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PHYSICAL AND CHEMICAL MUTAGENESIS IN *CATHARANTHUS ROSEUS* PLANT USING SCOT MARKERS

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SUMMARY

The current study comprising molecular identification of the mutagenic effects of gamma rays and sodium azide on *Catharanthus roseus* using scot markers took place at the Department of Biology, University of Kufa, Iraq. The seeds of two cultivars, i.e., 'victory carmine' and a local variety (pink variety) of *Catharanthus roseus* L (carmine var.) received irradiation with two doses of gamma rays (50 Gray and 75 Gray). Afterward, these got soaked in sodium azide with four concentrations (0.2%, 0.4%, 0.6%, and 0.8%) to induce the genetic variation in the cultivar. The non-treated seeds served as control treatments for comparison with treated ones. Accomplishing irradiation was at an average of 18 Gy/h using Cobalt-60. The effects of gamma rays and sodium azide treatments' evaluation were through 10 start codon targeted (SCoT) DNA markers. The higher number of distinctive fingerprints was three produced by primers SCoT-26, while primer SCoT-60 provided a distinct fingerprint for only one treatment. However, primer SCoT-40 failed to recognize any treatment with a distinctive fingerprint. The highest molecular size (2488 bp) came from primer SCoT-54, whereas the lower molecular size (143 bp) resulted in primer SCoT-12. Primer SCoT-33 provided the highest value for chief and unique bands, reaching 33 and 18, respectively. The highest number of amplified bands was 77 bands in primer SCoT-54. Primer SCoT-60 produced the highest value for monomorphic bands, primer SCoT-12 gave the highest value for polymorphic bands, polymorphism, and discriminatory (15, 68.18, and 20.83, respectively), and primer SCoT-9 produced the highest value for efficiency.

Keywords: *Catharanthus roseus*, SCoT primers, gamma rays, sodium azide, genetic variation

Key findings: In the presented molecular study, using ScoT markers were capable of assessing the genetic variations generated by gamma rays and sodium azide treatments with diverse doses and concentrations, respectively.

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INTRODUCTION

Catharanthus roseus (*C. roseus*) is an important medicinal plant belonging to the family Apocynaceae, and native to Madagascar (Paarakh *et al*., 2019). It has attracted increasing attention by possessing a wide range of phytochemicals with various biological activities, such as, antioxidant, antibacterial, antifungal, antidiabetic, and anticancer (Mishra and Verma, 2017; Pham *et al*., 2020; Kumar and Srivastav, 2021). Genetic diversity, considered an influential aspect used in improving crop plants, can be defined as a

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quantitative measure of the variability of a population, which reflects the equilibrium between mutation and the loss of genetic variations (Carvalho *et al*., 2019). Enhanced genetic diversity promotes the improvement of crop plants for yield and quality parameters (Aziz *et al*., 2016; Hlozakova *et al*., 2016; Mohsin *et al*., 2023).

Genetic diversity rose due to several factors, such as, mutation, gene flow, hybridization, and polyploidy in the genetic material (Begna, 2021). Naturally, spontaneous mutation can create a lot of genetic diversity in the crops, as well as, induce mutagenesis, used in the breeding of crop plants. A mutation is an effective tool in plant breeding and is highly successful in improving crop cultivars globally to provide the population's food requirements (Agrawal and Kumar, 2021). After increased genetic diversity due to mutation, qualitative mutation expresses itself through abrupt changes in morphological, anatomical, biochemical, and quantitative features. Micro-mutations also have smaller and more gradual effects, which accumulate over time and bring about changes through several chromosomal aberrations (Bhandari *et al*., 2017).

Mutagen is a tool for creating mutagenesis, which is an efficient process of generating mutation, which can occur spontaneously and can also be induced artificially by a mutagen (Chaudhary *et al*., 2019). Physical mutagens, such as, fast neutron and gamma rays, produce a vast magnitude of genetic variability and have played a significant role in plant genetics studies; however, gamma rays emerged as the most favored physical mutagen by mutation breeders, with extensive use in crop improvement programs (El-Shaer and Ibrahim, 2021; Al-Rehbawy, 2022). Sodium azide (NaN3) is an alkylating chemical mutagen, widely regarded as relatively safe to handle very efficient chemical mutagen, and inexpensive and non-carcinogenic (Salvi *et al*., 2014; Weldemichael *et al.*, 2021).

DNA markers have been used for mutation detection, with many molecular indicators used in mutation detection, such as, RAPD (Random amplified polymorphic DNA) (Wahyudi *et al*., 2020) and SCoT (Start Codon Targeted) (Amirmoradi *et al*., 2012). These DNA markers are very simple, inexpensive, do not need knowledge of the target sequence, and are easy to work with in data analysis (Collard and Mackill, 2009; Gorji *et al*., 2011).

The combination of medicinal and aromatic plants is an essential source of plant secondary metabolites, which play a vital role in human health care. The induced mutation is also an ultimate source of the genetic structure alteration of the crop plants that may not be possible through hybridization and other breeding procedures. Mutation-assisted plant breeding can play a crucial role in the development of desirable crop varieties of medicinal and aromatic crops (Kolakar *et al*., 2018).

MATERIAL AND METHODS

Breeding material

Provision of the *Catharanthus roseus* L. seeds of two cultivars, i.e., Victory carmine, were by SAKATA Company and the Local variety (pink variety) seeds by the local market (Figure 1).

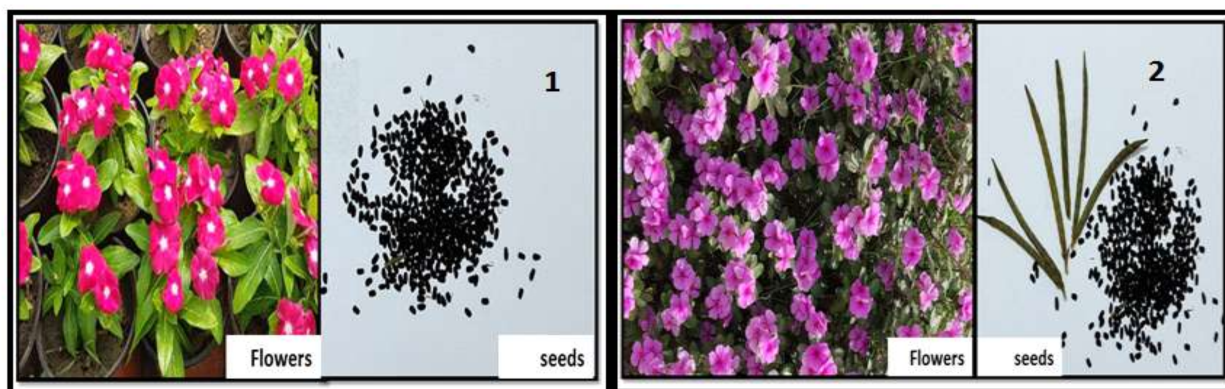

Figure 1. *Catharanthus roseus* L. 1) Victory carmine (flowers and seeds) and 2) Local variety (flowers and seeds).

Gamma irradiation treatment

Irradiation proceeded on 40 seeds of each cultivar of *C. roseus,* placed in Petri dishes for each treatment, with two different doses (50 Gy and 75 Gy). The control treatment had 0 Gy - non-irradiation. The accomplishment of an average of 18 Gy/h using Cobalt-60 as an irradiation source transpired at the Department of Physics, College of Sciences, Baghdad University, Iraq (El-Sharnouby *et al*., 2016).

Sodium azide treatment

Soaking the seeds of both cultivars of *C. roseus* in distilled water ensued for 12 h and then treated with four different concentrations of sodium azide (0.2%, 0.4%, 0.6%, and 0.8%) prepared in distilled water and kept for another 12 h. Afterward, washing the seeds in running tap water followed to completely remove the residual effects of mutagen sticking on the seed coat, with the seeds dried at room temperature (Ali *et al*., 2014).

Seed sowing

Seed sowing occurred at the Agricultural Orchid Unit, University of Kufa, Iraq, using plastic pots filled with peat moss till flowering, including the control treatments for both *C. roseus,* i.e., victory carmine and local variety, not exposed to any mutagen.

Primers

The SCoT primers came from Bioneer Corporation in lyophilized form, dissolved in TE buffer to obtain 100 pmol/µl as a final concentration (stock solutions). The prepared working solutions of 10 pmol/µl from stock solutions used 10 SCoT markers in applying SCoT with their nucleotide sequences (Vivodik *et al*., 2016) (Table 1).

DNA extraction

Fresh seedling leaves were used to take fresh apical leaves for genomic DNA extraction using Genomic DNA Mini Kit provided by Geneaid Biotech., Ltd.

PCR content and amplification program

Using PCR Pre Mix master mix, Bioneer Corporation, USA, consisted of 0.2 ml thin-wall 8-strip tubes with attached cup / 96 tubes (*Top* DNA polymerase - 1U) (dATP, dCTP, dGTP, and dTTP) (each 250 µM). Further, the Reaction Buffer used included 1.5 mM Mgcl2 (1X) and Stabilizer and tracking dye, 100 bp DNA ladder. According to the experimental protocol of AccuPower® TLA PCR PreMix, the PCR reaction mixture preparation consisted of 5 µl template DNA and 5 µl of primer (10 pmol/µl) added to each AccuPower® TLA PCR Pre Mix tube; Sterilized deionized distilled water added to AccuPower® TLA PCR PreMix tubes to the final volume of 20 µl. Performing PCR of samples: the amplified of each primer proceeded according to annealing temperatures and following the program of initial temperature at 94 ℃ for 3 min, 35 Cycles of denaturation at 94 ℃ for 1 min, annealing 50 ℃, extension at 72 ℃ for 2 min and final extension at 72 ℃ for 5 min.

Agarose gel electrophoresis

Electrophoresis methods were used, according to Sambrook and Russell (2001), using 1.2% agarose at 70 volts for two hours.

Table 1. SCoT primers used in the present study.

Primers	Sequence 5' 3'	Temperature	Reference
SCoT-9	CAACAATGGCTACCAGCA	50 ℃	Vivodik *et al*. (2016)
SCoT-60	ACAATGGCTACCACCACA	50 ℃	
SCoT-30	CCATGGCTACCACCGGCG	50 ℃	
SCoT-44	CAATGGCTACCATTAGCC	50 ℃	
SCoT-54	ACAATGGCTACCACCAGC	50 ℃	
SCoT-28	CCATGGCTACCACCGCCA	50 ℃	
SCoT-40	CAATGGCTACCACTACAG	50 ℃	
SCoT-26	ACCATGGCTACCACCGTC	50 ℃	
SCoT-6	CAACAATGGCTACCACGC	50 ℃	
SCoT-12	ACGACATGGCGACCAACG	50 ℃	

Statistical analysis

The use of photographs resulting from agarose gel electrophoresis scored data, with the presence of a product identified as '1' and the absence identified as '0.' Polymorphism, primer efficiency, and discriminatory values calculation for each primer used the equations described by Hunter and Gaston (1988) and Graham and McNicol (1995).

RESULTS AND DISCUSSION

The results of *Catharanthus roseus* L. seed irradiated with gamma rays induced significant variations through the appearance of polymorphic and unique bands in most treatments of both cultivars (El-Shaer and Ibrahim, 2021). The past results also established the marker's ability to reveal variations induced by gamma rays in ginger (Sharma and Thakur, 2021), *Atropa belladonna* (El-Shaer and Ibrahim, 2021), grape (Yue *et al*., 2019), and tomato (El-Fiki *et al*., 2021).

Results established that *C. roseus* L. seeds irradiated with gamma rays generate a sufficient quantity of induced mutations, and SCoT analysis offered a useful molecular marker for identifying mutants. Hence, gamma irradiation can start mutation and can change primer annealing sites. Earlier reports stated polymorphism could arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; or insertions that render the priming site too distant to support amplification (Fadoul *et al*., 2013). Thus, variations in DNA sequences lead to polymorphism, and greater polymorphism indicates greater genetic diversity (Goyat *et al*., 2016). In addition, sodium azide also is a good mutagen for causing point mutation (Al-Qurainy *et al*., 2011).

Chemical mutagens generally produce induced mutations in both cultivars of *C. roseus* L., which cause base pair substitutions, especially G.C→A.T, which results in alterations in amino acids, thereby modifying the function of proteins. However, they do not eliminate their functions, as occurs in deletions or frameshift mutations (Van-der-Veen, 1966). A higher number of distinctive fingerprints was three, produced by primer SCoT-26, while primer ScoT-60 provided only one treatment with a unique fingerprint (Table 2). Yet, the primer SCoT-40 failed to show any treatment with a distinct fingerprint. Primer ability to give unique fingerprint reflects its discriminatory values and its ability to produce exceptional bands, as shown in primers SCoT-33, SCoT-26, SCoT-6, SCoT-12, and ScoT-9, in addition to an increase in the number of amplified bands, as shown in Table 3 (Reddy *et al*., 2002; Tahir, 2014).

In addition, unique bands typically occur due to various alterations in the structural DNA of *C. roseus* L. (e.g., splits, transpositions, and deletions), resulting in modifications in amino acids and hence, a protein-shaped (Mondini *et al*., 2009). Previous predictions also said that the SCoT markers connect to functional genes and corresponding characteristics so that the amplicons can translate to gene target marker systems (Xiong *et al*., 2011; El-Shaer and Ibrahim, 2021). All the primers, except SCoT-40, successfully distinguished the control (untreated) treatments of both cultivars of *C. roseus* L., i.e., victory carmine and local variety. SCoT markers approved their ability to fingerprint in many crops, including *Artemisia herba-alba* (Omar *et al*., 2015), barley (Habiba *et al*., 2021), oat (Chnapek *et al*., 2022), Thymus (Alqahtani *et al*., 2020), and maize (Al-Tamimi, 2020).

Table 2. *C. roseus* treatment fingerprinting (DNA profile) using SCoT markers.

No.	Primer	Treatments	No. of Fingerprint
1	SCoT-33	1,2,3,4,5,6	6
2	SCoT-44	1,2,3	3
3	SCoT-54	1,2,5,6	4
4	SCoT-28	1,2	2
5	SCoT-40	None	0
6	SCoT-26	1,2,3,4,5,6,7,8	8
7	SCoT-6	1,2,3,4,5,6	6
8	SCoT-12	1,2,3,4,5,6	6
9	SCoT-9	1,2,3,4,5,6	6
10	SCoT-60	1	1

Table 3. Summarized results of SCoT markers amplification product include Amplified bands molecular size range in bp; Number of main, amplified, monomorphic, polymorphic, and unique bands, primer polymorphism (%), efficiency, and discriminatory value (%).

Primers	Molecular size	Main bands	Amplified bands	Mono-morphic band	Poly-morphic band	Unique bands	Poly-morphism (%)	Efficiency	Discrimi-natory value (%)
SCoT-33	1604-237	31	60	0	13	18	41.9	0.216	18.05
SCoT-44	1855-354	6	37	3	2	1	33.3	0.054	2.77
SCoT-54	2488-177	20	77	4	8	8	20	0.103	11.11
SCoT-28	1750-210	18	71	3	6	9	33.3	0.084	8.33
SCoT-40	1334-290	6	37	4	2	0	33.3	0.054	2.77
SCoT-26	987-194	15	66	5	6	4	40	0.09	8.33
SCoT-6	1135-253	15	45	2	6	7	40	0.133	8.33
SCoT-12	1631-143	22	63	0	15	7	68.18	0.238	20.83
SCoT-9	1758-226	21	44	0	14	7	66.66	0.311	19.44
SCoT-60	880-158	8	50	6	0	2	0	0	0

Total data for analysis of 10 SCoT primers indicated that the highest molecular size was 2488 bp produced by primer SCoT-54, whereas the lowest was 143 bp produced by primer SCoT-12. Since irradiation altered the DNA profile in most treatments of *C. roseus* L., this appeared clearly through arising polymorphic and unique bands. It is associated with the mutation due to insertions and deletions, which cause a change in primer annealing sites, and, consequently, change the size of the amplified fragment because it could change the distance between two annealing sites of the primer on target DNA (Fadoul *et al*., 2013; Al-Saadi, 2018). It will also enhance both amplified and main bands. Primer SCoT-33 produced the highest value for chief and unique bands, reaching 33 and 18, respectively.

Primer ability is to recognize a unique annealing site on the genome and successfully produce a unique DNA fingerprint for a particular genotype (Fadoul *et al*., 2013; Al-Ghufaili, 2017). In primer SCoT-54, the highest number of amplified bands was 77. Recognition of a high number of annealing sites by primer usually results in the highest number of main bands, as established in past studies on maize (Al-Saadi, 2018) and wheat (Tahir, 2014; Al-Ghufaili, 2017). It is also a fact that when primers show the highest amplification product (Figures 2, 3, and 4), which might be due to high homology between the primers' series and the examined plant genotypes (Verma and Agarwal, 2005). The increased binding site of a primer consequently enhances the number of amplified bands, which results in booting the chance to detect polymorphism among individuals (Al-Judy, 2004; Al-Ghufaili, 2017).

Primer SCoT-60 produced the highest value for monomorphic bands. The presence of monomorphic bands usually refers to genotypes that belong to one species, share their relatives in some genome sequences, and are conserved in the genome (Al-Judy, 2004; Al-Badeiry, 2013). The appearance of monomorphic bands may refer to a common character between the studied genotypes (Al-Tamimi, 2014) and might also be related to the sequences not affected by the mutagenic effects of gamma rays (Al-Rehbawy, 2022; Al-Saadi, 2022).

Primer SCoT-12 produced the highest value for polymorphic bands, polymorphism, and discriminatory (15, 68.18, and 20.83, respectively). Polymorphism value strongly associates with the primer ability to give polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995). These primers ably produced the highest amplified bands, which is a possibility always connected with the primer ability to generate the highest number of amplified bands. Primer SCoT-9 provided the highest value for efficiency since primer efficiency is related to the ratio between polymorphic and amplified bands of a particular primer (Hunter and Gaston, 1988; Graham and McNicol, 1995).

Other primers provided the lowest values for the studied criteria, including main, amplified, efficiency, and discriminatory values in the primers SCoT-44 and SCoT-40. However, the primers SCoT-33, SCoT-12, and SCoT-9 failed to produce any monomorphic bands. Primer SCoT-60 failed to give any polymorphic bands, which later affected its ability in its values for polymorphism, efficiency, and discrimination. Primer SCoT-54 provided the lowest value for polymorphism, reaching 20% (Table 3).

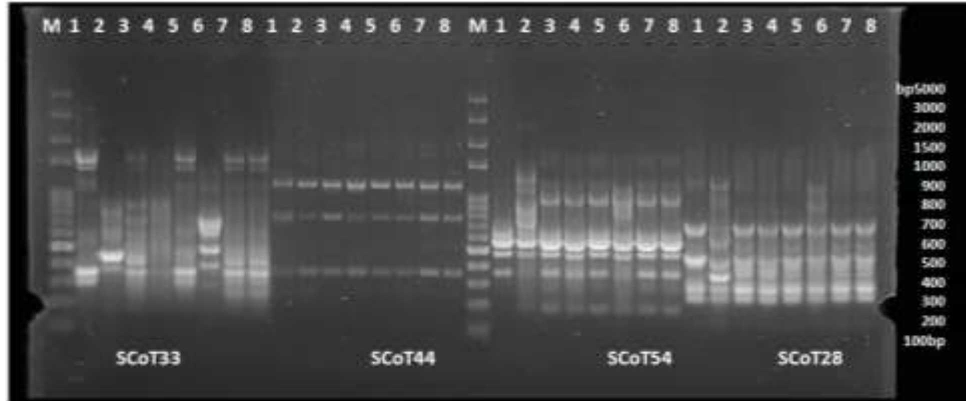

Figure 2. Amplification products of primers, viz., SCoT-33, SCoT-44, SCoT-54, and SCoT-28, M: DNA ladder, 1-Local variety (untreated), 2- Carmine variety (untreated), 3-50 Gy, 4-75 Gy, 5-0.2% S.A, 6-0.4% S.A, 7- 0.6% S.A, 8-0.8% S.A. (S.A.: Sodium Azide, Gy: Gray).

Figure 3. Amplification products of primers, viz., SCoT-40, SCoT-26, SCoT-6, and SCoT-12, M: DNA ladder, 1-Local variety (untreated), 2- Carmine variety (untreated), 3-50 Gy, 4-75 Gy, 5-0.2% S.A, 6-0.4% S.A, 7- 0.6% S.A, 8-0.8% S.A. (S.A: Sodium Azide, Gy: Gray).

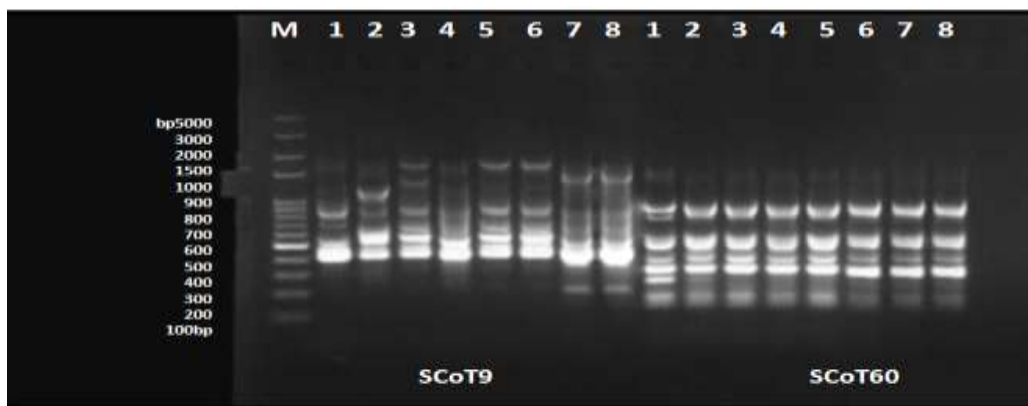

Figure 4. Amplification products of primers, viz., SCoT-9 and SCoT-60, M: DNA ladder, 1- Local variety (untreated), 2- Carmine variety (untreated), 3-50 Gy, 4-75 Gy, 5-0.2% S.A, 6-0.4% S.A, 7-0.6% S.A, 8-0.8% S.A. (S.A.: Sodium Azide, Gy: Gray).

CONCLUSIONS

Results indicated that both gamma rays and sodium azide were able to generate mutagenic effects through changing DNA profiles that were detected successfully using SCoT markers. Hence, the physical and chemical mutagens signified an excellent step in medicinal plant improvement through mutation breeding.

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