesis. The irradiated pollen can provide a stimulus to the egg cell to induce parthenogenetic haploids. The dose of irradiation is crucial in induced parthenogenesis; the inactivation rate of the generative nuclei increases with the increase in doses of radiation and thus promotes more parthenogenic embryo development and haploid induction (Aktas et al., 2023).

The successful induction of maternal haploids in *Tagetes erecta* L. using an unfertilised/unpollinated ovary culture (*in vitro gynogenesis*) approach has been described by Kumar et al. (2020). In the Asteraceae family, *in vitro* gynogenesis approach has been successfully utilised for haploid induction in sunflower (Aktas et al. 2023), gerbera (Li et al. 2020), niger (Bhat and Murthy 2007), and chrysanthemum (Miler et al. 2023). In most of these reports, very low haploid induction frequencies were observed. The induced parthenogenesis technique exploiting irradiated pollen has been efficiently used to improve the haploid induction frequency in several horticultural crops; however, to date, the potential of this approach has remained unexplored in *Tagetes erecta* L. In the Asteraceae family, γ -irradiated pollen of distantly related species/genera have been utilised to induce parthenogenetic haploids in *Chichorium* x *Lactuca*, *Chichorium* x *Cicerbita* (Dore et al. 1996), and *Lactuca* x *Helianthus* (Piosik 2016). In *Iris pseudacorus*, parthenogenetic haploids were successfully induced by utilising the X ray-irradiated pollen of *I. spuria*. Induced parthenogenesis through exploiting irradiated pollen of the same species has been successfully used in *Helianthus annuus* L. (Todorova et al. in 1997; Todorova and Ivanov 2000; Aktas et al. 2018; Wang et al. 2023), cyclamen (Tutuncu and Mendi 2022), *Rosa damascena* Mill. (Palvaneh et al. 2013), *Rosa* x *hybrida* (Meynet et al. 1994), *Dianthus caryophyllus* L. (Sato et al. 2000), and *Mimulus aurantiacus* Curtis (Murovec and Bohanec 2013).

So far, no research has been undertaken on the use of the induced parthenogenesis approach for haploid induction in the genus *Tagetes*. Hence, we have conducted an in-depth study on the role of different gamma irradiation doses on pollen viability, germination, *pseudo-fertilization* and haploid induction in *T. erecta* L. This is the first report in marigold where the gamma-irradiated pollen of a tetraploid French marigold was used for the induction of parthenogenetic haploids in diploid African marigold.

Materials and Methods

The primary goal of this work was to assess the effectiveness of gamma irradiation on haploid induction frequency in *Tagetes erecta* L. It was hypothesised that irradiated pollen loses its capability of fertilisation, which may be because mutation leads to disorganisation of the nuclei as well as breakage of the chromosome. Still, the pollen retains the ability to germinate until the lethal dose. The irradiated pollens trigger unfertilised egg cells to divide, and this leads to the development of haploid embryos by 'direct regeneration'. Further, we have chosen the distant species as a pollen source to reduce the risk of fertilisation. We have irradiated the pollen of tetraploid species ($2n = 4x = 48$) *T. patula* L. with different doses of γ ray (100, 200, 300, 400 and 500 Gy) in order to optimise the irradiation dose for parthenogenic haploid development in diploid ($2n = 4x = 48$) *Tagetes erecta* L.

Plant material and its raising

Two petaloid-type male sterile African marigold (*Tagetes erecta*) genotypes, DAMH-24 (OPMS-1 × AMS-3; dark orange) and DAMH-55 (YPMS-2 × AMS-18; lemon yellow) (Fig. 1a-b) has been chosen to undertake haploid induction studies, while a French marigold (*Tagetes patula* L.) genotype, 'Pusa Deep' (Fig. 2c) was used as pollen parent for γ irradiation. The seeds of all three genotypes were sown at the research farm of the Division of Floriculture and Landscaping, ICAR - Indian Agricultural Research Institute (IARI), New Delhi, India. The seeds were initially sown in plug trays containing a mixture of cocopeat, perlite, and vermiculite (1:1:1) under net house conditions during the rainy seasons of 2021–2022. After one month of sowing, the seedlings were transplanted to the open field conditions at the research farm of the Division of Floriculture and Landscaping, ICAR-IARI, New Delhi (28.08 °N and 77.12 °E at 228.61 m above mean sea level). All the good agricultural practices as prescribed for marigold crop by IARI were followed to raise the healthy crop. The plants were weekly sprayed with liquid fertilisers @ 3 g l⁻¹ (N: P: K = 19:19:19) during the vegetative and @ 3 g l⁻¹ (N: P: K = 13:0:45) during the flowering phase. No chemicals were used for plant protection in any form to avoid viability reduction of female gametophytes (Kumar et al. 2019).

Irradiation of Flowers

The plants of the pollen parent, 'Pusa Deep,' were bagged at the initiation of flowering to avoid pollen contamination. In the morning hour (9.00 am to 10.00 am), the bagged flowers of 'Pusa Deep' were collected one day before anther dehiscence and placed in small transparent polypropylene boxes. Each box containing 6–8 flowers was irradiated with different doses of γ rays, 100, 200, 300, 400, and 500Gy, along with control (0 Gy) at the Nuclear Research Laboratory (NRL), New Delhi, in γ chamber 5000 (GC-5000) using Co⁶⁰ isotope. After irradiation, the anthers from the irradiated flowers were allowed to dehisce in the laboratory. The irradiated flowers were placed in water-filled test tubes, and a translucent butter paper bag was placed on each test tube to maintain proper hydration. The dehisced pollen from four irradiated flowers, along with the control, were used for pollination of two petalous male sterile genotypes of African marigold, DAMH-24 and DAMH-55. Two irradiated flowers from each treatment, along with the control, were utilised for pollen study. This whole process was repeated three times in a season.

Pollen viability

The freshly dehisced anthers were utilised for pollen viability studies, which were done using two methods, *i.e.*, pollen staining and germination. Pollen staining was estimated using two colourimetric tests, that is, 2, 3, 5, Triphenyl Tetrazolium Chloride (TTC) and Potassium Iodide (KI). The TTC stain was prepared by minor modification into black colour-stained pollen (Fig. 2d ii). It was considered viable—the pollens, which were unable to take stain and remained yellow, were considered non-viable (Fig. (d i) Oberle

and Watson's (1953) media, which included 1g TTC and 6g sucrose in 20 ml of distilled water, and the pH of the solution was adjusted to 5.7. At the same time, the Potassium iodide stain was prepared by dissolving 1g Potassium Iodide (KI) and 0.5g Iodine (I) in 100 ml of distilled water (Baker and Baker, 1979). The freshly dehiscent pollen from the irradiated flowers (0, 100, 200, 300, 400, 500 Gy) were stained with TTC and KI solutions separately, and a cover slip was placed over them. For better absorption of the stain, the slides were kept in the dark for 30–60 minutes and then observed under an inverted light microscope. Three replications of ~ 40 pollen grains in each slide were observed (10X magnification). In the case of TTC, staining red-coloured pollen (Fig. 2d i) was considered viable, while in the case of KI reddish- blue, ii)). For *in-vitro* pollen germination studies, Brewbaker and Kwack (1963) media consisting of 20% sucrose, 100 ppm Boric acid, 300 ppm Calcium nitrate, 200 ppm Magnesium sulphate, 100 ppm Potassium nitrate, and 0.2% Agar (pH: 5.2–5.8) was used. Pollen germination was estimated using the hanging drop method. In this method, the germinating media was placed over a coverslip, and pollens were dusted over it using a needle. The cover slip was then placed on a cavity slide sealed with petroleum jelly, and the slide was turned to hang the media over the coverslip. The slides were kept for incubation at 25 °C under moist conditions for 12 hours for pollen germination. Three replications of ~ 100 pollen grains in each slide were observed (4X magnification). Pollen was considered germinated when its tube length was doubled the size of its diameter (Fig. 2 (d iii)).

Pollination and Pseudo-fertilization

The pollen of French marigold (*T. patula* L.) genotype 'Pusa Deep' irradiated with different doses of gamma irradiations was used to pollinate the petaloid type male sterile genotypes of African marigold 'DAMH-24' and 'DAMH-55'. The flower buds of African marigold genotypes DAMH-24 and DAMH-55 were covered with butter paper bags to prevent the chance of unwanted cross-pollination. The pollen from freshly dehiscence anthers of irradiated (0, 100, 200, 300, 400, 500 Gy) and non-irradiated (0 Gy) flowers of French marigold (*T. patula* L.) genotype 'Pusa Deep' were utilised to pollinate the male sterile flowers of 'DAMH-24' and 'DAMH-55'. A total of 60 flowers were pollinated in each genotype, *i.e.*, each treatment had 10 flowers (total of 6 treatments), and the experiment was repeated three times a season. The pollination was performed during the daytime (11:00 am to 1:00 pm) when the stigmas of female flowers were fully elongated, bifurcated and receptive. As marigold inflorescence (capitulum) consists of many florets and stigmas of different rows, they become receptive on other days; therefore, the pollination was repeated 2 to 3 times during 2 to 3 days' time tenure. The irradiated pollen retains the ability to germinate on the stigmas. However, the capabilities of the male gametes to undergo fertilisation is significantly reduced, and hence, it promotes parthenogenic embryo development in pseudo-fertilized ovaries. The confirmation of fertilisation and *pseudo-fertilization* was done after 30 days of pollination (DAP). These pseudo-fertilized ovaries failed to develop under field conditions. Ovaries showing dark black/brown colour are considered fertilised (triploid), while those showing cream to light purplish colour are deemed to be *pseudo-fertilized* (haploid). The percent of ovaries showing different colours was assessed, which showed the effectiveness of γ irradiation towards *pseudo-fertilization*.

In vitro embryo culture

To assess the role of γ irradiations on gynogenesis efficiency, the *pseudo-fertilized* ovaries were cultured on standardised media. As the pseudo-fertilised ovaries lacked endosperm and failed to support the development of parthenogenetic-induced embryos, hence ovary culture was attempted to rescue the haploid embryos. The pollinated and partially matured flowers (25–30 DAP) were plucked and sterilised as per the protocol described by Kumar et al. (2020). For surface sterilisation, the flower was treated with 0.2% HgCl_2 for 5 min, followed by 4–5 times with autoclaved double-distilled water to remove the traces of toxic remnants. Subsequently, the sterilised buds were dried on the autoclaved tissue papers. The *pseudo-fertilized* ovaries were carefully excised from the sterilised flowers with the help of forceps and inoculated on a standardised culture media containing MS, 0.75 mg l^{-1} TDZ, 0.2 mg l^{-1} NAA, 0.5 mg l^{-1} GA_3 , 125 mg l^{-1} PVP, 250 mg l^{-1} Casein hydrolysate, 50 ml l^{-1} Coconut water mix, 40 g l^{-1} Sucrose, 2.6 g l^{-1} Gelrite at 5.7 pH (Arzoo et al. 2022; Murashige and Skoog, 1962). A total of three Petri dishes, each containing ~ 30 ovaries, were cultured in each treatment, and each treatment consisted of three replications. The cultured plates were sealed with parafilm followed by phyta-wrap to avoid contamination and maintained at $25 \pm 1^\circ\text{C}$ temperature in the dark. After the induction of optimum responses (swelling/bulging of ovaries or embryo emergence), plates were moved to light conditions in a photoperiod of 16:8 hours of light and dark cycles under fluorescent white light ($47 \mu\text{mol/m}^2/\text{s}$).

Plant regeneration, proliferation, and hardening

The direct regenerated embryos, as well as the embryogenic calli induced from pseudo fertilised ovaries, were transferred to MS medium supplemented with 0.25 mg l^{-1} GA_3 , 0.1 mg l^{-1} NAA, 0.2 mg l^{-1} BAP, 125 mg l^{-1} Casein hydrolysate, 125 mg l^{-1} PVP, 40 g l^{-1} Sucrose and 8 g l^{-1} Agar for shoot formation and elongation. The regenerated micro-shoots were proliferated on MS medium supplemented with 0.25 mg l^{-1} KIN, 0.25 mg l^{-1} GA_3 , 0.1 mg l^{-1} NAA, 125 mg l^{-1} PVP, 40 g l^{-1} Sucrose with 8 g l^{-1} Agar and the well-developed shoots were transferred to $\frac{1}{2}$ MS medium supplemented with 0.5 mg l^{-1} NAA, 125 mg l^{-1} PVP, 45 g l^{-1} Sucrose with 8 g l^{-1} Agar for rooting. All the cultures were maintained at the same temperature and light condition as the embryo. The well-rooted plantlets were gradually acclimatized in a plastic pot covered with polythene containing sterile coco peat, perlite and vermiculite mixture (3:1:1) under controlled conditions (Arzoo et al. 2022).

Ploidy determination

Ploidy levels of plants induced from ovaries were determined using direct (chromosome counting and flow cytometry) and indirect (chloroplast number of the guard cells and morphological observations) methods of ploidy evaluation. The flow diagram in Fig. 1 below depicts all the four direct and indirect approaches used for ploidy determination.

Statistical Analysis

The experiments were laid out in a completely randomised design (CRD) to test the significance of the mean difference among treatments. The different treatments comprising irradiation doses (0, 100, 200, 300, 400, 500 Gy) in combination with genotypes were replicated three times. The percentage data were subjected to *Arc Sin* transformation. We had some data with zero (0) values, but for statistical analysis, we could not use zero values. Therefore, the values of the entire set of data were transformed through square root transformation. After the transformation of original values, statistical analysis was performed by using ANOVA. Further, the Karl Pearson correlation coefficient was computed by using the *cor()* and *corrplot mixed()* function in “*R software*” to study the relationship of irradiation doses with other variables under study.

Results and Discussion

Irradiated pollen induces parthenogenesis or apogamy and can also lead to efficient haploid induction in several economically important crops (Wang et al. 2023). At a specific dose, the capabilities of the irradiated pollen (male gametophytes) to undergo fertilisation are significantly reduced. However, it retains its ability to provide stimulus on the stigma for fertilisation that leads to cell division as well as embryo development from egg cells. Hence, an effective dose of gamma irradiation can enhance the production of parthenogenic embryos rather than zygotic embryos and lead to the development of haploid plants (Tran et al. 2023).

Effects of gamma irradiation on pollen viability and pollen germination

The pollen viability of irradiation and non-irradiation (control) flowers of *Tagetes patula* L. genotype ‘Pusa Deep’ was assessed through staining and *in vitro* germination tests. In both stains, the number of pollens obtaining stains reduced drastically when the exposure time to the irradiations (dose) was enhanced. In the Tetrazolium chloride (TTC) staining, the control (non-irradiated) pollen exhibited maximum staining, revealing maximum pollen viability. As the dose was increased from 100 to 200 Gy, there was a sharp decline (~ 68 percent) in the percentage of pollen taking the stain, and only 18.34% of the pollen remained viable on exposure to 200 Gy. The percentage of pollen taking up stain reduced to below 5.0% when the flowers were exposed to 400 and 500 Gy doses of gamma irradiations, indicating the drastic reduction in pollen viability on exposure to higher doses of gamma irradiation (Fig. 3). Similarly, with Potassium Iodide (KI) staining the maximum pollen staining ~ 95.0% was observed in non-irradiated pollen, which reduces to almost 60.0% on exposure to 100 Gy. The exposure of flowers to higher doses of gamma irradiations, i.e. 300 and 400 Gy, led to the reduction in pollen viability to as low as 5.0% (Fig. 3). A negative correlation between radioresistance and pollen viability was observed earlier by Guler *et al.* (2017). In both TTC and KI stains, exposure of flowers to 400 Gy of gamma irradiation led to the maximum reduction in staining and hence resulted in the least viability of pollen (0.84% in TTC & 3.33% in KI); therefore, this dose can be considered as the optimum dose for parthenogenic haploid induction in marigold genotypes. Among different parts of a flower, pollen is regarded as the most

sensitive to irradiation and reduction in pollen viability on exposure to irradiation can be attributed to pollen dehydration (Kundu and Dubey, 2022). One of the reasons behind this is that the treatment of pollen grains by ionising radiation (X-ray, γ -ray) may also cause mutations, breakage of the chromosomes and disorganisation of the nucleus (Froelicher et al. 2007).

We have also assessed the effects of gamma irradiations on *in vitro* pollen germination of irradiated and non-irradiated (control) flowers of Pusa Deep. The maximum *in vitro* pollen germination (71%) was found in the non-irradiated flowers (Fig. 4). A sharp decline in pollen germination percentage was observed when gamma irradiation was applied to the flowers. A reduction of 85.0% germination was observed in pollen when flowers were subjected to 100Gy of gamma radiation (only 10.0% pollen germinated), while further increasing the irradiation doses to 300Gy reduced the germination to as low as 5.0%. The effects of irradiation on viability and *in vitro* germination of pollen were earlier studied by Thaneshwari et al. (2019) in Marigold, who noticed that pollen treated with 300Gy gamma irradiations exhibited minimum pollen viability and pollen germination. Salehian et al. (2023) also proved that with increasing doses of gamma rays, pollen viability, germination, and tube elongation of cucumber continuously decreased compared to non-irradiated pollen. Similar revelations were earlier proposed by Blasco et al. (2016) in Loquat and Kurtar et al. (2009) in Pumpkin, where the pollen grains completely failed to germinate under *in vitro* conditions when flowers were irradiated with 400Gy. Hence, this dose was considered as the desirable dose for gynogenic haploid induction in marigold. Similar to γ rays, X-rays also reduced the pollen viability and germination in *Iris spuria* at a comparable dose (Grouh et al. 2015). The loss of viability and inability of the pollen to germinate on irradiation exposure is attributed to the loss of cytoplasmic fluid inside the pollen that keeps the generative cells wet and alive inside the pollen (Pacini and Franchi, 2020). In our experiments, we have also observed the difficulties in anther dehiscence on exposing the flowers to 500 Gy, signifying the sensitivity of marigold genotypes to irradiations. Meynet et al. (1994) have also revealed a similar problem with the higher dose of irradiations ~ 500Gy dose in Rose. The exposure of flowers to higher doses of γ rays leads to pollen dehydration. Which in turn gives rise to poor germination (Fig. 4). The irradiated pollen technique can be effectively used to improve the haploid induction frequency. Still, before applying this technique on a commercial scale, the dose of γ irradiation that can inactivate the generative nuclei in a maximum number of pollen grains and thus reduce the pollen viability to as low as zero must be optimised.

Effects of gamma irradiation on fertilisation

The fertilisation, as indicated by a change of the ovary colour to black, was severely hampered by irradiation. Pollination of African marigold genotypes DAMH-24 & DAMH-55 with the irradiated pollen of French marigold genotype 'PD' exhibited only a slight change in the colour of the ovaries to purplish red, indicating resulted in *pseudo-fertilization*. The earlier studies have established the fact that the irradiated pollen retains its capability to germinate on the stigma and can grow within the style to reach the embryo sac. Still, it cannot fertilise the egg cell and the polar nuclei (Froelicher et al. 2007). The irradiated pollen thus stimulates the division of the egg cell and apogamic/parthenogenic embryo development, which was evident from the change in the colour of the ovaries in the marigold. The full penetration of pollen

tubes into the ovaries (even in the absence of fertilization) can sufficiently trigger seed sets (Tran et al. 2023). Aslam et al. (2023) delineated that seeds produced in irradiated crosses were 45% lighter compared with seeds of control (non-irradiated pollen). Both colour change and weight loss of seed were because the irradiated pollen stimulated cell division from haploid cells of the embryo sac other than the zygote formation (diploid cell) and induced haploid embryos (Falque, 1994). The maximum frequency of fertilisation (indicated by blackening of ovaries; 93.67%) was observed when the African marigold flowers were pollinated with the non-irradiated pollen of PD (Fig. 5). Pollination with 400 Gy irradiated pollen (Fig. 5) resulted in the lowest percentage of fertilised ovaries (1.83%). This treatment was found to be statistically similar to 500 Gy (Fig. 5). Similar observations have been noticed by Kundu and Dubey (2022) in citrus on *pseudo-fertilized* seed development pattern when *C. grandis* was pollinated with γ irradiated pollen (100–500 Gy) of *C. limetta*. They observed maximum *pseudo fertilisation* at 300 Gy dose. The degree of *pseudo-fertilization* was dependent on both the genotype and irradiation doses. The genotypes $\times$ treatments interaction revealed that the minimum percentage (1.67%) of *pseudo-fertilization* was recorded in DAMH-24 $\times$ PD when pollinated with the 400 Gy. Thus, 400 Gy has been considered the optimum dose for induced parthenogenesis-mediated haploid production in marigold. (Fig. 5). Godbole et al. (2012) also successfully induced parthenogenesis in Snapmelon with 250 Gy of γ -irradiation, whereas low (150 and 200 Gy) or high (300 and 350 Gy) irradiation doses were not found desirable for parthenogenesis. Contrary to our results, Wang et al. (2023) achieved the highest frequency of the parthenogenetic haploid induction in sunflower with a lower dose of irradiation, i.e. with 100 Gy. The irradiation doses for induced parthenogenesis were found to be highly crop and genotype-specific. The lower doses of γ irradiation were found effective for parthenogenic embryo development in citron watermelon (200 to 250 Gy; Kurtar et al. 2025), bottle gourd (100 to 125 Gy; Zhao et al. 2023) and in Winter Squash (50 and 100 Gy; Kurtar and Balakaya, 2010). In Muskmelon, Cuny et al. (1993) noticed that a dose less than 1600 Gy was not adequate for *pseudo-fertilisation*, while pollen irradiated at 2500 Gy showed a maximum number of aborted/*pseudo-fertilised* fruits.

Effects of gamma irradiation on haploid induction through induced parthenogenesis

The irradiation doses significantly affected the response and embryo regeneration (direct or indirect) in both the genotypes of African marigold (Fig. 6–9 & Table 1). The minimum days to visible response (swelling/bulging of ovaries and embryo emergence) was shown by ovaries pollinated with non-irradiated pollen, which may be due to ease in fertilisation and embryo development. In contrast, with the increase in irradiation doses, the days to visible response from the *pseudo-fertilized* ovaries increased significantly up to 300Gy. However, we observed a slight reduction in days to response at 400Gy, indicating higher efficiency of *pseudo-fertilization*. The maximum response from cultured ovaries (24%) was observed when African marigold genotypes were pollinated with non-irradiated pollen, while the minimum response was observed with 200 Gy dose (2.76%). We have observed 400Gy as the optimum dose for induced parthenogenesis in marigold. It led to the optimum pseudo-fertilization as reflected by the highest percentage of direct regeneration in the shortest possible time. Besides direct regeneration, we have also observed indirect regenerations (callusing). The maximum percent callusing was recorded

with 0 Gy (control) and 100 Gy doses. Kundu et al. (2017) have revealed that pollination of *Citrus grandis* with gamma-irradiated (300 and 400 Gy doses) pollen of *C. limetta* and *C. sinensis* (respective doses) stimulated the process of parthenogenesis and haploid induction. At the same time, doses below 300 Gy were found to be ineffective in inducing haploids in either of the cross combinations. A high irradiation dose of 500Gy was not found suitable for regeneration (either direct or indirect) because higher irradiation prevents parthenogenic or zygotic embryo formation. These results are supported by the findings of Salehian et al. (2023) in Sunflower, where they could not induce any haploids at 500 Gy because of higher penetration energy. They have standardised 300 Gy as an optimum dose for parthenogenic embryo development and haploid induction. The optimum dose of γ irradiation for parthenogenic haploid induction was found to vary among the flower crops: 250 Gy in *Rosa damascena* (Palvaneh et al. 2013), 300 Gy in Sunflower (Todorova et al. 1997), and 500 Gy in *Rosa x hybrida* (Meynet et al. 1994).

The number of induced embryos was significantly reduced with the increase in the dose of irradiations. The embryo response and regeneration were not only related to the irradiation doses but also to the genotypes. DAMH-24 x PD showed the maximum direct regeneration (Fig. 6; Table 1), while no direct regeneration and only indirect regeneration (callogenic embryo) was observed in DAMH-55 x PD (Fig. 7; Table 1). The maximum regeneration for efficient haploid induction was found when 400 Gy irradiated pollen of PD were used to pollinate DAMH-24. Keles et al. (2016) also observed varietal variations for haploid induction in Spinach; out of three varieties, haploid embryo formation was observed in Favorit F1 when pollinated with 400 Gy irradiation pollen of the same variety. Contrary to these, Aktas et al. (2023) obtained efficient haploid induction in Sunflower when a higher dose of irradiation, i.e. 750 Gy, was used. They obtained haploid induction only in three genotypes (K3AD SN:8, IMI 069 and IMI 044) out of the 16 tested genotypes. Similarly, Zhao et al. (2023) observed the highest embryo induction bottle gourd genotype, BG-4, when 50 Gy irradiated pollen of the same variety was used for pollination.

Correlation among different variables of in vitro cultured pseudo fertilised ovaries of and irradiation doses

For a better understanding of the relationship between haploid induction through induced parthenogenesis and γ irradiation doses, a correlation matrix was established between different variables of haploid induction and irradiation doses. In Fig. 10, a correlation matrix scale showing the values ranging from $r = 1$ to -1 represents the strength and direction of the correlation between variables; the brown colour denotes a negative/indirect correlation, while the blue colour depicts a positive/direct correlation between both the parameters (Bakhshandeh et al. 2023). All the parameters are correlated after taking the average of both crosses (DAMH-24 $\times$ PD and DAMH-24 $\times$ PD) with different gamma-ray doses. According to our study, as the irradiation dose increased, the time taken for the response ($r = 0.85$) and days to direct regeneration ($r = 0.73$) showed a strong direct correlation. In contrast, percent direct regeneration ($r = 0.58$) showed a moderate direct correlation and days to callus initiation ($r = 0.42$) exhibited a direct correlation. Similarly, with some other parameters as doses increase the percentage of ovaries showing colour change ($r = -0.90$) exhibited a robust indirect correlation while percent response

($r = -0.58$) and percent callusing ($r = -0.52$) exhibited a moderate indirect correlation. Similar to the present findings, Ulukapi and Ozmen (2018) also studied the correlation effects of various levels of γ -rays (100 to 500 Gy; 50 Gy interval) on the survival rates of the seedlings and vegetative traits in Common bean (*Phaseolus vulgaris* L.) plants. They found a significant positive correlation between root length and shoot length ($r = 0.90$), between root fresh weight and shoot fresh weight ($r = 0.667$), and between leaf length and chlorophyll index in M1 plants of the F16 variety.

Ploidy determination of gynogenically developed plants

Determination of the ploidy level is a vital step during haploid and doubled haploid production and their utilisation in breeding programmes. The methods for ploidy determination in marigold have been described earlier by Kumar et al. (2019). A total of 52 plants, regenerated from the cultured ovaries of DAMH-24 $\times$ PD and DAMH-24 $\times$ PD) were assessed for their ploidy confirmation along with the mother plants. The ploidy was initially assessed by indirect and later confirmed by direct methods (Fig. 11; Table 2). The morphological evaluation of regenerated plants revealed the prevalence of albino plants at higher doses, ~ 400 & 500 Gy. At higher irradiation doses, haploid embryo induction might have resulted due to induced parthenogenesis. Hence, more plants with physical deformities were obtained. The haploid plants were mostly albino and exhibited high mortality rates, and if they survived, they also then failed to flower; this phenomenon may be due to the 'Hertwig effect' (Pandey and Phung, 1982). The number of chloroplasts in guard cells of stomata varied from 3–7 in haploids, 10–14 in diploids and 16–20 in triploid plants (Fig. 11). The plants of different ploidy levels, including haploids, diploids, and mixoploids have also been reported during the androgenesis and gynogenesis in marigold by Kumar et al. (2018) and Kumar et al. (2020) and Bhatia et al. (2017) in cauliflower. The cytological analysis (chromosome counting) further revealed that all the regenerants from 0 Gy and a few from 100, 200 and 300 Gy were triploid showing chromosome number $2n = 3x = 36$ in their root cells (Fig. 11k). A total of 8 haploids ($2n = x = 12$; 15.38% Fig. 11c), 27 diploids ($2n = 2x = 24$; Fig. 11g) and 17 triploids ($2n = 3x = 36$; Fig. 11k) were identified. The maximum haploid induction regenerants were identified in DAMH-24 $\times$ PD. The maximum haploid induction was observed within DAMH-24 $\times$ PD with 400 Gy (7 out of 12) followed by 200 Gy (1 out of 5), while no haploid was obtained in DAMH-55 $\times$ PD even on irradiations. The higher dose of gamma irradiation might have led to the inactivation of male gamete in the pollen; however, it supported the direct embryogenesis from haploid cells of the embryo sac and subsequent haploid production. In this study, we have obtained a large number of diploid regenerants, which could be a result of regeneration from the diploid cells of the ovary wall/ embryo sac. The triploid plants could have regenerated as a result of successful fertilisation between diploid African marigold and tetraploid French marigold species. Both these species are cross-compatible, and successful interspecific hybrids among these two species have been reported by Zhang et al. (2022). Some of these diploids could also be doubled haploids that might have resulted from spontaneous chromosome doubling of haploid embryos. Spontaneous chromosome doubling is a common phenomenon during haploid production and has been in a large number of plant species, including Brassica vegetables (Bhatia et al. 2016 & 2018). After chromosome counting, the ploidy levels of the regenerants were further confirmed by flow cytometry analysis. Here, we have haploids have C DNA content with $\sim 54K$ mean Pi A (Fig. 11d), diploids with 2C

DNA content with ~ 108K mean Pi (Fig. 11h), and triploids with 3C DNA content with ~ 154K mean Pi (Fig. 11i). The flow cytometry analysis also revealed that out of the 52 randomly selected plants 8 were haploids (15.38%), 27 were diploid (51.92%) and 17 were triploid (32.69%) (Fig. 12). The haploid plants were successfully recorded using induced parthenogenesis approach in cucumber (Pradeep et al. 2024). This study has proved the utility of the irradiated pollen technique (induced parthenogenesis) in haploid induction in African marigold. In the future, this approach can be utilised to achieve high throughput haploid induction in different marigold genotypes.

Conclusion

For the first time, we have conducted an in-depth study to determine the role of gamma irradiations on haploid induction in marigold genotypes. The irradiated pollen of tetraploid marigold species, *Tagetes patula* genotype Pusa Deep ‘PD’ (2n = 4x = 48) was used to induce the haploids in the diploid marigold species, *T. erecta* genotypes ‘DAMH-24’ and DAMH-55’ (African marigold; 2n = 2x = 24). The irradiated pollen technique was found effective in improving the haploid induction frequency in marigold. An irradiation dose of 400 Gy was found to be the optimum dose for efficient pollen inactivation and high-throughput induction of haploids. This research also provided insight into the effectiveness of gamma irradiation on successful haploid induction in marigold. This protocol will be instrumental in strengthening the F₁ hybrid breeding and genetic and genomic research in marigolds.

Abbreviations

Gy	Gray (=100 rad)
KI	Potassium Iodide
TTC	2, 3, 5, Triphenyl Tetrazolium Chloride
DHs	Doubled Haploids
DAP	Days after Pollination
DAI	Days after Inoculation
PVP	Polyvinylpyrrolidone
TDZ	Thidiazuron
QTLs	Quantitative Trait Locus

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Author contributions

RB comprehended and supervised the experiments. RB, KPS, SP, and AT designed the experiments. EA and KD executed field/lab experiments and collected experimental data. EA and BS conducted the gamma irradiation experiment. EA, MF and RA have done pollen and pollination studies. EA, KD, and MR have determined the ploidy. RB, EA, and B did data analysis and interpretation. EA wrote the first draft of the manuscript, and RB has made all the corrections. All other authors have proofread and approved the final manuscript.

Availability of data and materials

All the data and other details used for this study can be requested from the corresponding author.

Conflict of interest

All the authors declare no competing interests regarding the publication of this study.

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Tables

Table 1 Effect of different doses of gamma irradiation on direct and indirect regeneration response from *pseudo-fertilized* ovaries.

Treatments	Co ⁶⁰ Doses (Gray)	Percent direct regeneration		Percent callusing	
		DAMH-24 x PD	DAMH-55 x PD	DAMH-24 x PD	DAMH-55 x PD
T0	0 Gy	-	-	6.67	3.33
T1	100Gy	-	-	1.11	8.89
T2	200Gy	3.33	-	-	-
T3	300Gy	-	-	-	2.22
T4	400Gy	4.44	-	2.22	6.67
T5	500Gy	2.22	-	1.11	3.33

Table 2 Ploidy estimation of *in vitro* regenerated plants of DAMH-24 x PD and DAMH-55 x PD by different indirect and direct methods.

Mother/Crosses	Doses	Total regenerates	Haploids (x)	Diploids (2x)	Triploids (3x)
DAMH-24	Mother plant	2	0	2	0
DAMH-24 x PD	0 Gy	7	0	0	7
	100 Gy	6	0	4	2
	200 Gy	5	1	3	1
	300 Gy	0	0	0	0
	400 Gy	12	7	5	0
	500 Gy	4	0	4	0
DAMH-55	Mother plant	2	0	2	0
DAMH-55x PD	0 Gy	5	0	0	5
	100 Gy	7	0	5	2
	200 Gy	0	0	0	0
	300 Gy	3	0	3	0
	400 Gy	2	0	2	0
	500 Gy	1	0	1	0
Total regenerants		52	8	27	17

Figures

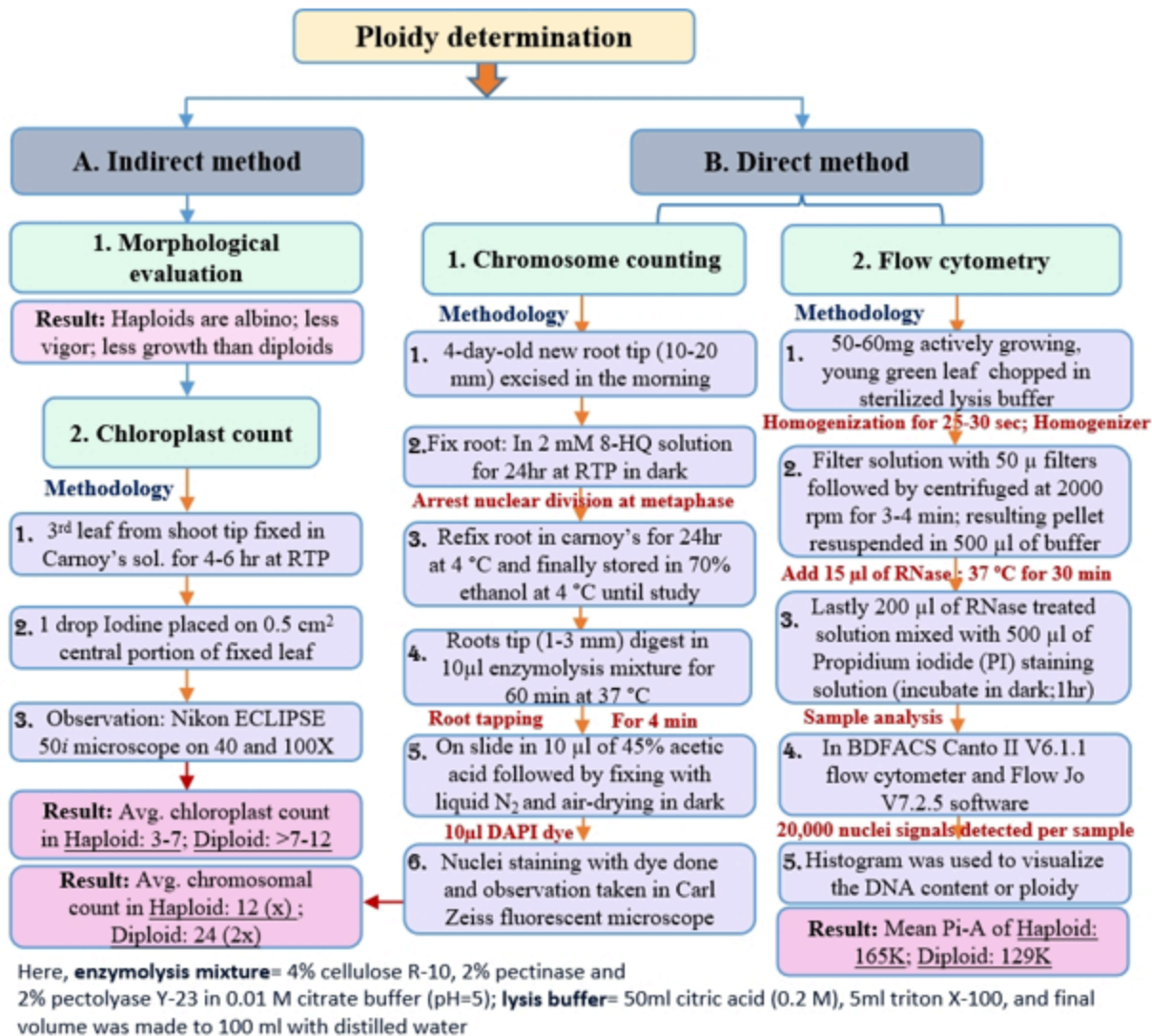


Figure 1

Different methods of ploidy determination.

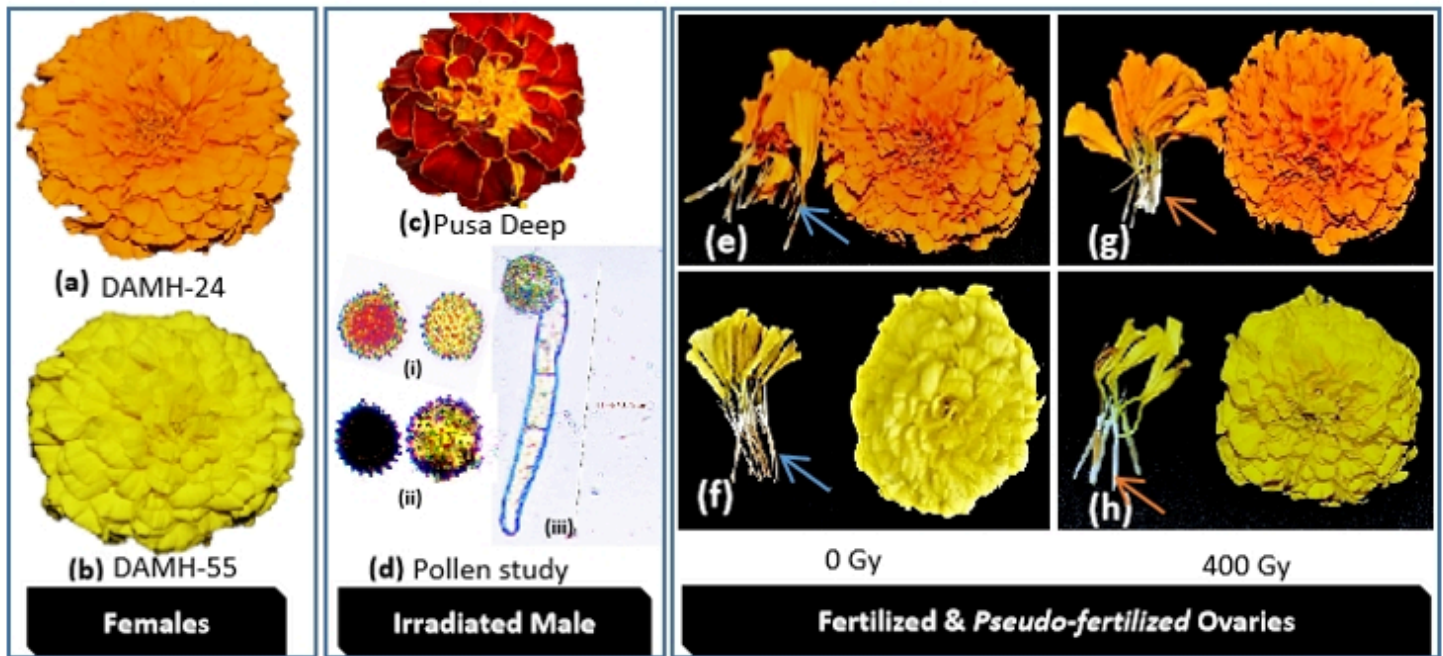


Figure 2

Effect of gamma irradiation on pollen viability and fertilisation. Female parents: (a) DAMH-24, b DAMH-55 and pollen parent (c) Pusa Deep. (d) Pollen study: viable pollen (LHS) - (i) red stained (Tetrazolium chloride), and (ii) zed blue stain (Potassium Iodide), while non-viable pollen (RHS) (i-ii) without stain; (iii) viable germinated pollen. Fertilised and *pseudo-fertilized* ovary 30 DAP: (e) DAMH-24 × PD (0 Gy) and (f) DAMH-55 × PD (0 Gy), (g) DAMH-24 × PD (400 Gy) and (h) DAMH-55 × PD (400 Gy).

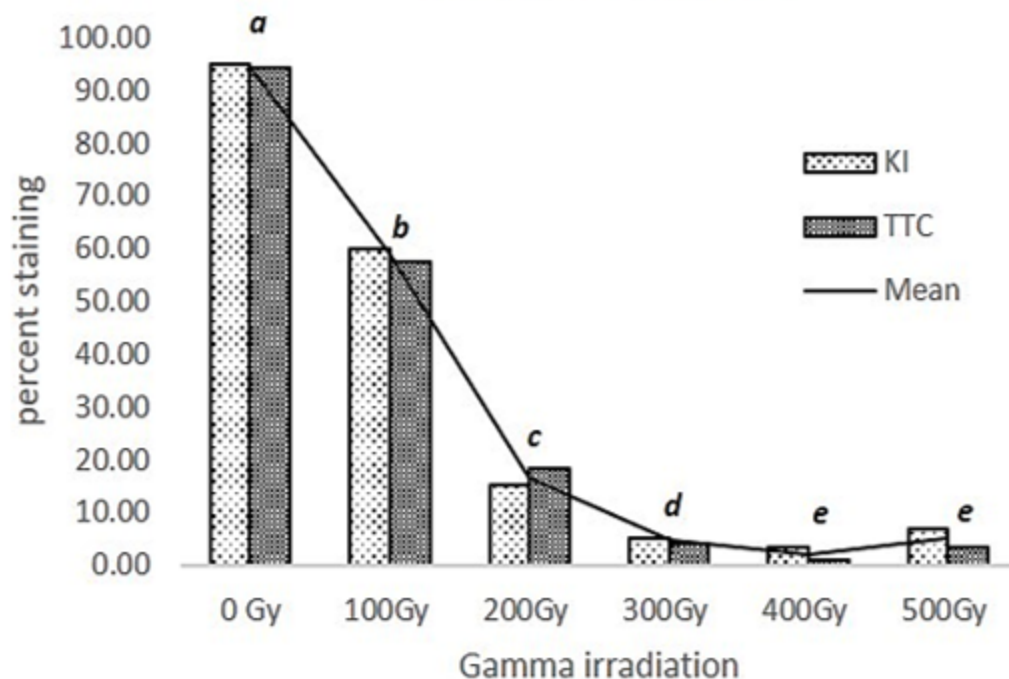


Figure 3

Effect of different doses of gamma irradiation on *in vitro* pollen staining and pollen viability in *Tagetes patula* cv. Pusa Deep.

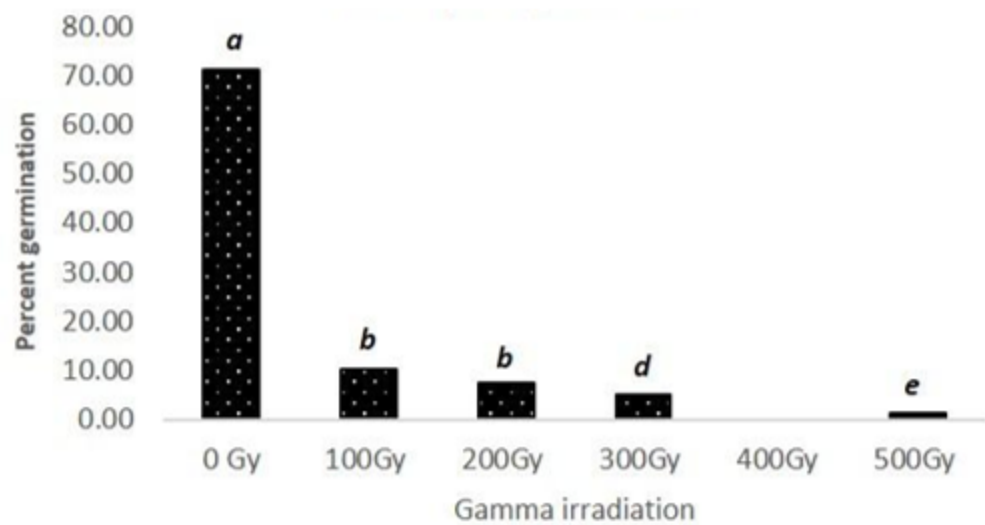


Figure 4

Effect of different doses of gamma irradiation on *in vitro* pollen germination in *Tagetes patula* cv. Pusa Deep.

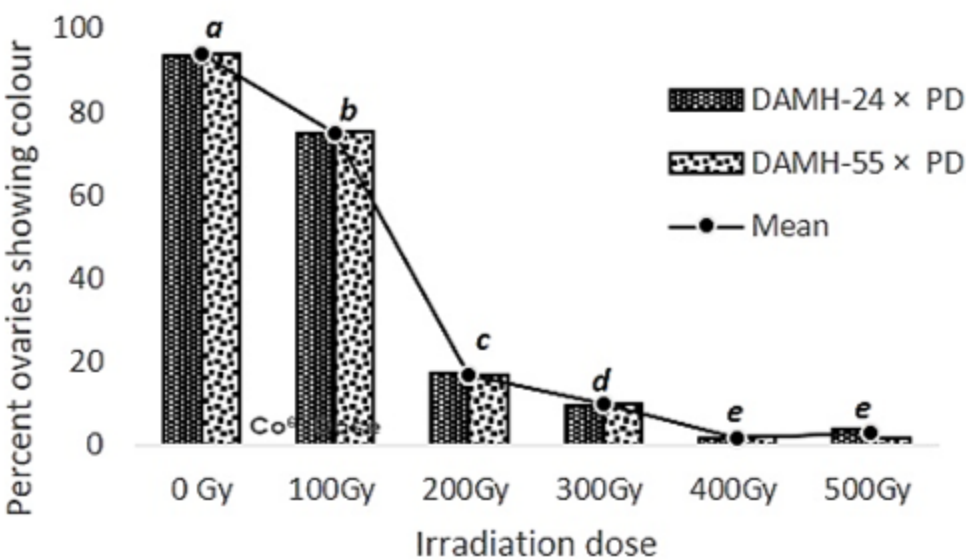


Figure 5

Effect of different doses of gamma irradiation on fertilisation/ pseudo fertilisation in other crosses.

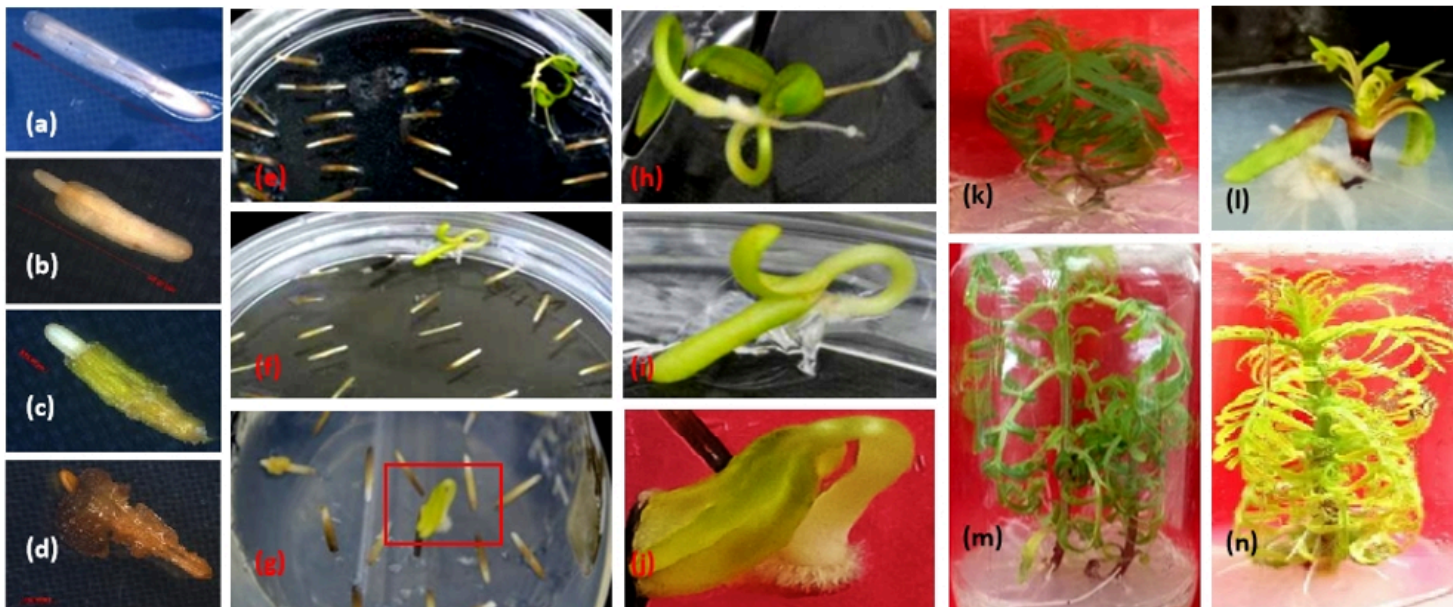


Figure 6

Effect of gamma irradiation on DAMH-24 x PD (direct regeneration from *in vitro*cultured ovaries) on pollination with different doses of γ irradiated pollen: **(a)** healthy ovary on the day of culture; **(b)** embryo emergence 5 days after culture (100 Gy); **(c)** embryo emergence from **the pseudo-fertilised**ovary (400 Gy), **(d)** dried/degenerated ovary (500 Gy); e-j represents direct regeneration with true cotyledonary leaves: 200 Gy **(e & h)**; 400 Gy **(f & i)**; 500 Gy **(g & j)**; **k- n**depicted the regenerated plants induced via parthenogenesis: **(k)** 20 days old shoots (200 Gy); **(m)** 40 days old shoors (200 Gy); **(l)** 20 days old shoots (400 Gy), **(n)** 40 days old shoots (400 Gy).

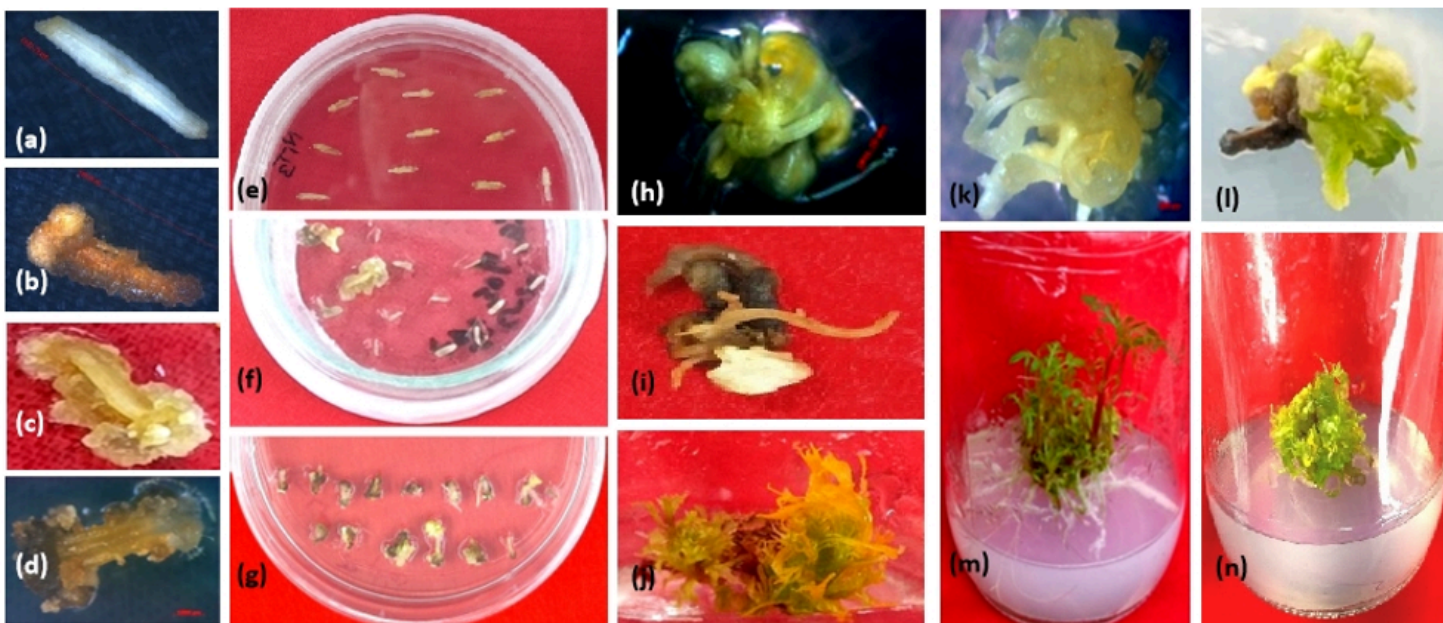


Figure 7

Effect of gamma irradiation on DAMH-55 x PD (indirect regeneration/Callogenic embryogenesis from *in vitro* cultured ovaries) on pollination with different doses of γ irradiated pollen: **(a)** healthy ovary on the day of culture; **(b) & (e)** embryogenic callus induction from ovaries (100 Gy); **(c) & (f)** healthy vigorous embryos (400 Gy), **(d) & (g)** dried/degenerated embryo at higher gamma irradiation (500 Gy); **(h)** globular shaped embryo (300 Gy), **(i)** heart shape embryo (400 Gy), **(j)** embryo with mutated orange coloured shoot (500 Gy); **(k-n)** Cotyledonary embryo formation from ovary: **(k)** 200 Gy and **(l)** 400 Gy; **(m-n)** Proliferated Shoot from parthenogenically induced ovaries (40 DAI): **(m)** 200 Gy, and **(n)** 400 Gy.

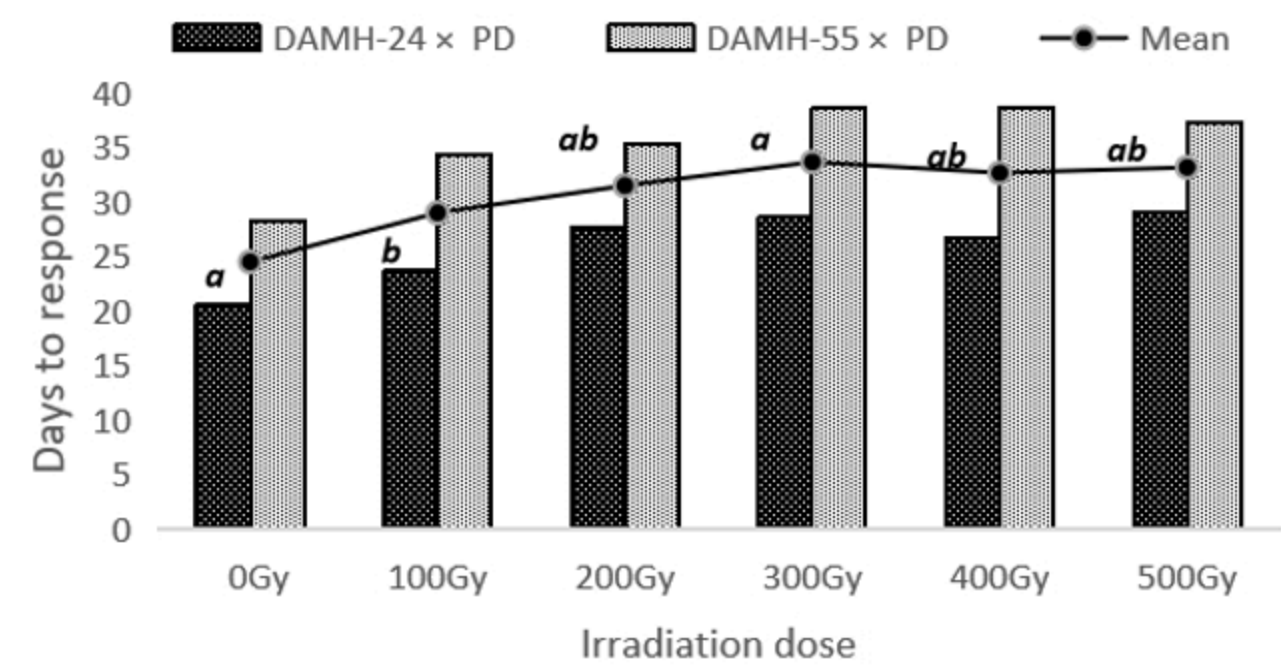


Figure 8

Effect of different doses of gamma irradiation on the number of days for visible response in *pseudo-fertilized* ovaries.

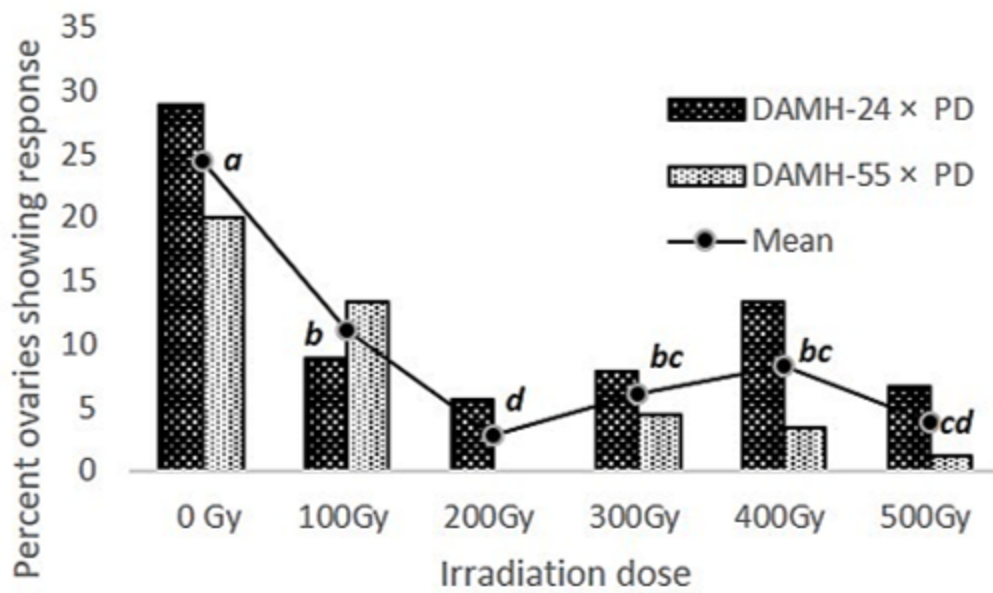


Figure 9

Effect of different doses of gamma irradiation on *in vitro* response of *pseudo-fertilized* ovaries.

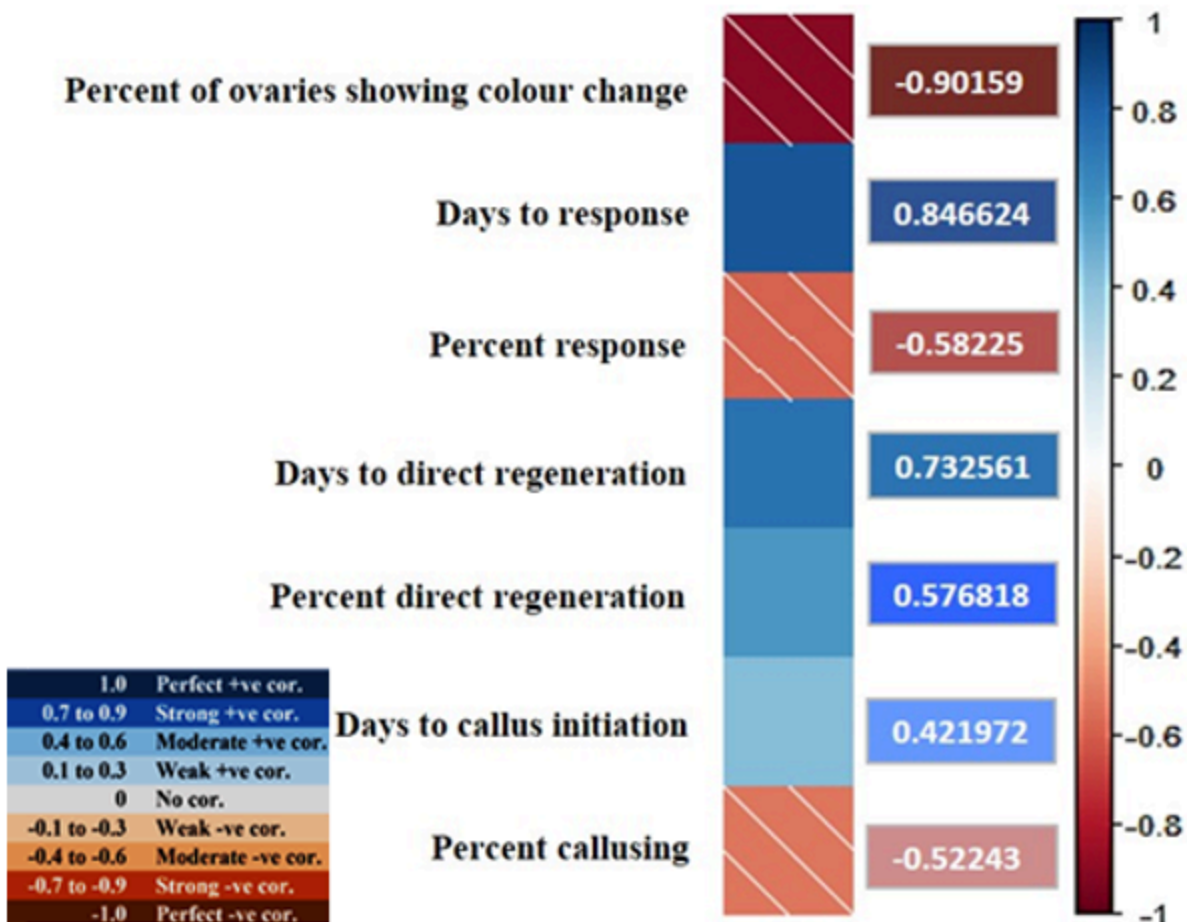


Figure 10

Correlation among different variables and doses of gamma irradiation from *in vitro* cultured *pseudo-fertilized* ovaries.

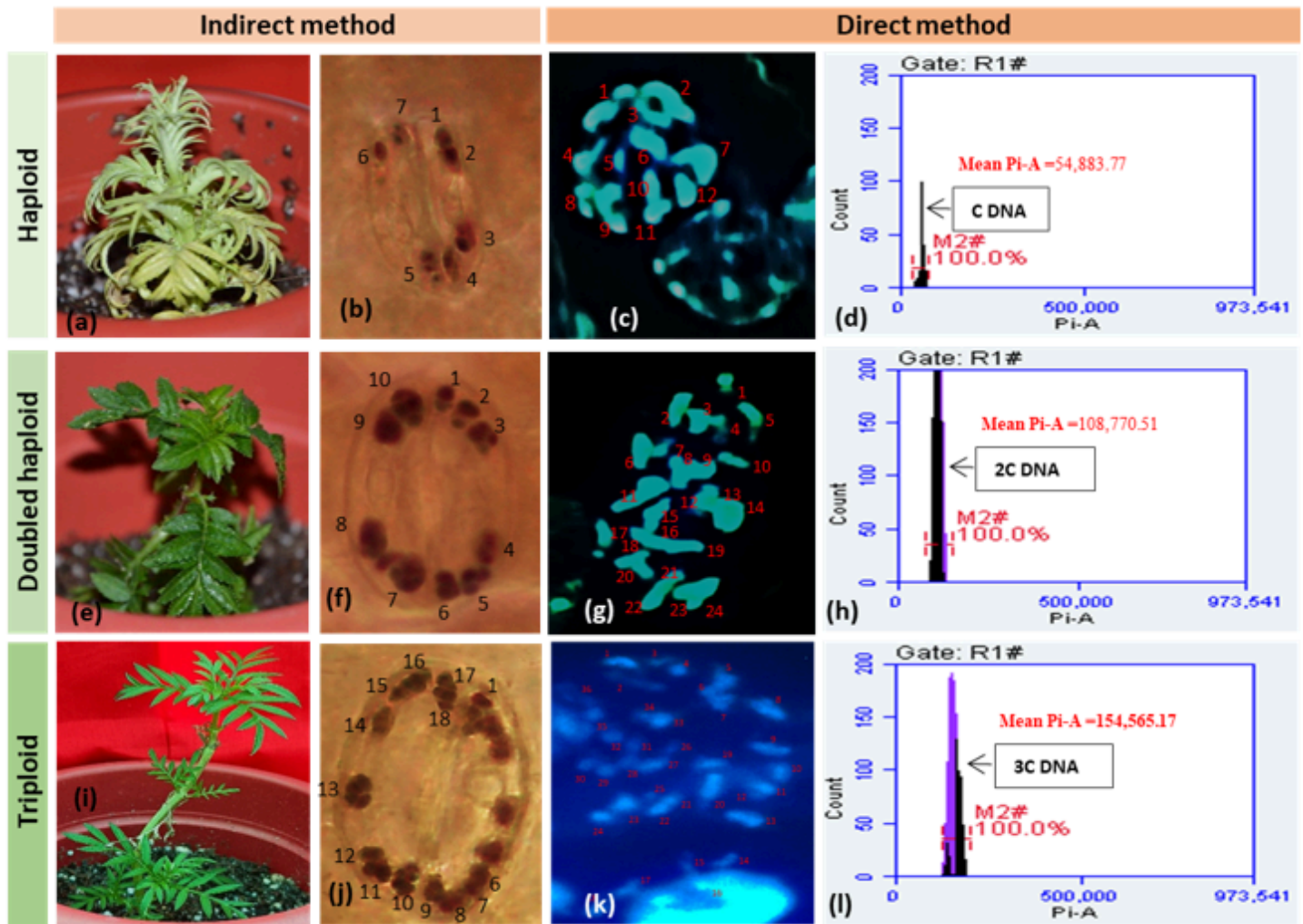


Figure 11

Comparison of the ploidy level of *in vitro* induced regenerated haploid (a-d; *pseudo-fertilized* parthenogenically induced), doubled haploid (e-h) and triploid (i-l) plants of DAMH-24 x PD on pollination with different doses of γ irradiated pollen. Here, (a), (e) & (i) shows morphological; (b), (f) & (j) show chloroplast count; (c), (g) & (k) show chromosomal count; and (d), (h) & (l) depicted flow cytometry analysis of haploid, doubled haploid as well as triploid respectively.

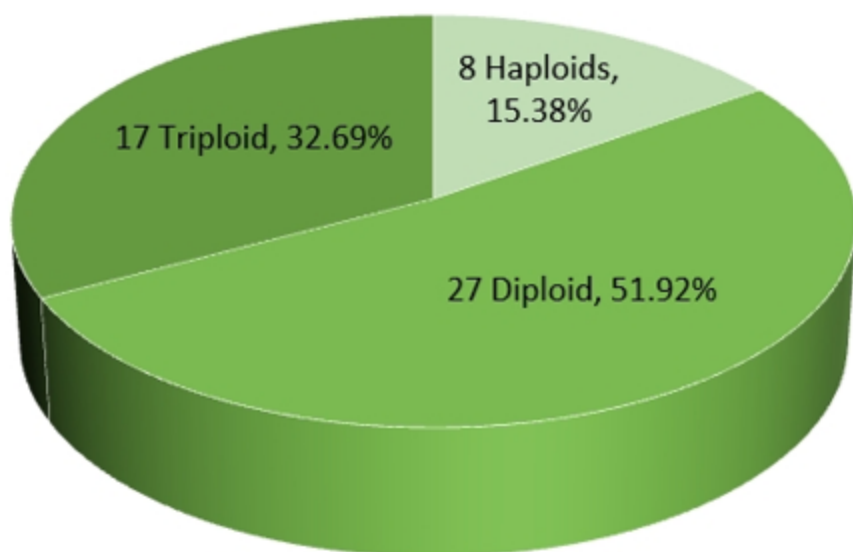


Figure 12

Pie chart showing the overall percentage of regenerated plants. Out of 52 plants, 15.38% were haploids, 51.92% were diploids, and 32.69% were triploids.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.png](#)