Enhancement of regeneration potential and variability by γ-irradiation in cultured cells of *Scilla indica*

B. CHAKRAVARTY* and S. SEN

Centre of Advanced Study, Department of Botany, University of Calcutta, Calcutta - 700019, India

Abstract

Induced mutagenesis in callus tissues was studied in the medicinal plant *Scilla indica* irradiated with different doses of γ-radiation ranging from 2.5 to 20 Gy. Low doses accelerated the cell division and growth rate of the tissues whereas high doses repressed growth rate and resulted in lethality of tissues. Various cytological and chromosomal abnormalities were observed in the irradiated calli, the degree of which depended upon the dosage. Low doses of irradiation also promoted the regenerating capacity of the calli tissues and plants regenerating from them exhibited better growth and vigour compared to normal plants. High doses led to loss of regenerating capacity and promoted formation of malformed and stunted plants. Cytological study of regenerants revealed both diploid and mixoploid plants but no tetraploids were obtained.

Additional key words: callus, dosage, diploids, mixoploids.

Introduction

Mutagenesis induced *in vitro* by γ-radiation has special advantages as nuclear and cytoplasmic alterations in mutated cells, can be easily analysed and suitable media enable recovery of the entire plants (Moustafa *et al.* 1989, Ibrahim *et al.* 1998). The study of irradiated cells *in vitro* can also provide information regarding cell growth and differentiation, chromosome variations as well as physiological and biochemical changes (Eapen 1976, Chagvardieff *et al.* 1989). The effects of irradiation may be stimulating or inhibiting (Liu 1982, Zhang *et al.* 1994). Morphological variants can also be obtained by regeneration from irradiated callus (Bouharmont 1994, Kuksova *et al.* 1997).

The objective of this study was to irradiate established callus cultures of *Scilla indica*, a medicinal plant, with different dosages of γ-radiation to induce variations *in vitro* since genetic variations in normal callus tissues are rare in this species (Chakravarty and Sen 1987, 1989). The mitotic behaviour of chromosomes *in vitro* following irradiation was also studied in successive subcultures to ascertain the extent of damage and recovery of the irradiated cells.

Material and methods

Bulbs of *Scilla indica* (Roxb.) Baker obtained from western coastal belts of India were replanted in experimental plots of the University garden. Leaf-tip explants (5 - 6 mm length) excised from sprouting bulbs were sterilized in 0.1 % mercuric chloride for 15 min followed by rinsing 3 or 4 times in sterile distilled water. The explants were inoculated into glass culture tubes (18 × 2.5 cm) containing Murashige and Skoog's (1962) basal medium (MS) supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 15 % (v/v) coconut milk. Cultures were incubated under fluorescent tubes (irradiance 32 μmol m^{-2} s^{-1} for 16-h photoperiod) at a temperature of 25 ± 1 °C and 55 % relative humidity. Subculturing onto the fresh medium was carried out after every 30 d.

Received 20 March 2000, *accepted* 1 November 2000.
Acknowledgements: The first author is grateful to CSIR, New Delhi for financial assistance.
*Present address: 1510, Sector-29, Noida - 201303, India, phone: (+99) 118 4450219, e-mail: bipasha123@rediffmail.com

Two months old calli were cut into equal pieces of approximately 200 mg each and inoculated into fresh medium in culture tubes. The tubes were exposed to varying dosages of γ-radiation (2.5, 5, 10 and 20 Gy; 1 Gy = 1 J kg^{-1}) from ^{60}Co (supplied by *BARC*, Bombay, India) for 6.4 s at 28 °C. To avoid the effects of irradiated medium, the calli were immediately transferred onto fresh medium. For each dosage, twenty culture tubes were used and the experiment was repeated twice. Ten culture tubes with untreated callus were kept as controls.

The callus pieces were harvested 10 d after transfer to the fresh medium after each subculture (when growth rate was at maximum) and fixed in chilled Carnoy's fluid (ethanol:chloroform:acetic acid 6:3:1) for overnight at 10 - 12 °C. The callus pieces were hydrolyzed and stained in 2 % aceto-orcein:HCl (9:1), squashed and observed under the microscope following the method of Sharma and Sharma (1980). Similar method of staining and squashing of root tips was followed for observation of cytology of regenerated plants. The percentage of dividing cells (mitotic index) and percentage of mitotic abnormalities were noted in the fixed and stained cells. The mean and standard deviation values were scored from 1000 cells in each case.

For regeneration, callus pieces were transferred to MS medium containing 2 mg dm^{-3} α-napthalene acetic acid (NAA) and 15 % (v/v) coconut milk. Regenerated shoots were transferred to half-strength MS medium for rooting and further growth. Completely regenerated plants were transferred to the soil in pots and grown in greenhouse. The percentage of shoots and roots regenerating from each piece of callus (500 mg mass) and the minimum days required for regeneration were noted for each dose of radiation. Means and standard deviations were calculated from 20 replicates.

Results

The growth of callus was strongly influenced by the radiation dose: more vigorous growth as compared to control occurred in two following weeks after 2.5 Gy dose. After 5 and 10 Gy doses, calli growth were similar to control whereas 15 and 20 Gy doses reduced the growth of callus compared to control. After 10 weeks, the growth rate of the low dose irradiated cultures were all similar to control whereas high dose irradiated cultures did not show further growth. Calli were pale-yellow and friable after lower doses (2.5, 5 and 10 Gy). On the other hand, high doses (15 and 20 Gy) induced hard and compact calli with browning and necrosis.

Table 1. Cytological study of callus cells of *Scilla indica* in different days of culture after irradiation with various doses of γ-radiation. Means ± SE, $n = 20$. M.I. - mitotic index, M.A. - mitotic abnormalities [%].

Dose	20 d		40 d		70 d		100 d		130 d	
[Gy]	M.I.	M.A.	M.I.	M.A.	M.I.	M.A.	M.I.	M.I.	M.I.	M.A.
2.5	1.82 ± 0.3	15.1 ± 1.9	4.1 ± 0.7	23.4 ± 1.2	3.0 ± 0.8	40.4 ± 2.6	1.25 ± 0.06	51.3 ± 3.8	0.87 ± 0.05	60.3 ± 4.7
5.0	0.97 ± 0.6	22.2 ± 1.5	2.8 ± 0.7	43.5 ± 2.3	2.1 ± 0.9	45.2 ± 3.7	1.16 ± 0.08	52.2 ± 5.1	0.97 ± 0.06	68.2 ± 5.5
10.0	1.23 ± 0.8	38.6 ± 2.0	2.6 ± 0.4	61.5 ± 2.7	1.3 ± 0.4	60.9 ± 5.1	0.97 ± 0.05	63.6 ± 6.2	0.55 ± 0.03	72.4 ± 3.9
15.0	1.14 ± 0.7	47.3 ± 1.7	1.3 ± 0.5	65.4 ± 3.1	1.2 ± 0.7	63.1 ± 3.3	0.83 ± 0.07	64.7 ± 5.6	0.48 ± 0.03	73.6 ± 5.8
20.0	0.56 ± 0.1	86.4 ± 2.3	0.6 ± 0.3	87.2 ± 3.4	0.4 ± 0.03	90.4 ± 4.7	–	–	–	–

Table 2. Occurrence of different types of mitotic abnormalities [%] in callus cells of *Scilla indica* irradiated with different doses of γ-radiation after 40 d of culture. Means ± SE, $n = 1\ 000$.

Dose [Gy]	Stickiness	Spindle disturbances	Breaks	Clumping	Micronuclei
2.5	13.2 ± 2.1	11.4 ± 1.5	–	–	1.5 ± 0.8
5.0	5.6 ± 1.3	14.3 ± 2.0	10.8 ± 1.9	11.3 ± 1.6	2.7 ± 1.8
10.0	–	19.6 ± 1.9	12.2 ± 2.6	23.6 ± 1.7	3.3 ± 0.6
15.0	–	8.9 ± 1.1	22.5 ± 2.7	33.7 ± 2.2	3.8 ± 1.1
20.0	–	1.2 ± 0.9	37.7 ± 3.2	40.2 ± 2.4	5.1 ± 1.3

The mitotic index of callus cells in all doses was low for 10 first days after irradiation (Table 1). After the first subculture (30 d) an increase in the mitotic indices to above 2 % was seen after 2.5, 5 and 10 Gy doses

(Table 1). This was followed by a fall after 70 d. Subsequent subcultures showed a gradual decline in mitotic indices to below 1 % after 100 d culture. The callus revealed different types of mitotic abnormalities, the degree of which depended upon the γ-radiation dosage applied (Table 2). In lower doses (2.5 and 5 Gy) the percentages of abnormalities were low and these were mainly due to disturbances of the mitotic spindle (Table 2). In higher doses, the abnormalities were mainly breakage and clumping of chromosomes, amitotic budding, increase in cell-size, shrinkage of cytoplasm and formation of micronuclei (Table 2). The percentage of abnormalities increased in later subcultures and cultures irradiated with high doses (20 Gy) could not be maintained further after 60 d in culture.

Table 3. Regenerating capacity of 60-d-old callus of *Scilla indica* irradiated with different doses of γ-radiation. Means ± SE, $n = 20$.

Dose [Gy]	Minimum number of days for shoot formation	Number of shoots per callus	Minimum days for root formation	Total regeneration capacity [%]
Control	60	20.7 ± 3.4	120	38.9 ± 3.6
2.5	60	20.5 ± 2.8	125	40.4 ± 5.1
5.0	70	32.3 ± 1.9	130	60.3 ± 5.4
10.0	70	15.8 ± 1.5	140	23.5 ± 2.9
15.0	80	7.7 ± 1.3	140	18.6 ± 3.7

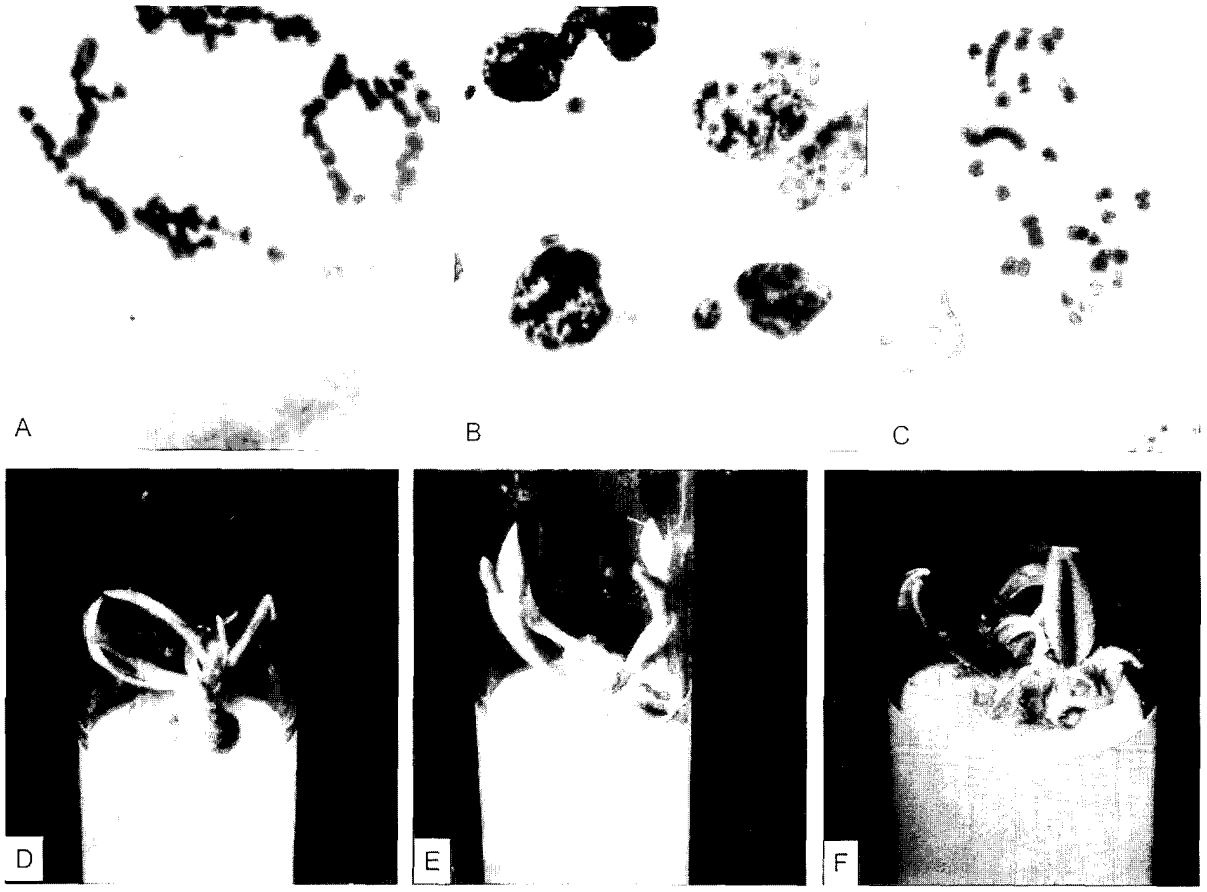

Fig. 1*A*. Cell from 15 Gy irradiated callus showing chromosomal condensation and breakage after 100 d.
Fig. 1*B*. Cell from 20 Gy irradiated callus showing micronucleus formation and bridge formation after 100 d.
Fig. 1*C*. Cell from root-tip of regenerant from 10 Gy irradiated callus showing the diploid (2n=30) chromosome number.
Fig. 1*D*. Regenerated plantlet from 15 Gy irradiated callus showing stunting and hypertrophy.
Fig. 1*E*. Regenerated plantlet from 10 Gy irradiated callus showing abnormal leaves.
Fig. 1*F*. Regenerated plantlet from 2.5 Gy irradiated callus showing normal leaves.

Calli of all doses except 20 Gy on transfer to the regeneration medium showed the development of shoot-buds. In calli irradiated with lower doses, (2.5, 5, and 10 Gy) regeneration could be noted within 60 - 70 d which was similar to the control (Table 3). Regeneration after 15 Gy dose took a longer period (80 d). Further development of leaves and shoots occurred within three weeks. After 4 - 5 weeks these shoots were transferred to half-strength MS medium for rooting.

Plants regenerating from 10 and 15 Gy irradiated calli showed morphologically abnormal shoots and roots. The plants exhibited stunted growth with thick, stout roots (Fig. 1). Rosette-like structures and hypertrophied structures with multiple shoots were common after higher doses.

The total number of plants regenerating per callus was similar to control in 2.5 Gy irradiated calli whereas this regenerating capacity was seen to be higher in calli with 5 Gy dose (Table 3). But higher doses (10 and 15 Gy) induced a progressive decrease in the regenerating capacity. Calli irradiated with 20 Gy did not show any regeneration of shoots. The regeneration potential also decreased with age in culture and young cultures showed more shoot-buds per calli than older cultures. Callus tissues after 240 d in culture could only regenerate thick, stout roots but no shoot formation was seen. Morphological variants were also common in regenerants from older cultures and cytological study of root-tips of these abnormal plants revealed mixoploidy and presence of chromosomal aberrations.

Table 4. Cytological status of regenerated plants from irradiated calli of *Scilla indica*.

Dose [Gy]	Number of plants regenerated	analyzed	Percentage of diploids	mixoploids
Control	35	20	20	–
2.5	32	20	20	–
5.0	40	20	16	4
10.0	20	10	7	3
15.0	12	8	3	5
20.0	None	–	–	–

Cytological analysis of root-tips of regenerated plants showed variation in the ploidy level. Plants regenerating from 2.5 Gy irradiated calli revealed only diploid number ($2n = 30$) (Table 4). On the other hand, plants regenerating from 5 and 10 Gy irradiated calli contained both diploid and mixoploid plants. Cytological study of the mixoploid plants from 5 Gy calli showed some cells with chromosome numbers above 2n (2.1 %) and some with 4n (7.4 %) cells along with diploid cells and the mitotic abnormalities were 20 %. Regenerants from 10 and 15 Gy irradiated calli showed a higher number of mixoploids cells (Table 4) with mitotic abnormalities being 28.6 and 34.5 %, respectively. No exclusively tetraploid plant was seen. The regenerated plants could be transferred to the soil with 50 % of survival. Most of the plants that survived retained the same morphological characters and cytological status as observed in culture.

Discussion

Low dosage of γ-radiation induced active cell proliferation as also noted in other species (Batra and Arya 1974, Liu 1982). The extent to which the stimulatory effects are results of increase in cell division or cell-expansion has not been ascertained. High doses resulted in inhibition of cell division and depression of growth rate (Moustafa *et al.* 1989). This is due to depression of DNA, RNA and protein synthesis.

Cytological analysis of callus tissues after irradiation has clearly demonstrated the disruption of mitotic spindle. This was evident by presence of chromatin bridges, laggards, multipolar spindles, unequal separation and multinucleate cells, particularly at lower doses. The direct effect on the chromatin was seen in chromosome breaks and disintegration of chromatin due to high doses. A depression in cell division was more prominent in the first few days after irradiation probably due to shock effect which was followed by a recovery.

Decrease in mitotic index and increase in mitotic abnormalities with increasing age in culture was observed in both control and irradiated callus cells. This may be due to accumulation of toxic metabolites in the system. This effect might be further enhanced by irradiation (Kar and Sen 1985, Kharkwal 1998).

Simultaneous occurrence of altered and normal cells suggests that cells may behave differently to the same dosage due to effect at different stages of nuclear cell-cycle with certain stages being more susceptible to damage by irradiation than others (Eapen 1976). In addition, primary cultures with less polyploid is more radio-sensitive than long-term cultures with more polyploids since polyploidy has a protective effect on cells (Opatrný 1974).

An optimum dose was beneficial for improving regeneration from callus. This behaviour finds parallel in low doses of γ-radiation which help in accelerating seed germination and also in improving the seed quality in different systems (Abo-Hegazi and Ragab 1986, Prasad *et al.* 1986). This study has thus shown that irradiation with γ-radiation can be used to increase regeneration

capacity and produce more vigorous plants *in vitro* in *Scilla indica*. The genetic variants produced can be utilized for generating tolerant or resistant plants for commercial uses (Spiegel-Roy and Kochba 1973). The extent to which variability in regenerants can be transmitted to the next generation is yet to be studied.

References

Abo-Hegazi, A.M.T., Ragab, A.I.: A programme for improving oil seed in Egypt through mutation breeding. - In: Prasad, A.B. (ed.): Mutagenesis, Basic and Applied. Pp. 123-128. Printhouse, Lucknow 1986.

Batra, A., Arya, H.C.: Effect of gamma irradiation on cells of *Arachis hypogaea* L. and *Dolichos lablab* L. grown in culture. - Indian J. exp. Biol. **12**: 67-71, 1974.

Bouharmont, J.: Use of mutations induced by *in vitro* culture in crop plants. - Bull. Séances Acad. roy. Sci. outre Mer **40**: 523-529, 1994.

Chakravarty, B., Sen, S.: *In vitro* regeneration from callus cultures of *Scilla indica* (Roxb) Baker. - Curr. Sci. **56**: 960-962, 1987.

Chakravarty, B., Sen, S.: Regeneration through somatic embryogenesis from anther explants of *Scilla indica* (Roxb) Baker. - Plant Cell Tissue Organ Cult. **19**: 71-75, 1989.

Chagvardieff, P., Dimon, B., Carrier, P., Triantaphylides, C.: Cell division arrest by gamma-irradiation in photoautotrophic suspension culture of *Euphorbia characias*: Maintenance of photosynthetic capacity and overaccumulation of sucrose. - Plant Cell Tissue Organ Cult. **19**: 141-149, 1989.

Eapen, S.: Effect of gamma and ultraviolet irradiation on survival and totipotency of haploid tobacco cells in culture. - Protoplasma **89**: 149-155, 1976.

Ibrahim, R., Mondelaers, W., Debergh, P.C.: Effect of X-irradiation on adventitious bud regeneration from *in vitro* leaf explants of *Rosa hybrida*. - Plant Cell Tissue Organ Cult. **54**: 37-44, 1998.

Kar, D.K., Sen, S.: Effect of gamma irradiation on callus of *Asparagus racemosus*. - Nucleus **28**: 52-55, 1985.

Kharkwal, M.C.: Induced mutations in chickpea (*Cicer arietinum* L.). I. Comparative mutagenic effectiveness and efficiency of physical and chemical mutagens. - Indian J. Genet. **58**: 129-168, 1998.

Kuksova, V.B., Piven, N.M., Gleba, Y.Y.: Somaclonal variation and *in vitro* induced mutagenesis in grapevine. - Plant Cell Tissue Organ Cult. **49**: 17-27, 1997.

Liu, M.C.: Effect of gamma irradiation on growth of callus and it's differentiated plantlets in sugarcane. - Nucl. Sci. J. **19**: 208-215, 1982.

Moustafa, R.A.K., Duncan, D.R., Widholm, J.M.: The effect of gamma irradiation and N-ethyl-N-nitrosourea on cultured maize callus growth and plant generation. - Plant Cell Tissue Organ Cult. **17**: 121-132, 1989.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Opatrný, Z.: Effect of gamma irradiation on the growth of primary and long-term tissue cultures of *Nicotiana tabacum* L. - Biol. Plant. **16**: 418-425, 1974.

Prasad, A.B., Verma, N.P., Jha, A.M.: Seed protein content and protein pattern in gamma-ray induced *Phaseolus* mutants. - In: Prasad, A.B. (ed.): Mutagenesis. Basic and Applied. Pp. 159-184. Printhouse, Lucknow 1986.

Sharma, A.K., Sharma, A.: Chromosome Techniques. Theory and Practice. - Butterworth, London 1980.

Spiegel-Roy, P., Kochba, J.: Stimulation of differentiation in orange (*Citrus sinensis*) ovular callus in relation to irradiation in the medium. - Radiat. Bot. **13**: 97-103, 1973.

Zhang, J.J., Fan, K.H., Zhang, Z.H.: Study on variation in rice (*Oryza sativa* L.) somaclones from *in vitro* irradiated somatocytes. - Acta Agr. Shanghai **10**: 6-11, 1994.