

***In Vitro* Mutation Induction and Selection of Chrysanthemum (*Dendranthema Grandiflora* Tzelev) Lines with Improved Resistance to *Septoria Obesa* Syd**

B. Kumar, S. Kumar*, M. Thakur

Department of Biotechnology, University of Horticulture & Forestry, Solan, Himachal Pradesh, 173230, India

Abstract This paper describes *in vitro* mutation selection technique for improved resistance in chrysanthemum (*Dendranthema grandiflora* Tzelev) cv. Snow Ball against culture filtrate of *Septoria obesa*, a leaf spot pathogen. The callus was initiated from leaf explant on MS medium supplemented with 10 mg/l kinetin and 1 mg/l NAA. Optimal doses of gamma radiation and culture filtrate on the per cent survival of calli were standardized. The optimal dose of gamma radiation was 20 Gy and that of culture filtrate was 15%. The selection of the resistant calli was made at 15% culture filtrate concentration and the calli were subjected to two more cycles of selection (30 day cycle) to obtain resistant cell lines. 100% of the plants raised from cuttings had acquired resistance against the pathogen in the pot in greenhouse. The plants were maintained for four years without any symptoms with regular inoculation of fungal spore suspension. No phenotypic variation was observed in the resistant plants.

Keywords Culture filtrate, Growth regulators, *In vivo* testing, Leaf spot pathogen, Resistance

1. Introduction

Chrysanthemum is one of the most important commercial cut flowers of the world and is the second largest in demand after rose, among the ornamental plants traded in the global flower market[1]. Economic returns from cut flowers mainly depend upon the quality of the flowers[2]. Garden chrysanthemums are the number one herbaceous perennial in United States with whole sale farmgate value of \$141.8 million in 2005[3]. Diseases caused by fungi, bacteria, mycoplasma, and viruses are responsible for poor quality of flowers and reduction in yield[4].

Septoria obesa Syd is one of the most important pathogens of chrysanthemum. This pathogen causes leaf spots, resulting in 15-20% yield loss. The control of leaf spot pathogen by fungicides is difficult because fungicides are often ineffective as the pathogen spreads rapidly under favourable conditions. On the other hand, major destructive fungi are developing resistance to most classes of fungicides and environmental pollution caused by these chemicals is a serious problem[5,6]. Genetic resistance in *D. grandiflora* is therefore of considerable importance. Mutation techniques have been widely applied to improve crop yield, quality, disease and pest resistance[7]. About 2335 varieties were released

through mutagenesis in the world, in which ornamental crops and decorative crops are 552[8]. In chrysanthemum mutant cultivars can account for almost 40% of the Dutch flower market and about 236 officially announced commercial mutants have been obtained through X- or γ -ray mutation breeding[9]. A large number of germplasm with novel, desired traits have been produced through mutation techniques[10-13]. Combining mutation technique with *in vitro* selection, allow the selection of traits controlled by their recessive or dominant genes, can improve recovery of the desired mutant. *In vitro* mutation and selection has advantages over traditional breeding methods as these are more efficient and cost effective. In order to screen target characters in a large number of mutagenised cells, it is essential to have efficient selection agents[7]. Toxic culture filtrate and purified toxins have been used in *in vitro* selection of disease resistant plants[14-16]. It has been attempted at the plant level where a large population of plants, raised through *in vitro* callus cultures, have been screened for resistance in the field capacity or via a more targeted approach of regeneration of disease resistant plants through resistant callus culture selected against fungal toxins[17]. Kumar et al.[16] reported resistant cell lines of chrysanthemum against *Septoria obesa*. In this paper the development chrysanthemum cv. Snow Ball with an improved resistance to *Septoria obesa*, is described.

2. Materials and Methods

2.1. Plant Materials

* Corresponding author:

skuh07@yahoo.co.in (S. Kumar)

Published online at <http://journal.sapub.org/plant>

Copyright © 2012 Scientific & Academic Publishing. All Rights Reserved

Chrysanthemum (*Dendranthema grandiflora* Tzelev) cv. Snow Ball, growing in the Department of Biotechnology, University of Horticulture and Forestry, Solan, Himachal Pradesh, India was propagated from cuttings and maintained under greenhouse at $24\pm 2^\circ\text{C}$ with 80% relative humidity.. Young leaves (upper 3-4 leaves) isolated from the one-year-old plants washed thoroughly under running water, cut into small segments (0.2-0.5 cm) and were used as explants. The explants were surface sterilized with 5% sodium hypochlorite and washed 3-4 times with sterile water prior to culturing. The callus cultures were initiated by culturing the leaf explants on MS[18] medium containing 3% sucrose (w/v), 10 mg/l kinetin and 1 mg/l NAA. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was solidified with 0.8% (w/v) agar and autoclaved at 121°C for 15 min. Erlenmeyer conical flasks (100 ml) were cast with 30 ml media and sealed with cotton plugs made from non-absorbent cotton. The cultures were incubated under cool fluorescent lamps at a light intensity of $50\text{-}60\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ with a light/dark cycle of 16/8 h at $24\pm 2^\circ\text{C}$. All the cultures were transferred to the fresh medium at 30 days interval.

2.2. Determination of Optimal Dose of Gamma Ray

To determine the suitable dose of gamma ray calli (50 mg fresh weight) were cultured on MS medium and exposed to gamma irradiation of 0, 10, 20 and 30 Gy (cobalt 60). About 50 calli pieces were tested for each dose. The surviving calli were subcultured onto fresh MS medium supplemented with growth regulators used for callus induction and growth. (After calli irradiation, did you allow to grow on the same medium or transferred to the fresh medium for further growth). The calli were observed for their growth after 30 days recovery period, after which surviving calli were counted, weighed and LD50 (half lethal dose) of gamma rays was estimated. (What is the parameter you used for the determination of LD50 dose)

IAEA, Vienna has been carrying out work on induced mutations. You have not quoted.

2.3. Maintenance and Multiplication of the Pathogen Cultures

The pure cultures of *S. obesa* were obtained from the department of Mycology and Plant Pathology, University of Horticulture and Forestry, Solan (H.P.), India. The isolate was multiplied on PDA medium at 25°C and maintained at 4°C on the same medium. The pH of the PDA medium was adjusted to 6.0-6.5. After inoculation, the cultures were incubated at 25°C in the dark for 21 days until a uniform fluffy mycelial growth was obtained.

2.4. Preparation of Culture Filtrate

The culture filtrate was prepared by inoculating $1\ \text{mm}^2$ of the fungal mycelium in 100 ml liquid Czapek dox medium. The culture was incubated in a BOD incubator shaker at 25°C in dark. After 21 days the fungal cultures were fil-

tered through Whatman filter paper no. 1 and the filtrate centrifuged at 20,000 rpm for 30 min. The supernatant was then filtered through sintered glass filter (G-5 grade, $0.25\ \mu\text{m}$ pore size) to produce transparent culture filtrate.

2.5. Determination of Optimal Concentration of Culture Filtrate for Screening

To determine the effects of culture filtrate on survival and growth of calli, one hundred pieces of mutated calli (20 mg fresh weight) per culture filtrate treatment, in ten flasks (100 ml), each having 10 pieces, were inoculated on MS medium containing 0%, 2.5%, 5.0%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% (v/v) culture filtrate and the growth regulators used for callus induction and the medium was solidified with 0.8% agar. In control cultures an equal volume of Czapek dox medium replaced with culture filtrate. The cultures were maintained at light intensity of $50\text{-}60\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ under light/dark cycle of 16/8 h at $24\pm 2^\circ\text{C}$ for four weeks. The calli obtained from the selection medium containing 15% culture filtrate, the highest concentration of culture filtrate at which only few calli survived, were subcultured on the same medium for two more cycles (30 day cycle) to select resistant calli. The surviving calli were cultured on MS medium supplemented with 10 mg/l kinetin and 1 mg/l NAA for multiplication. The pH of the medium was adjusted to 5.8.

2.6. Plant Regeneration from Calli

After determining the optimal concentration of culture filtrate, the surviving calli were induced to regenerate plantlets. For shoot regeneration, calli were transferred onto MS medium supplemented with 0.2 mg/l BA, 0.2 mg/l NAA and 1 mg/l GA₃ without culture filtrate. After shoot formation, they were separated from the callus mass and cultured on MS medium containing 0.3 mg/l IBA and 0.2% activated charcoal for rooting. The plantlets were maintained at $24\pm 2^\circ\text{C}$ under 16/8 h (light/dark) photoperiod. The plants with well developed root systems were transferred to pots containing soil:sand (1:1) mixture and grown in the greenhouse. The average number of shoots and roots produced were recorded to examine the effect of culture filtrate concentration on plant regeneration.

2.7. Leaf Spot Resistance of Selected Regenerated Plants

Hundred plants (one plant in each pot) were tested for resistance against leaf spot pathogen. The plants were subjected to infection by spraying spore suspension (40 spores/ml) of *S. obesa* and were observed daily for the development of symptoms. Disease severity was recorded according to 0-4 rating scale[16]. Two hundred cuttings (5-6 cm long) were collected from one-year-old resistant plant and treated for 24 h with 100 mg/l IBA solution for rooting. The rooted cuttings were transferred to pots containing soil:sand (1:1) and maintained in greenhouse for three months before spraying with spore suspension (40 spores/ml) of *S. obesa*. The plants were observed daily for the appearance of symptoms. The resistant plants were maintained for four years in the greenhouse and inoculated regularly by spraying

spore suspension of the pathogen and observed for the development of symptoms.

2.8. Statistical Analysis

The experiment was repeated three times with similar trends of results using completely randomized design [19]. The significance of treatment effects on various parameters was determined using analysis of variance (ANOVA).

3. Results and Discussion

To determine the optimal dose of gamma rays (optimal dose not concentration), the calli were exposed to 10, 20 and 30 Gy gamma ray doses. The percentage of surviving calli decreased with the increasing gamma ray doses (Figure 1). There was no calli recovered at gamma ray dose 30 Gy, about 22.2% of the calli survived at 20 Gy dose of gamma irradiation. Therefore subsequent physical mutation was carried out only with 20 Gy of gamma irradiation. To determine the effects of culture filtrate, survival of the calli decreased with the increase in the culture filtrate concentration, reaching a 0% survival at 17.5% culture filtrate (Figure 2). A significant negative correlation was found between culture filtrate concentration and average survival of calli. The cell selection was therefore carried out at the 15% culture filtrate level to exert maximum selection pressure that allowed the recovery of resistant lines. The selected calli were subcultured on culture medium in the absence of culture filtrate for three subcultures to get rid of physiological adaptations and again cultured at 15% culture filtrate for two more cycles (30 day cycle) before the plants were regenerated.

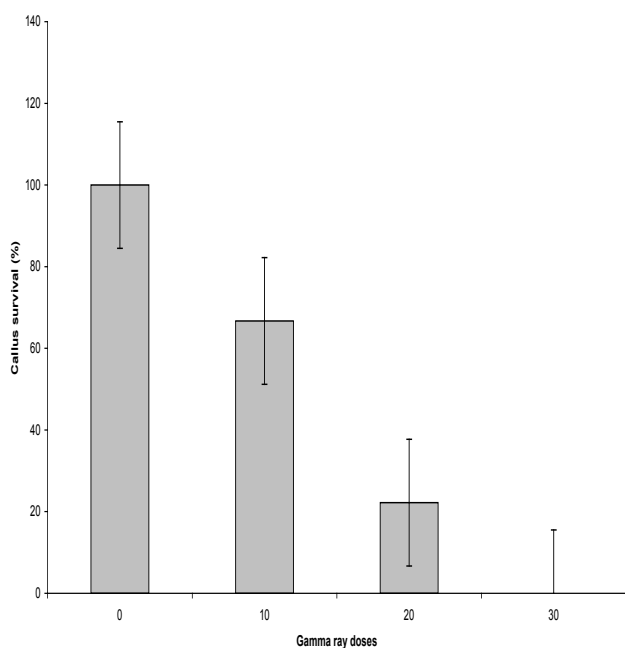


Figure 1. Effect of gamma ray doses on average survival of calli (%). Vertical bars represent LSD ($P \leq 0.05$).

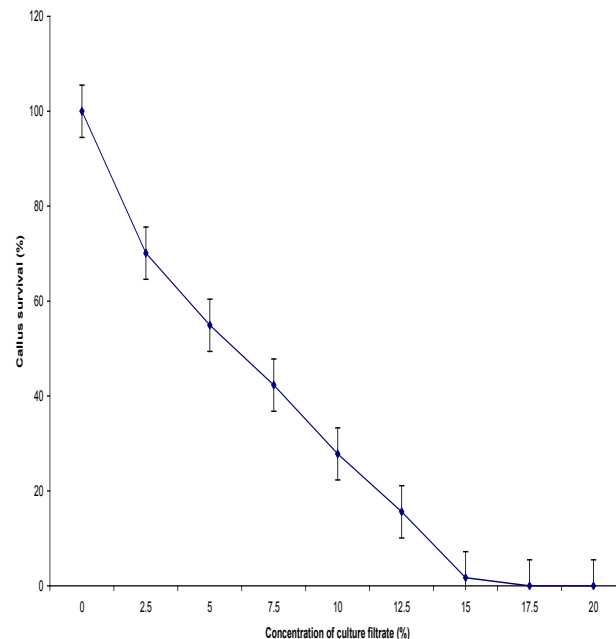


Figure 2. Effect of different concentrations of culture filtrate on percent survival of calli. Vertical bars represent LSD ($P \leq 0.05$).

The results presented in this paper describe the development of protocol for selecting resistance to leaf spot using *in vitro* mutation and selection. Earlier [16] it has been reported that about 70 to 80% of the plants raised from resistant cell lines showed resistance to *S. obesa*. Radiation mutation breeding with tissue culture technique have made a significant contribution to plant breeding. They have introduced new techniques for inducing genetic variation by improving selection technology and by accelerating breeding time. This method can produce large population of calli for *in vitro* mutation-selection, which increases the probability of obtaining mutants with the desired traits. Liu et al. [7] reported that *in vitro* mutation-selection method with calli prove especially useful with genotypes for which it is difficult to obtain large number of embryos from microspore culture.

The present study revealed the production of chrysanthemum plants with greater resistance to leaf spot disease than the lines produced by other methods [17,20,21]. The improvement in leaf spot disease resistance using this protocol over a period of four years was more than achieved earlier [16]. Symptoms were observed in the control plants (Figure 3a). 100% resistance to leaf spot pathogen was observed in all the plants raised through cuttings from resistant cell lines under greenhouse (Figure 3b). About 150 resistant plants were maintained in the greenhouse. No morphological variations were observed in the plants. Liu et al. [7] also observed improved resistance to *Sclerotinia sclerotiorum* in doubled-haploid *Brassica napus*.

The variations arising from *in vitro* mutation-selection may be the result of calli developed from a variety of diploid and polyploids [22]. The development of resistance *in vitro* in callus cultures against the phytotoxins has been correlated with the secretion of extracellular proteins by the host in culture, which may be antifungal hydrolases or pathogene-

sis-related proteins[23]. The callus tissue offers opportunities for a variety of selection, including tolerance to phytotoxins and may allow recovery of desired variants, related to recessive or dominant genetic changes.

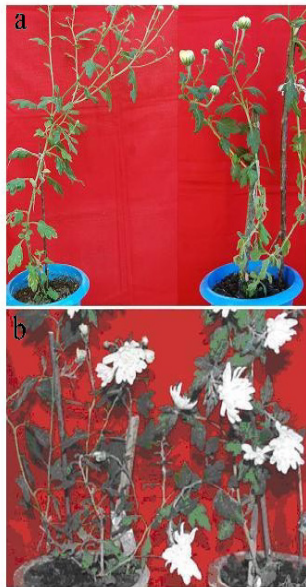


Figure 3. control (a) and resistant plants (b) of chrysanthemum in pots under glasshouse

The present investigation describes the development of a protocol for *in vitro* mutations and selection of resistant plants of chrysanthemum to culture filtrate of *Septoria obesa*, a leaf spot pathogen.

The genetic stability of selected resistant plants is not shown in the subsequent breeding lines.

REFERENCES

- [1] Kumar, S., Prasad, K.V. and Choudhary, M. L. 2006. Detection of genetic variability among chrysanthemum radiomutants using RAPD markers. *Current Science*, 90: 1108-1113
- [2] Kaul, A., Kumar, S., Thakur, M. and Ghani, M. 2011. Gamma ray induced *in-vitro* mutations in flower colour in *Dendranthema grandiflora* Tzelev. *Floriculture and Ornamental Biotechnology*, 5: 71-73
- [3] Anderson, N., Ascher, P., Gesick, E., Klosner, L., Eash, N., Frita, V., Hebel, J., Popppe, S., Reith-Rozelle, J., Wagner, R., Jacobson, S., Wildung, D. and Johnson, P. 2008. Winter-hardy mammoth series garden chrysanthemum 'Red Daisy', 'White Daisy' and 'Coral Daisy' sporting a shrub plant habit. *HortScience*, 43: 648-654
- [4] Hammond, J., Hsu, H.T., Huang, Q., Jordan, R. Kamo, K. and Pooler, M. 2006. Transgenic approaches to disease resistance in ornamental crops. *Journal of Crop Improvement*, 17: 155-220
- [5] Plimmer, J.R. 1984. *Chemical for agriculture*. IAEA Bulletin, 26: 13-16
- [6] Mohan Babu, R., Sajeena, A., Seetharaman, K., Reddy, M.S. 2003. Advances in genetically engineered (transgenic) plants in pest management – an overview. *Crop Protection*, 22: 1071-1086
- [7] Liu, S., Wang, H., Zhang, J., Fitt, B.D.L., Xu, Z., Evans, N., Liu, Y., Yang, W. and Guo, X. 2005. *In-vitro* mutation and selection of doubled-haploid *Brassica napus* lines with improved resistance to *Sclerotinia sclerotiorum*. *Plant Cell Reports*, 24: 133-144.
- [8] Mba, C., Afza, R., Lagoda, P.J.L. and Dargiw, J. 2005. Strategies of the joint FAO/IAEA programme for the use of induced mutations for achieving sustainable crop production in member states. *Proceedings of the second international seminar on production, commercialization and industrialization of plantin, Manizales, Colombo, August 28- September, 2*, 281-291
- [9] Teixeira da Silva, J.A. 2004. Ornamental chrysanthemum: improvement by biotechnology. *Plant Cell, Tissue and Organ Culture*, 79: 1-8
- [10] Chatterjee, J., Mandal, A.K.A., Ranade, S.A., Teixeira da Silva, J.A. and Datta, S.K. 2006. Molecular systematics in *Chrysanthemum x grandiflorum* (Ramat) Kitamura. *Scientia Horticulturae*, 110: 373-378
- [11] Jain, S.M. and Spencer, M.M. 2006. Biotechnology and mutagenesis in improving ornamental plants. In: ed. J.A. Teixeira da Silva, *Floriculture, ornamental and plant biotechnology: advances and topical issues*. Global Science Books, Isleworth, UK. pp. 589-600
- [12] Jain, S.M. 2010. Mutagenesis in crop improvement under the climate change. *Rumanian Biotechnological Letters*, 15: 88-106
- [13] Micke, A., Donini, B. and Maluszynski, N. 1990. Induced mutations for crop improvement. In: *Mutation Breeding Review (FAO/IAEA)*, no. 7, Vienna (Austria), IAEA, pp. 41
- [14] Thakur, M., Sharma, D.R. and Sharma, S.K. 2002. *In-vitro* selection and regeneration of carnation (*Dianthus caryophyllus* L.) plants resistant to culture filtrate of *Fusarium oxysporum* f. sp. *dianthi*. *Plant Cell Reports*, 29: 825-828.
- [15] Svábová, L. and Lebeda, A. 2005. *In-vitro* selection of improved plant resistance to toxin-producing pathogens. *Journal of Phytopathology*, 153: 52-54
- [16] Kumar, S., Sunil Kumar, Negi, S.P. and Kanwar, J.K. 2008. *In-vitro* selection and regeneration of chrysanthemum (*Dendranthema grandiflorum* Tzelev) plants resistant to culture filtrate of *Septoria obesa* Syd. *In Vitro Cellular and Developmental Biology-Plant*, 44: 474-479
- [17] Saxena, G., Verma, P.C., Rahman, L., Banerjee, S., Shukla, R.S. and Kumar, S. 2008. Selection of leaf blight-resistant *Pelargonium graveolens* plants regenerated from callus resistant to culture filtrate of *Alternaria alternata*. *Crop Protection*, 27: 558-565
- [18] Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum*, 15: 473-479
- [19] Gomez, K.A. and Gomez, A.A. 1984. *Statistical procedures for agricultural research*. John Wiley, New York
- [20] Nasir, A., Ahmed, I. and Seikh, R. 2008. *In-vitro* selection for *Fusarium* wilt resistance in gladiolus. *Journal of Integrated*

Plant Biology, 50: 601-612

- [21] Löffler, H.J.M. and Mouris, J.R. 1992. Fusaric acid: phytotoxicity and in-vitro production by *Fusarium oxysporum* f. sp. *lilli*, the casual agent of basal rot in lilies. Netherlands Journal of Plant Pathology, 98: 107-115
- [22] Hansen, D.L., Lambertini, C., Jampeetong, A. and Brix, H. 2007. Clone-specific differences in *Phragmites australis* effects of ploidy level and geographic origin. Aquatic Botany, 86: 269-279
- [23] Jayasankar, S. and Litz, R.E. 1998. Characterization of embryogenic mango cultures selected for resistance to *Colletotrichum gloeosporioides* culture filtrate and phytotoxin. Theoretical and Applied Genetics, 96: 823-831