Effect of gamma ray irradiation and cryopreservation on pollen stainability, in vitro germination, and fruit set in \textit{Citrus}

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Abstract: The present work analyzed the stainability and in vitro germinability of the gamma-ray–irradiated fresh and cryopreserved pollens of 3 \textit{Citrus} species: \textit{C. limon}, \textit{C. limetta}, and \textit{C. sinensis}. Fruit set after pollinating 75 flowers of \textit{C. grandis} with irradiated and nonirradiated (control) fresh and cryopreserved pollen was also assessed. Results showed that in solid and liquid culture media the highest in vitro germination values were detected in fresh (61.45\% and 62.53\%, respectively) as well as cryopreserved (53.17\% and 55.36\%, respectively) \textit{C. sinensis} nonirradiated pollens. However, at a higher irradiation dose of 500 Gy in both culture media, the minimum reduction of germinability of fresh (30.85\% and 28.01\%) and cryopreserved pollens (33.37\% and 31.06\%) was found in \textit{C. limon}. Contrary to in vitro germination, the minimum reduction in stainability of fresh and cryopreserved pollens, as assessed by acetocarmine (9.51\% and 11.85\%, respectively) and FDA tests (10.78\% and 12.73\%, respectively), was recorded in \textit{C. limetta}. Regardless of irradiation dose, the highest fruit setting in \textit{C. grandis} at 40 days after pollination was detected when it was pollinated with both fresh (38.02\%) and cryopreserved (34.92\%) \textit{C. limetta} pollen grains.

Key words: Acetocarmine, cryopreservation, fruit setting, irradiation, pollen, in vitro germination, stainability

1. Introduction

In \textit{Citrus} as well as in other woody species, a long reproductive cycle, heterozygosity, large canopy size, and self-incompatibility pose major problems for breeding and genetic research (Germanà and Chiancone, 2001). To overcome them, the production of haploid progenies is of utmost importance. Haploids have significant potentiality in \textit{Citrus} breeding because homozygous plants, which are very important for genetic analysis and breeding, are easily obtained by doubling the chromosomes of haploid progenies. In \textit{Citrus}, haploids have been produced by anther culture (Hidaka et al., 1979; 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 that alternative methods should be developed to produce haploids in recalcitrant genotypes and improve the induction rate in haploid genotypes that are already producing. The irradiation pollen technique (UV, gamma rays, and X-rays) is currently used to induce in situ haploid plants. The irradiated pollen is genetically inert, physiologically active, and can be easily germinated on the stigma, but it is not able to fertilize the egg cell and the polar nuclei. Hence, these pollens might be used to stimulate parthenogenesis, including gynogenetic haploid production and overcoming minor cross-incompatibilities, and in physiological studies of incompatibility (Savaskan and Toker, 1991; Todorova et al., 2004), gene transformation (Pandey, 1978), and nucleus substitution (Raquin et al., 1989). Gamma rays are commonly used in haploidy programs because of their simple application, good penetration, reproducibility, high mutation frequency, and lower number of disposal problems (Chahal and Gosal, 2002). The major constraint is the sensitivity of \textit{Citrus} pollen to dehydration; hence, they may lose their viability when treated with gamma ray irradiation. Therefore, in studies on pollen stainability, in vitro germinability and fruit set in this species. Furthermore, flowering times of \textit{Citrus} species are different from each other, thus preventing the use of these species as pollen parents in any successful interspecific hybridization program. In a further breeding program regarding the efficiency of gamma irradiation on haploid production
in different *Citrus* species, the storage of irradiated pollen grains is of the utmost importance. Storing pollen in a viable condition is one way of guaranteeing that pollen will be available when the stigmatic receptivity is maximal. However, the storage of pollen at low temperatures (+4 °C to −20 °C) is not ideal as viability can rapidly decline over a short period of time (Khosh-Khui et al., 1976). On the other hand, Engelmann (2004) reported that cryopreservation or the storage of biological material at an ultra-low temperature, usually that of liquid nitrogen (−196 °C), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of different types of germplasm. However, literature on cryopreservation of irradiated *Citrus* pollen is scanty. This study aimed to examine the stainability, in vitro germinability, and fruit set of gamma-irradiated fresh and cryopreserved pollen grains in different *Citrus* species.

2. Materials and methods

2.1. Materials

Three different *Citrus* species, viz. sweet orange (*Citrus sinensis* 'Mosambi,' lemon (*C. limon*) 'Kagzi Kalan,' and sweet lemon (*C. limetta*), were selected as pollen sources for the experiment based on our previous preliminary study on pollen viability. Pummelo (*C. grandis*), a monoembryonic diploid *Citrus* species, was selected as seed parent to test the fruit setting after pollinating with fresh and cryopreserved irradiated and nonirradiated (control) pollen grains.

2.2. Pollen collection and irradiation

Twenty unopened flowers approaching anthesis were separately collected from mature plants of each of these 3 species in the morning (0900 to 0930). After removal of petals and stigma, the anthers with filament were put in glass petri dishes and kept under sunlight for proper dehiscence. Irradiation was performed on these anthers in a gamma chamber by Cobalt 60 gamma rays (NRL, IARI, New Delhi, India) at 100, 300, and 500 Gray (Gy). To evaluate the effect of gamma-irradiated pollens on fruit set, female flowers before anthesis were emasculated on the same day (0900 to 1030; up to 5 flowers per cluster) and bagged in white muslin cloth bags (40 × 40 cm) to prevent uncontrolled pollination. After removing the bags, hand pollinations of these emasculated flowers were carried out on the same day (1030 to 1300) by brushing the stigmas with both irradiated and nonirradiated pollen grains (control). The bags were removed 10 days after pollination (DAP).

2.3. Cryopreservation of irradiated and nonirradiated pollen

Anthers with filaments, from each species at each exposure dose, were dehydrated for 24 h at room temperature in anhydrous CaCl₂ desiccators. For each species at each exposure dose, 2 samples of pollen grains, extracted from dehiscent anthers, were placed in polypropylene vials and stored for 1 year (March 2011 to March 2012) at an ultralow temperature (−196 °C) in a liquid nitrogen cylinder. After this storage period, pollen samples were taken out of the cylinder and rehydrated for about 3 h in a controlled environment at 20 °C and 95% relative humidity, prior to use in the stainability and in vitro germination tests as well as the controlled pollination.

2.4. Viability test

To test pollen viability, the stainability and in vitro germinability of irradiated as well as nonirradiated pollen (control) was carried out on the day of irradiation treatment. Pollen stainability was estimated by acetocarmine crushing (Nassar et al., 2000) and fluorescein diacetate (FDA) test (Heslop-Harrison and Heslop-Harrison, 1970).

For the acetocarmine test, after uniform distribution on a glass slide, the pollen grains were dyed with 1% acetocarmine solution and observed by light microscope (40× magnification). The grains with abnormal size, light coloring, and reduced and/or absent protoplasm were considered nonstained pollens, while those with intact exine and strongly colored protoplasm were classified as stained pollens. For FDA test, a stock solution (2 mg/mL) in acetone of fluorescein diacetate was prepared. A 10% sucrose solution was used to prevent the bursting of pollen grains, while 300 mg/mL of calcium nitrate was added to improve their response. After putting 2–5 mL of this solution in a small glass vial, drops of FDA stock solution were added until the resulting mixture showed persistent turbidity. After taking a drop of sucrose-FDA mixture on a micro slide, pollen grains were suspended in the drop and incubated in a humid chamber (>90% RH) for 5–10 min. After that, a cover glass was lowered, and the preparation was observed under a fluorescent microscope (Leica DM 5000B). Pollen grains that fluoresced brightly were counted as viable.

In vitro germination tests of pollen grains were performed in 2 different composition media: an agar-solidified growing medium, according to Sahar and Spiegel-Roy (1980), and a liquid culture medium, according to Cavalcante et al. (2000).

In the solid medium, both irradiated and nonirradiated pollen grains were dusted on a thin layer of growing media on a slide and incubated for 24 h in the dark at 26 °C. Thereafter, they were observed under an optical light microscope (40× magnification). In liquid media, the hanging drop technique was followed. To get uniform samples, pollen grains were thoroughly mixed in 1 mL of liquid germination media, and a 15 μL aliquot of this mixture was placed on a glass slide with a ring of 8 mm in diameter inside. Slides were inverted, placed on a rack in a polycarbonate-sealed container lined with moistened
blotting paper, and incubated in the dark at 26 °C. Thereafter, pollen grains, observed by a light microscope (40× magnification), were considered germinated when the length of the pollen tubes was equal to or greater than the grain diameter. For each irradiation dose, the pollen stainability and in vitro germination rates were evaluated on 200 pollen grains in each replication with 5 repetitions.

2.5. Effect of pollination with gamma-irradiated pollen on fruit set
Seventy-five flowers of Citrus grandis were hand pollinated with the pollens of 3 Citrus species (C. sinensis, C. limon, and C. limetta) for each exposure rate along with nonirradiated pollen as control. The number of fruit set by these irradiated pollen grains was counted at 10, 25, and 40 DAP.

2.6. Statistical analysis
The experiment was laid out in a complete randomized design with 5 replications; statistical analysis was performed using Microsoft Excel (2007) and statistical analysis software (SAS software), and the means were compared using Duncan’s multiple range test (DMRT).

3. Results
3.1. Pollen viability
The results of stainability observed in fresh pollen by acetocarmine and FDA tests indicate a significant variation among the species (Figures 1 and 2). Regardless of irradiation doses, higher stainability by acetocarmine crushing was recorded in Citrus sinensis (85.60%); in FDA tests the maximum value was observed in C. limon (83.83%), which was statistically at par with C. sinensis (83.30%). However, the lowest stainability was observed in C. limetta in both test methods (83.74% and 82.64%, respectively). Regardless of the species, stainability values decreased with increasing concentrations of gamma irradiation. Among different species, at higher irradiation doses the minimum reduction of pollen stainability, as compared to respective controls (acetocarmine and FDA

![Graphs showing pollen stainability and in vitro germination](Panel A and B)

**Figure 1.** Viability of fresh pollen of 3 different Citrus species as evidenced by stainability (acetocarmine and FDA) tests (Panel A) and in vitro germination in solid and liquid media (Panel B). Vertical bars indicate the mean value ± standard deviation. LSD at P ≤ 0.05 for stainability in acetocarmine, 2.86; in FDA 3.16 and in vitro germination in solid media, 3.16; and in liquid media, 3.20.
tests), was recorded in *C. limetta* (9.51% and 10.78%, respectively). *Citrus* species also showed differences with regard to in vitro germination (Figures 1 and 2). Without considering the effect of gamma irradiation, lower germination of fresh pollen was recorded in *C. limetta* in both solid (43.02%) and liquid media (44.14%). A similar trend was found for the other 2 species with higher and at par values under both culture media. Irrespective of species, in vitro *Citrus* pollen germinability declined with increasing gamma irradiation doses. Contrary to the stainability tests, at higher irradiation levels the minimum reduction was observed in *C. limon* (30.85% in solid and 28.01% in liquid mediums).

### 3.2. Cryopreservation

A similar trend to fresh pollen viability was observed in cryopreserved pollens (Figures 3 and 4). Regardless of irradiation doses, the maximum stainability in acetocarmine (80.20%) and in FDA (79.18%) tests was recorded in *Citrus limon*, at par with *Citrus sinensis* in the acetocarmine test (79.48%). In both these tests, the stainability of cryopreserved pollens declined significantly with increasing doses of gamma irradiation. At higher doses the minimum reduction of stainability was recorded in *C. limetta* for both acetocarmine (11.85%) and FDA (12.65%) tests. After comparing the viability of fresh and cryopreserved pollen grains (paired t-test), it was evident that pollen stainability after 1 year of storage at ultralow temperature significantly decreased in all species at different irradiation doses, as depicted by acetocarmine and FDA tests. In the acetocarmine test the maximum stainability decrease in cryopreserved pollen over fresh was observed in 500-Gy-gamma-ray–treated *C. sinensis* pollen (8.67%) followed by 100-Gy–treated *C. sinensis* pollen (8.42%) while in the FDA test the maximum was recorded in nonirradiated *C. sinensis* pollen (8.52%). The minimum decrease in the acetocarmine test was detected in 100-Gy–treated *C. limetta* pollen (3.59%) followed by 300-Gy–treated and nonirradiated cryopreserved pollen of *C. limetta* (4.60% and 4.68%, respectively); in the FDA test the minimum was in 100-Gy–treated *C. limetta*
pollen (3.64%). Similarly, after 1 year of cryopreservation of irradiated as well as nonirradiated pollen, in vitro germinability was significantly lower compared to fresh pollen. The maximum decrease of in vitro germination in cryopreserved pollen compared to fresh in solid medium was in 500-Gy–treated C. limon (16.05%) pollen followed by 500-Gy–treated C. limetta pollen (15.06%); the minimum decrease was recorded in 100-Gy–treated C. limetta pollen. In liquid medium the minimum decrease of in vitro germination was observed in 500-Gy–treated C. sinensis pollen (4.93%) followed by 500-Gy–treated C. limon pollen (6.22%).

3.3. Fruit setting
Regardless of species, fruit setting significantly decreased with increasing doses of gamma irradiation on fresh pollen (Table). Without considering the effect of irradiation, the highest fruit setting at 40 DAP (38.02%) was found in Citrus grandis × Citrus limetta crosses. Furthermore, the minimum reduction in fruit set at 40 DAP was recorded in 100-Gy–treated C. grandis × C. sinensis pollen (41.36%), which had at par value with C. grandis × C. sinensis (42%) as with the respective controls. However, in nonirradiated pollen the maximum fruit setting at 40 DAP was detected in C. grandis × C. sinensis crosses (44.83%), at par with C. grandis × C. limetta crosses (43.07%). Furthermore, with respect to fruit setting, trends similar to fresh pollen were observed in cryopreserved pollens.

In comparison with fresh pollen (paired t-test) fruit setting in cryopreserved pollen decreased significantly during the entire period of observation. The maximum reduction over fresh pollen was observed when C. grandis flowers were pollinated with 300-Gy–treated C. sinensis pollen grains (14.53%) followed by 100-Gy–treated C. limon pollen grains (13.29%); the minimum reduction was recorded in pollination with 100-Gy–treated C. sinensis pollen (2.04%) and nonirradiated C. limetta pollen (2.23%).

The fruit setting gradually decreased with increasing DAP in all treatments, and it was the highest in all the species at 10 DAP and significantly decreased thereafter.
The maximum reduction in fruit setting by nonirradiated fresh pollen was recorded in *C. grandis × C. limon* crosses (56.72%), while the minimum was observed in *C. grandis × C. sinensis* crosses (48.45%) at 40 DAP, as compared to 10 DAP. Similarly, at a higher irradiation dose (500 Gy) the maximum reductions in fruit set were also detected in *C. grandis × C. limon* crosses (70.45%), at 40 DAP.

4. Discussion

4.1. Pollen viability

Regardless of irradiation dose, differences in pollen viability in the form of stainability and in vitro germination among different *Citrus* species are mainly due to the genotypic variation, which confirms the earlier findings of Soost (1963) in *Citrus*, Chalak and Legave (1997) in kiwi fruit, and Ali et al. (1998) in pomegranate. On the other hand, the results confirmed that the in vitro pollen germination test is a better and more reliable indicator of viability than stainability tests; in the latter, pollen grain viability was overestimated. A similar overestimation was observed by other authors for fresh, irradiated, nonirradiated, and cryopreserved pollens in different crops (Falque et al., 1992, in cocoa; Panella et al., 2009, in sugarbeet; Chaudhary et al., 2010, in *Murraya koenigii*; Yahata et al., 2010, in *Citrus*). Acetocarmine stainability, considered the simplest technique for pollen viability analysis, is the less sensitive test because it only confirms the presence and stainability of the cytoplasm (Dafni and Firmage, 2000), whereas the FDA test ensures 2 pollen parameters: esterase activity and plasmalemma integrity. Pollen grains degenerated due to necrosis (pollen with shrunken cytoplasm) or programmed cell death (pollen with cytoplasm sticking to the plasmalemma) remained nonstained in the FDA test (Nepi and Franchi, 2000). In
our experiment, due to gamma irradiation treatments, sensitive pollen grains may be degenerated in the above-mentioned patterns and found nonviable by FDA test; however, they may be stained by acetocarmine, resulting in a higher viability by this less sensitive test. On the other hand, between the 2 different growing media used for in vitro germination tests, the liquid medium contains a higher concentration of calcium nitrate, which increased pollen grain germinability; in fact, a higher calcium concentration gives more rigidity to cell walls (Brewbaker and Knawck, 1964), resulting in fewer ruptured pollen tubes and increased germination rate. These results are in agreement with the earlier findings reported by Cook and Walden (1965) and Pfahler (1968) in maize.

Moreover, pollen viability not only depends upon the genotype, pollen maturity, plant physiological status, and growing media composition (Barnabas and Kovacs, 1997; Ferri et al., 2008), but also upon the internal conditions of pollen grains in relation to environmental factors like air temperature, humidity, grain water content, reserve substances, gametic maturity, and their interactions (Franchi et al., 2002; Pacini et al., 2006). On the basis of all these factors, the pollen grains of different crop species have been classified into 2 categories: partially hydrated pollen, with a water content $>$30% (like recalcitrant seeds) and partially dehydrated pollen, with a water content $<$30% (like orthodox seeds) (Kermade and Finch-Savage, 2002).

Unfortunately, in the literature there is scarce evidence regarding the interaction between the water content of pollen grains and irradiation doses, especially in Citrus. According to their morphological similarities with pollen grains of other Angiosperm species classified by Franchi et al. (2002), it is possible to consider Citrus pollen grains partially dehydrated pollen. However, the exposure to different gamma irradiation doses may reduce the capability of Citrus pollens to interconvert carbohydrate reserves; this is strictly linked to a change in the state of the cytoplasmic water of pollen grains, resulting in abnormal meiosis, irregular gametes that may cause significant changes in pollen properties (Nepi and Pacini, 1993), and a consequent reduction in their viability. The earlier findings reported by Zhang and Lespinasse (1991) in apple and Kurtar (2009) in pumpkin and winter squash have been confirmed.

### 4.2. Cryopreservation

Due to scanty literature about the effect of cryopreservation on Citrus pollen, it is very difficult to understand the reasons for the significant decrease in pollen viability after this treatment. In these species the partially dehydrated pollens could be less difficult to store at low temperatures, as required for long-term storage in liquid nitrogen. Due to the freezing effect, however, Citrus pollens may be unable to maintain cytoplasmic water content within a certain range by mobilizing carbohydrate reserves, resulting in

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<th>Treatment</th>
<th>10 DAP Fresh pollen</th>
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<th>25 DAP Cryo pollen</th>
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<td>87.10$^{bcd}$</td>
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Different letters in the same column indicate significant differences at $P \leq 0.05$ (Duncan’s multiple range test); DAP: days after pollination.
their death. Moreover, this significant reduction in pollen viability after 1 year of cryopreservation may also be due to the production of cellular lesions during freezing and thawing (Martinez-Gomez and Gradzki, 2002). This finding is in agreement with the earlier findings reported by Luza and Polito (1985) in walnut, Martinez-Gomez and Gradzki (2002) in almond, and Alba et al. (2011) in olive.

4.3. Fruit setting

Results showed that fruit setting is much higher at 10 and 25 DAP for all Citrus species at every irradiation dose: in both cases fruit setting values were higher than with in vitro germination. However, at 40 DAP this value was quite low and was also lower than the in vitro germination percentage. This is mainly due to the fact that during the early stage of pollination generally there is good growth of the ovary before fertilization; however, if this does not continue the fruits do not retain and drop down within a short period of time, and only the fertilized fruits are retained on the tree. In Citrus, the unfertilized flowers may drop down 25 DAP. Hence, only the data at 40 DAP give the actual value, while those at 10 and 25 DAP may overestimate the fruit setting percent. On the other hand, the higher fruit setting by nonirradiated pollen at every time period is mainly due to the higher germination capacity of this pollen, as mentioned above. The rapid reduction in fruit setting, with gradual increases in gamma irradiation, might be due to the sensitivity of pollen grains to this treatment which reduced their viability at a faster rate, as evidenced in this experiment. Similarly, lower fruit setting by cryopreserved pollen than fresh is also due to the lower viability of pollen stored at ultralow temperatures. Furthermore, C. *limon* pollen showed a higher in vitro germination rate but comparatively low fruit setting. This may be due to a more rapid degeneration of the pollen tube within this floral style as compared to the pollen tubes of the other 2 species.

The results showed that, among different techniques, the in vitro germination test is the better and more reliable indicator of pollen viability, as acetocarmine and FDA tests sometimes overestimate this parameter. On the other hand, at the higher irradiation dose of 500 Gy the pollen of Citrus *limetta* showed higher resistance to gamma-ray irradiation than the other 2 species, as evidenced by stainability, in vitro germinability, and fruit setting data. Hence, although C. *limon* and C. *sinensis* could also be used in haploid breeding programs, C. *limetta* is the better and more reliable species to be employed in these studies. Moreover, the results of this research have highlighted the operational effectiveness of Citrus pollen cryopreservation. This technique, however, should be improved further in order to optimize several parameters. Particular attention should be devoted to the prefreezing treatments, especially in relation to pollen water content. Care must be taken to avoid ice crystallization during the freezing process, which could cause physical damage to the pollen grain tissues. Moreover, further research is needed to evaluate the effects of gamma-irradiated fresh and cryopreserved pollens on seed development and haploid production in Citrus. Furthermore, large variability exists in citrus; thus, the response of different important genotypes to irradiation doses should be studied in detail in future studies.

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