RESEARCH ARTICLE

Induction of powdery mildew resistance in gerbera (*Gerbera jamesonii*) through gamma irradiation

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Abstract In this study, the effect of gamma irradiation in inducing resistance/tolerance towards powdery mildew disease was investigated in *Gerbera jamesonii* cv. ‘Harley’. In vitro shoot cultures were established through capitulum explants on Murashige and Skoog medium supplemented with 22.2 μ M 6-benzyladenine (BA) and 2.53 μ M indole acetic acid (IAA), followed by gamma irradiation of regenerated shoots (3–5 cm). Activity of four antioxidant enzymes i.e. superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase increased significantly as compared to the control and reached to highest level at the most stringent doses of mutagen. Ninety randomly selected irradiated plants (6 months old) and 100 control plants were inoculated with fungal conidial suspension, to screen for tolerance/resistance against powdery mildew. The severity of the disease was recorded on 0–4 scale with ‘0’ indicating highly resistant; ‘1’ indicating resistant; ‘2’ indicating medium resistance; ‘3’ indicating susceptible and ‘4’ indicating highly susceptible. Three plants (3.33%) irradiated with 5 Gy were found to be tolerant to powdery mildew as these plants showed slight and delayed development of fungal colonies on the leaves. The random amplified polymorphic DNA characterization showed that the irradiated plants had DNA patterns that were different from the control and mother plants.

Keywords Antioxidant enzymes · Gamma rays · Gerbera · Mutation · Fungal resistance · RAPD markers

Introduction

Barberton daisy (*Gerbera jamesonii*) is fifth most important ornamental plant with respect to demand and revenue generated in global cut flower trade (Terri et al. 2006; Bhatia et al. 2009). Flowers have unique forms and are available in a wide range of colours. It is valued for its unique and attractive flower forms and bright colours. The demand of floricultural products especially ornamental flowers has increased consistently over the years. In order to meet the increased demand of gerberas, there is a need for formulation of robust and reliable multiplication system. Although, both sexual and asexual propagation methods are available for multiplication, but these are insufficient to keep up with the huge demand (Murashige et al. 1974). Micropropagation technology promises rapid and large scale multiplication and dependable propagation protocols have been established with different explants of gerbera (Altaf et al. 2009; Ghani et al. 2014). Like any other flowering plant, gerbera is also affected by a number of diseases, leading to poor flower quality and heavy monetary losses to the growers. Powdery mildew caused by *Erysiphe cichoracearum* and *Oidium crysiphoides* f. sp. *gerbera* is a major production problem in gerbera (Ramchandarn et al. 2004). The disease spreads rapidly on polyhouse grown gerberas, thereby necessitating frequent applications of fungicides. Beside increasing the cost of production, use of fungicides leads to other problems, such as, development of fungicide resistance, phyto-toxicity and environmental pollution (Gullino and Wardlaw 1999). Additionally, the increase in consumer awareness further

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calls for search of alternative disease control measures (Moyer and Peres 2008).

In vitro mutation technique in combination with tissue culture method is one such approach that provides a powerful technology for improvement of clonally propagated plants. Irradiation of in vitro raised cultures, followed by multiplication under aseptic conditions has proved to be an invaluable method for stimulation of desired variations such as disease resistance (Liu et al. 2005). Over the year, this technique has been used extensively (Ali et al. 2007; Jain 2010, 2012; Shin 2011) and release of more than 3000 improved mutant varieties of different crops clearly demonstrates the potential of this technique (Kharkwal and Shu 2009; Jain and Suprasana 2011).

Reactive oxygen species (ROS) are formed in response to any kind of biotic or abiotic stress and these ROS oxidize photosynthetic pigments, membrane lipids, proteins and nucleic acids. The antioxidant defense system in the plant cell comprises of two components enzymatic [superoxide dismutase (SOD), ascorbate oxidase (APX), catalase (CAT), peroxidase (POD), glutathione reductase (GR) etc.] and non-enzymatic responses (Sudhakar et al. 2001). There are many lines of evidence showing how the anti-oxidant enzymes protect the plants against the harmful effects of the ROS accumulated in the plants under stress (Helaly and Hanan El-Hosieny 2011; Tarroum et al. 2011; Bhoyar et al. 2011). Level of antioxidants in cell act as determinant for the cellular resistance and therefore the efficacy of resistance is also dependent upon the level of antioxidant enzyme activity (Lee and Lee 2000).

In this study, the potential of gamma radiations in inducing mutations in in vitro raised shoots of *G. jamesonii* is explored. The effect of stress on the activity of antioxidant enzymes is studied. Further, the induction of tolerance/resistance towards powdery mildew disease due to induced mutations is investigated at visual as well as genetic level.

Materials and methods

Source of plant material and culture establishment

Thirty young capitulum (flower buds) derived from the plants of *Gerbera jamesonii* cv. 'Harley' growing in the glasshouse of Department of Biotechnology, University of Horticulture and Forestry, Solan (Himachal Pradesh), India were used as explants for shoot establishment. Disinfection of explants was carried out with fungicide SAFAL (Indofil Chemical Company, Mumbai, India), 0.2% (w/v) solution for 15 min and washing under tap water. Thereafter, surface sterilization was done for 2.5 min with 0.1% (w/v) HgCl_2 , followed by washing

thoroughly with autoclaved distilled water and blotting on sterilised filter paper. Sterilised explants were cultured on MS medium (Murashige and Skoog 1962) supplemented with 22.20 μM 6-benzyladenine (BA), 2.53 μM indole acetic acid (IAA) and 3% (w/v) sucrose. The cultures without growth regulators served as control. The pH of the medium was set to 5.8 with 1 N NaOH and/or 1 N HCl, and 0.8% (w/v) agar was added before autoclaving at 121 °C at a pressure of 1.1 kg cm^{-2} for 15 min. Cultures were incubated under cool fluorescent light at an intensity of 50–60 $\mu\text{mol cm}^{-2} \text{s}^{-1}$ with a light/dark cycle of 16/8 h at 24 ± 2 °C. The percent uncontaminated explants and explants producing calli were recorded. After 6 weeks, the shoots regenerated from a single callus lines were sub-cultured on shoot multiplication medium consisting of MS salts supplemented with 4.44 μM BA and 2.53 μM IAA.

Gamma irradiation of in vitro raised shoots

120 well-developed in vitro shoots (3–5 cm long) regenerated from callus were selected for irradiation with different doses of γ -irradiation (0, 1.5, 2, 2.5, 5, 10, 15, 20, 30 Gy/h, source Cobalt⁶⁰). Each treatment consisted of five replicates (culture flasks) containing four shoots per culture flask (total 20 shoots). Untreated shoots served as control. After irradiation, the shoots were transferred to shoot multiplication medium consisting of MS salts supplemented with 4.44 μM BA and 2.53 μM IAA and incubated at 24 ± 2 °C under 16/8 h (light/dark) photoperiod with light intensity of 50–60 $\mu\text{mol cm}^{-2} \text{s}^{-1}$. Those shoot cultures that survived after 4 weeks of irradiation were transferred to MS medium supplemented with 15.60 μM indole butyric acid (IBA) for root initiation. Shoots with well developed root system were shifted to pots containing sterile mixture of FYM: soil:sand in 1:1:1 (w/v) ratio, and placed in glasshouse with 80–90% relative humidity (RH) for hardening and acclimatisation.

Enzyme extraction

Fine powder was made from frozen leaf samples (0.3 g) in liquid nitrogen with a pre-chilled pestle and mortar. The extraction buffer (3 ml) used for sample homogenization consisted of 1 mM ethylenediaminetetra acetic acid (EDTA) and 1 mM ascorbate dissolved in 50 mM potassium phosphate buffer (pH 7.8). The slurry was transferred to a tube and centrifuged at 20,000 rpm for 20 min at 4 °C. One part of supernatant was used directly for APX and CAT assay, and the other part (1 ml) was transferred to a dialysis membrane, dialysed at 4 °C and used for SOD estimation.

Enzyme estimation

APX and GR activity was measured spectrophotometrically following the method of Tanaka et al. (1988), CAT and SOD was measured following the method of Wayne et al. (1987). The enzyme activities were expressed as enzyme units per mg protein.

Bioassay

Fungal inoculum was prepared following the method of Kitao and Doazan (1990). Fungal colonies (3 weeks old) from the infected plant were washed in sterile distilled water containing Tween 20 in order to remove conidia from conidiophores. The concentration of the conidia was adjusted to the level of 3×10^4 conidia/ml. 100 ml of the conidial suspension was sprayed gently on the plants (control and irradiated) maintained in sterile enclosure in the glasshouse at 27 ± 2 °C with 80% relative humidity. Observations on the development of colonies were taken daily. The scale of 0–4 was used for defining the severity of disease. The disease severity was assessed on the basis of leaf area covered under fungal colonies. Plants on which no infection occurred were designated '0' (highly resistant), with less than 10% leaf area under colonies were designated '1' (resistant), with 30–40% leaf area covered were designated '2' (medium resistance), with 50% leaf area covered under colonies were designated '3' (susceptible), and those with more than 50% leaf area covered with fungal colonies were designated '4' (highly susceptible).

Molecular analysis

Genomic DNA was isolated from young leaves of 10 randomly selected irradiated plants following the method of Virscek Marn et al. (1999), with some modifications. Ten RAPD primers (Operon Technologies, Inc., USA) were used for initial screening. The amplification reaction volume was 25 µl, consisting of 1U of Taq DNA polymerase, Taq DNA polymerase buffer (1 X) containing 1.5 mM MgCl₂, 10 pmol of decamer primer, 2.5 mM MgCl₂, 2.5 mM of deoxynucleotide triphosphate (dNTP) and 30–40 ng of template DNA. Reaction mixtures were exposed to the following conditions: preliminary denaturation of DNA at 94 °C for 4 min followed by denaturation at 94 °C for 30 s (40 cycles). Primer annealing at temperature specific to primer for 1 min and amplification at 72 °C for 2 min. After 40 cycles, there was a final extension step of 8 min at 72 °C followed by 4 °C. 1 kb DNA ladder (Fermentas, Lithuania) was used as the size marker. The amplification products were analysed on 1.4% agarose gel prepared in 1 X TAE buffer and visualised under UV light in gel documentation system (Alpha Imager, USA).

Statistical analysis

Each experiment was repeated thrice and data were analysed statistically using completely randomized design. Statistical analysis based on mean values per treatment was made using ANOVA. LSD ($P \leq 0.05$) was used to determine difference between doses of gamma rays.

Results

Out of 30 explants used, callus initiation started after 1 week from cut ends of twenty (66.7%) capitulum explants on MS + 22.20 µM BA + 2.53 µM IAA medium and complete callus formation took place within 4 weeks of culturing. After another 6 weeks, shoots regeneration took place on seventeen (85%) out of these twenty explants that produced calli. Shoots so obtained were multiplied on MS medium containing 4.44 µM BA and 2.53 µM IAA with an average number of 9.8 shoots per explants until 3–5 cm long shoots were obtained. Shoots irradiated with 20 and 30 Gy could not survive in cultures (Fig. 1). The mutated shoots after two cycles of multiplication were transferred to the rooting medium consisting of MS salts supplemented with 15.60 µM IBA. It was observed that root formation occurred on 82.9% plants treated with 1.5 Gy gamma rays in average 34.1 days whereas only 18.6% plants irradiated with 5 Gy dose showed rooting in 42.9 days (Table 1). However 100% rooting took place in the control shoots in 32 days of transferring to root induction medium.

The data recorded on survival of plants upon hardening showed similar trend. A significant decrease in the percent survival of plants with the increase in irradiation dose was observed after 8 weeks. Only 70.9% plants treated with 1.5 Gy and 43.1% plants treated with 5 Gy dose could survive during hardening (Table 1). Enzyme activity studies showed increase in mean activity of SOD, APX, CAT and GR, respectively in gamma irradiated plants (Table 2).

Further, thirty irradiated plants (6 months old) in three replicates (total 90 plants) were screened for resistance/tolerance to powdery mildew. Only 3 plants irradiated with 5 Gy gamma ray doses were found to be moderately resistant or tolerant with only 35% leaf area covered under fungal colonies. The plants were healthy and produced new leaves. None of the control plant showed resistance (Table 3, Fig. 2). All the plants treated with 1, 1.5, 2 and 2.5 Gy gamma radiation were susceptible to powdery mildew. RAPD analysis produced distinct and scorable bands in the size range of 250–2000 bp (Table 4). The number of scorable bands ranged from 2 to 6 depending upon the primer. A total of 46 scorable bands

Fig. 1 Effect of gamma radiations on percent survival of in vitro shoots after 2 weeks of treatment. Figures within parenthesis represent arc sine transformed values. Vertical bars represented LSD ($P \geq 0.05$)

Table 1 Effect of gamma radiation on rooting and survival percentage of plantlets

Treatment (Gy)	Number of days required for root induction	Percent rooting	Survival of plantlets (%)
0 (control)	32.0	100.0 (90.00)	85.01 (67.22)
1.5	34.1	82.9 (65.58)	70.90 (57.35)
2	35.8	75.6 (60.37)	61.09 (51.41)
2.5	40.8	45.9 (42.64)	57.90 (49.54)
5	42.9	18.6 (25.53)	43.11 (41.04)
10	0	0	0
15	0	0	0
20	0	0	0
30	0	0	0
LSD _{0.05}	4.40	10.16	3.64

Data were recorded after 30 days of transfer to pots. Figures within parentheses are arc sine transformed values

Table 2 Effect of gamma rays on the activities of antioxidant enzymes

Treatment (Gy)	Enzyme activity (units per mg protein)			
	SOD	APX	GR	CAT
0 (control)	29.29	2.67	33.56	17.80
1.5	33.22	3.34	40.32	22.03
2	34.30	3.50	43.08	22.77
2.5	39.93	3.83	46.22	25.14
5	42.67	4.33	47.90	27.34
Mean	37.53	3.75	44.40	24.32
LSD _{0.05}	1.84	0.53	3.47	2.06

Data were recorded after 2 weeks of exposure to gamma irradiation

were obtained with average number of 4.6 bands per primer. All the banding profiles of irradiated plants were polymorphic showing 100% polymorphism when

Table 3 Identification of disease resistant/tolerant plants in glasshouse

Dose Gy	Disease severity					Number of plants tested
	0	1	2	3	4	
Irradiated	0	0	3	12	75	90
Control	0	0	0	0	100	100

Scale: '0' = no infection occurred, '1' = less than 10% leaf area covered under fungal colonies, '2' = 30–40% leaf area covered under fungal colonies, '3' = 50% leaf area covered under fungal colonies, '4' = more than 50% leaf area covered under fungal colonies

compared with control and parent plants (Fig. 3). In RAPD analysis, three primers (OPB-6, OPB-12 and OPC-01) showed distinct new profiles. To confirm that these

Fig. 2 After 4 weeks of inoculation **a** 6 months old plant showing development of few fungal colonies in mutated plant. **b** Control showing well developed fungal colonies on the entire plant. Arrows indicate developed fungal colonies

Table 4 Nucleotide sequence and RAPD amplification results of the primers used in the PCR amplification

Primer	Sequence	Total bands	Monomorphic bands	Polymorphic bands	Fragment size (kb)
OPA-05	TGCGCCCTTC	5	0	5	2000–500
OPA-06	CAAACGTCGG	4	0	4	1500–600
OPA-20	GTTGCGATCC	5	0	5	1500–500
OPB-06	GGTCCCTGAC	4	0	4	2000–500
OPB-11	GTAGACCCGT	4	0	4	1500–250
OPB-12	CCTTGACGCA	5	0	5	1200–250
OPB-19	ACCCCCGAAG	2	0	2	1500–500
OPB-20	GGACCCCTAC	6	0	6	2000–250
OPC-01	TTCGAGCCAG	6	0	6	1200–250
OPC-05	GATGACCGCC	5	0	5	1100–600
Total		46	0	46

Fig. 3 RAPD amplification profile of gerbera revealed by primer OPB-06. PCR was carried out with genomic DNA isolated from the leaves of control and mutated plants. 1: mother plant, 2: control plants, 3–12: mutated plants, 13: 1 kb DNA marker. Arrow indicates distinct band

molecular markers were linked to resistance needs further molecular analysis.

Discussion

Successful establishment of in vitro shoot cultures is the first and critical step towards any mutagenesis experiment. Many factors like choice of explant, type, combination and concentration of growth regulators play a determining role in shoot regeneration. Many researchers have reported successful shoot regeneration from capitulum explant (Hussein et al. 2008; Bhatia et al. 2011). Similar to the

results obtained in this study, Warar et al. (2008) and Bhargava et al. (2013) have also reported efficiency of BA and IAA in in vitro shoot induction from capitulum explant. The dose of radiation at which 50% survival or mortality is obtained is called lethal dose LD₅₀. In this study, the calculated LD₅₀ was found to be 6.5 Gy. Therefore, the doses were selected carefully, keeping LD₅₀ in the middle of mutation spectrum. Following irradiation, the shoots were multiplied and after two cycles of multiplication, these shoots were transferred to rooting medium. The decline in the survival after irradiation and in rooting percentage may be due to the fact that the in vitro shoots were exposed to mutation stress and needed time to recover from it. Similar results were reported by many workers in different crops (Barakat et al. 2010; Kaul et al. 2011). Lu et al. (2007) reported high mortality of Chinese narcissus exposed to gamma rays. Inverse relationship between radiation dose and overall plant survival and well being has been reported earlier by Ahmad et al. (2006) and Dehgahi and Joniyasa (2017) in gamma irradiated cultures of *Oncidium lancearum* and *Dendrobium* cv 'sonia-28', respectively. Abou Dahab et al. (2017) also reported the highest survival of in vitro gamma irradiated plants of *Eustoma grandiflorum* at lowest dose of radiations (10 KR). Irradiation has a pronounced effect on physiological and biochemical processes in plant system. Under natural conditions, plants are inevitably exposed to different types of stresses, which lead to the production of reactive oxygen species (ROS), and so the plants have naturally evolved a number of mechanisms to eliminate or reduce ROS. Antioxidant enzymes play an important role in conferring the innate stress tolerance and improving ability of the plants to withstand the stressful conditions.

Chakrabarty and Datta (2008) and Varshney and Anis (2011) reported enhanced activities of antioxidant enzymes during early acclimatization of plants. Enhancement of antioxidant enzyme activity under stress conditions has been studied extensively. Increased activity of antioxidant enzymes under cold stress in Caper and Citrus fruit was documented by Manish et al. (2011) and Mohammadian et al. (2012), respectively. Tarroum et al. (2011) investigated the response of *Hordeum vulgare* to drought stress in terms of the antioxidant enzyme activity and reported an increase in the activity of these enzymes. Helaly and Hanan El-Hosieny (2011) also reported increase in antioxidant enzyme activity in gamma irradiated plants of *Citris limon*. Enhancement of antioxidant enzyme activity in plants lead to build up of resistance in plants against attack of disease causing organisms. Development of red rot (*Colletotrichum falcatum*) resistant sugarcane (*Saccharum officinarum*) by induced mutation was reported by Ali et al. (2007). Similarly, chrysanthemum plants resistant to

Septoria obesa were obtained by Kumar et al. (2012) after gamma irradiation.

There are many studies which support the fact that gamma radiations interact with the target plants at genetic level. RAPD markers have been used by many workers to ascertain the effect of gamma irradiation at genetic level. In this study, 100 percent polymorphism was observed. Earlier, Kaul et al. (2011) also reported 100% polymorphism in chrysanthemum cv. 'Snow Ball' after gamma irradiation. Studies conducted by Badr et al. (2006) and Kumar et al. (2012) indicated that RAPD markers can be used effectively for genetic diversity studies among radio-mutants. Similarly, occurrence of polymorphism was reported by Minisi et al. (2013) in gamma irradiated seeds of *Molucella laevis*. In another study conducted by Sianipar and Maarisit (2015), it was reported that RAPD markers are efficient in determining genetic variation arising due to irradiation in *Typhonium flagelliforme* Lodd.

Conclusions

This study presents a reliable protocol for in vitro mutation induction in *G. jamesonii*. The studies on antioxidant enzyme activity in mutated plants clearly demonstrate the role played by these enzymes in conferring the resistance to plants under stress conditions. Further, three plants were identified that were found to be moderately resistant to the powdery mildew causing fungus. Molecular analysis of plants revealed that the gamma radiations interact with plants at genetic level. However, whether the unique bands obtained in this study are associated with disease resistance need further studies.

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