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ASSESSMENT OF VARIATIONS IN *CATHARANTHUS ROSEUS* L. INDUCED BY GAMMA RAYS AND SODIUM AZIDE USING RAPD MARKERS

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SUMMARY

The study of genetic variations in *Catharanthus roseus* L. induced by gamma rays and sodium azide using RAPD markers transpired in 2021–2022 at the Department of Biology, University of Kufa, Iraq. The seeds of two cultivars of *Catharanthus roseus* L., i.e., victory pure white and local (pink variety), received two doses of gamma rays (50 and 75 Gray) and four sodium azide concentrations (0.2%, 0.4%, 0.6%, and 0.8%). Non-treated seeds of both cultivars also served as a control for comparison. Evaluating the effects obtained through the exposure of *C. roseus* seeds to gamma rays and sodium azide employed DNA markers, including 16 primers used in applying RAPDs. DNA extraction, accomplished from fresh apical leaves, underwent molecular markers application. RAPD markers could detect the generation of genetic variation induced by gamma rays and sodium azide treatments through the appearance of polymorphic bands. Using 16 RAPD primers, the largest molecular size was 2,543 bp produced by primer OPB-17, with the lowest 114 bp produced by primer OPC-08. The highest number of main and polymorphic bands was 18 and 10, respectively, in primer OPA-10. The highest number of monomorphic bands was eight in primer OPX-17, which affected its ability to give the lowest value for polymorphism. The highest number of amplified bands was 78 bands in primer OPC-09. Primer OPA-10 provided the highest number of polymorphic and unique bands, as well as, the highest values for discrimination and primer efficiency. Primer OPC-09, with seven treatments resulting in a unique fingerprint, is superior to all other primers.

Keywords: *Catharanthus roseus* L., RAPDs, gamma rays, sodium azide

Key findings: In the presented molecular study, RAPD markers assessed genetic variations generated by both gamma ray exposure and sodium azide treatments with diverse doses and concentrations..

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INTRODUCTION

Catharanthus roseus L. (synonym of *Vinca rosea* L.) is native to Madagascar, belonging to the family Apocynaceae, with 411 genera and 4,650 species (Maher *et al*., 2021), and the majority are of decorative and medicinal values (Salama and Ali, 2016). Its current cultivation has reached globally, including Iraq, for its therapeutic and attractive ornamental features. Its decorative aesthetic appearance refers to its green variegation and prolific flowers in splendid colors (Lahuf, 2019). In general, *C. roseus,* as an important medicinal plant, treats various diseases with its active chemical constituents including volatile and phenolic

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compounds (Lahare *et al*., 2020), alkaloids (Maher *et al*., 2021), ajmalicine and serpentine (Van-der-Heijden *et al*., 2004), reserpine, catharanthine, and anthocyanin (Sain and Sharma, 2013), and carbohydrate, flavonoid, and saponin (Sain and Sharma, 2013).

The mutation is a term describing the sudden heritable changes that occur in the genotypes and is the ultimate source of all the genetic changes that provide the raw material for evolution and a valuable approach for the improvement of the economic characteristics of the crop plants (Zakir, 2018). Thus, mutation breeding employs many types of mutagenesis, including irradiation as gamma ray, which is ionizing radiation with its multiple forms, used in inducing mutations in seeds, cuttings, pollen, and tissue-cultured calli (Xie *et al*., 2019; Kurucz *et al*., 2022) and treatment with chemical mutagens, including sodium azide, which is a chemical mutagen that has become an essential tool to enhance agronomic traits of crop plants by creating point mutation in the plant's genome (Dubey *et al*., 2017).

In *C. roseus*, mutation breeding conducts precisely the enhancement of contents of total root and leaf alkaloids and anticancer leaf alkaloids, vincristine, and vinblastine, which may lead to the development of high anticancer and antihypertensive containing 'ideochemocultivars,' which helps in decrease extraction cost and the market value of these alkaloids (Verma *et al*., 2013). Among many tools for mutation detection, DNA markers are the most valuable tool, with many marker systems, widely used in mutation detection, such as, RAPD (Random amplified polymorphic DNA) (Wahyudi *et al.,* 2020). It is also simple, inexpensive, does not need knowledge of the target sequence, and is easy to implicate and analyze data (Bahadur *et al.,* 2015).

Medicinal and aromatic plants are vital sources of secondary metabolites, which are crucial for human health care. Secondly, the induced mutation is the ultimate source to alter the genetics of crop plants that may be difficult to bring through cross-breeding and other breeding procedures using different mutagens. Mutation-assisted plant breeding can play a crucial role in generating desirable crop varieties to address the threats and challenges of the present and future needs of medicinal and aromatic crop plants (Kolakar *et al*., 2018). Therefore, the presented study aimed to analyze the genetic variations in *Catharanthus roseus* L. induced by gamma rays and sodium azide through RAPD markers.

MATERIALS AND METHODS

Seed samples

Seeds of *Catharanthus roseus* L. cv. 'pure victory white' came from SAKATA Company, with the *C. roseus* local variety (pink variety) seeds obtained from the local market (Figure 1).

Gamma irradiation treatment

Individually, 40 seeds of each cultivar, placed in a Petri dish, received each treatment. Irradiation proceeded for the white cultivar at two doses (50 Gy and 75 Gy). The control treatment was 0: not irradiated white cultivar, as proposed by El-Sharnouby *et al*. (2016) at an average of 18 Gy/h using Cobalt 60 as irradiation source, which transpired at the Department of Physics, College of Science, Baghdad University, Iraq.

Figure 1. *Catharanthus roseus* L., 1) victory pure white (flowers and seeds) and 2) Local variety (flowers and seeds).

Sodium azide treatment

Seeds of the white cultivar got soaked in distilled water for 12 h, afterward, received treatment with four sodium azide concentrations (0.2%, 0.4%, 0.6%, and 0.8%) prepared in distilled water and left for another 12 h. Immediately, washing the seeds with running tap water removed any residual effects of mutagen sticking to the seed coat. Drying the seeds followed at room temperature till sowing (Ali *et al*., 2019).

Seed sowing

For further studies, seed sowing progressed in 2021–2022 at the Orchid of Agriculture Division at the University of Kufa, Iraq, using plastic pots filled with peat moss till flowering. The inclusion of control treatment was also for both cultivars of *C. roseus,* viz., the victory white and local variety, with the seeds of the control treatments unexposed to any physical and chemical mutagen.

Primers

The primers came from Bioneer Corporation in lyophilized form, dissolved in TE buffer to obtain 100 pmol/µl as a final concentration (stock solutions). Working solutions of 10 pmol/µl, prepared from stock solutions, and 16 primers were used in the application of RAPDs (Carelli *et al*., 2006; Abd-El-Hady *et al.*, 2010; Ezekiel *et al.,* 2011; El-Assal and Gaber, 2012), with their nucleotide sequences and the primer names provided in Table 1.

Table 1. RAPD primers used for analysis in the study.

Primer Name	Sequence 5' 3'	Temperature	Primer name	Sequence 5' 3'	Temperature
OPA-01	CAGGCCCTTC	40 °C	OPB-06	TGCTCTGCCC	37 °C
OPA-02	TGCCGAGCTG	40 °C	OPB-17	AGGGAACGAG	37 °C
OPA-03	AGTCAGCCAC	40 °C	OPC-05	GATGACCGCC	37 °C
OPA-04	AATCGGGCTG	40 °C	OPC-08	TGGACCGGTG	37 °C
OPA-10	GTGATCGCAG	40 °C	OPC-09	CTCACCGTCC	37 °C
OPA-14	TCTGTGCTGG	37 °C	OPC-19	GTTGCCAGCC	40 °C
OPA-15	TTCCGAACCC	37 °C	OPH-01	GGTCGGAGAA	37 °C
OPA-17	GACCGCTTGT	37 °C	OPX-17	GACACGGACC	40 °C

PCR content and amplification program

PCR Pre-Mix master mix, Bioneer Corporation USA (0.2 ml), thin-wall 8-strip tubes with attached cup, and 96 tubes were used (*Top* DNA polymerase [1U]), (dATP, dCTP, dGTP, dTTP) (each 250 µM). Using the Reaction Buffer with 1.5 mM MgCl2 (1X) and Stabilizer and tracking dye included 100 bp DNA ladder. Fresh seedling leaves, were used to take fresh apical leaves for genomic DNA extraction, employed the Genomic DNA Mini Kit provided by Geneaid Biotech.

According to the experimental protocol of AccuPower® TLA PCR PreMix, preparing the PCR reaction mixture was as follows: adding 5 µl template DNA and 5 µl of primer (10 pmol/µl) to each AccuPower® TLA PCR Pre Mix tube containing 5 µl, and adding sterilized deionized distilled water to AccuPower® TLA PCR PreMix tubes to the final volume of 20 µl. For performing PCR of the samples, the amplified of each primer proceeded according to annealing temperatures and following the program of initial temperature at 94 ℃ for 3 min, 40 cycles of denaturation at 94 ℃ for 1 min, annealing: variable, extension at 72 ℃ for 1 min, and final extension at 72 ℃ for 5 min.

Agarose gel electrophoresis

The gel electrophoresis methods followed the technique by Sambrook and Russell (2001), using 1.2% agarose at 70 volts for two hours.

Statistical analysis

Using photographs resulting from agarose gel electrophoresis scored data, with the presence of a product identified as '1' and the absence as '0.' Polymorphism, primer efficiency, and discriminatory value calculations for each primer followed the formulas of Hunter and Gaston (1988) and Graham and McNicol (1995).

RESULTS AND DISCUSSION

Results indicated that gamma irradiation and sodium azide treatments induced significant variation through the appearance of polymorphic bands (absence and appearance of bands) in most treatments, generally originating through mutation. Dehgahi and Joniyasa (2017) and Mistry *et al*. (2022) established that gamma irradiation and sodium azide could induce plant mutations, especially with increased doses and concentrations, respectively. Chemical mutagen generally produces induced mutation, which leads to base pair substitutions, especially GC-AT resulting in amino acid changes, which change the function of proteins but do not abolish their roles as deletions that frameshift mutation mostly does. These chemo-mutagens induce a broad variation in morphological and yield parameters compared with normal plants (Khan *et al*., 2009).

In addition, gamma irradiation-induced mutation and altering primer bind sites. A report stated that, in general, polymorphism among the treatments could arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; and insertions that render the priming site too distant to support amplification (Fadoul *et al*., 2013). Gamma rays and sodium azide mutagenesis established effects in many plant species, including cowpea (Raina *et al*., 2022), peanuts (Okasha *et al*., 2021), sweet potatoes (Orji *et al*., 2021), rice (Lo *et al*., 2021), and barley (Dyulgerova and Dyulgerov, 2022). RAPD markers recorded their ability to detect chemical and physical mutagens in many crops, including rice (Ikhajiagbe and Omoregie, 2020), potato (Mahfouze, 2012), fenugreek (Al-Saadi, 2022), coriander (Al-Rehbawy, 2022), and *C. roseus* (Salama and Ali, 2016; EL-Sayed, 2013).

Primer OPC-09 successfully gave a unique fingerprint in seven treatments, with the rest of the primers proving to fingerprint in lesser treatments, including OPA-17 and OPA-15 (one unique fingerprint for treatment four) (Table 2). However, the primers OPA-14 and OPB-06 failed to reveal the unique fingerprint. Primers ably produce polymorphic and unique bands, especially with varied treatments both polymorphic and unique alleles inside the genotypes enhance the chances of making unique fingerprints (Al-Saadi, 2022; Al-Rehbawy, 2022).

Past studies established the ability of RAPD markers in fingerprinting by exposing *C. roseus* to gamma rays (Ibrahim *et al.*, 2013; Salama and Ali, 2016; Saadedin, 2018; Salama *et al*., 2020). Sodium azide also induced the mutation successfully, as detected by RAPD markers in rice (Ikhajiagbe and Omoregie, 2020). Figures 2-8 illustrate the amplification products of agarose gel electrophoresis. Table 3 further illustrates the total data for analysis of 16 RAPD primers, indicating the largest molecular size was 2,543 bp produced by primer OPB-17 and the lower one was 114 bp produced by primer OPC-08.

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

No.	Primer	Treatments	No. Fingerprint
1	OPA-03	1/7	2
2	OPA-04	1/2/4/7	4
3	OPA-02	2/4	2
4	OPA-14	0	0
5	OPB-06	0	0
6	OPA-17	4	1
7	OPB-17	1/4/8	3
8	OPA-10	1/3/4/6	4
9	OPC-08	2/5/8	3
10	OPA-15	4	1
11	OPC-09	1/2/3/4/5/6/8	7
12	OPH-01	¾	2
13	OPC-05	1/2/3/4	4
14	OPC-19	1/2/4	3
15	OPX-17	1/2/4	3
16	OPA-01	1/2/5	3

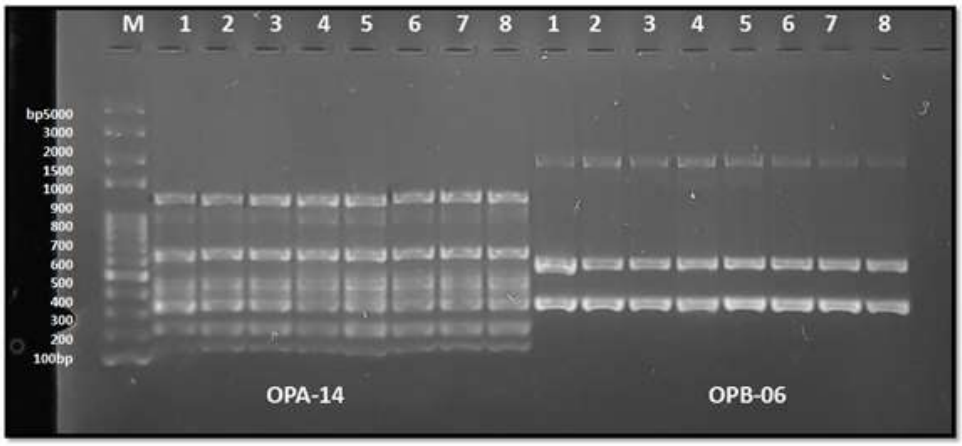

Figure 2. Amplification products of primers OPA-14 and OPB-06, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

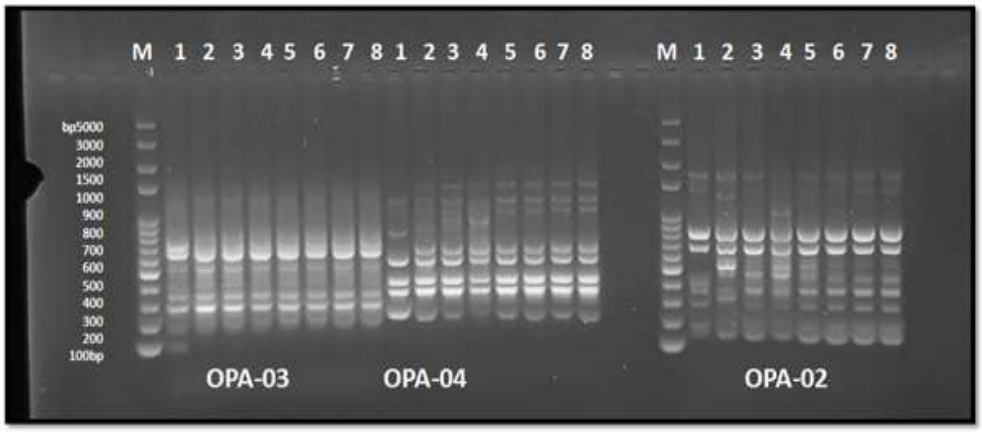

Figure 3. Amplification product of primers OPA-03, OPA-04, and OPA-02, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

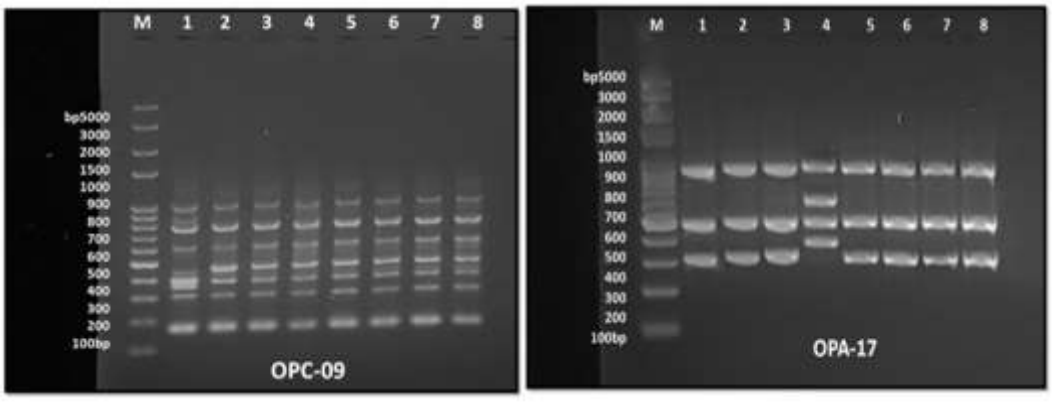

Figure 4. Amplification product of primers OPA-17 and OPC-09, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

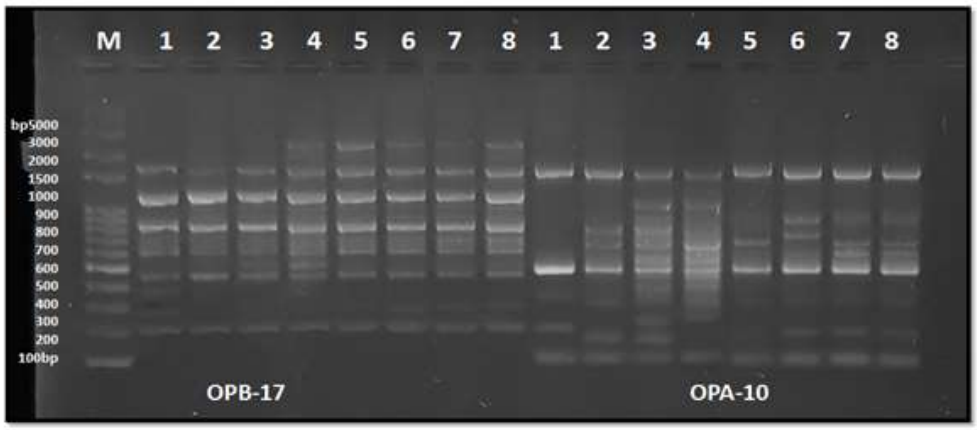

Figure 5. Amplification product of primers OPB-17 and OPA-10, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

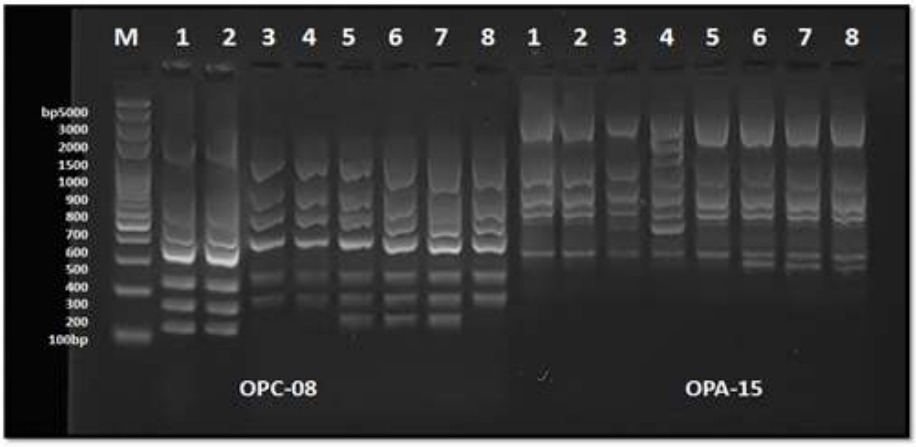

Figure 6. Amplification product of primers OPC-08 and OPA-15, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

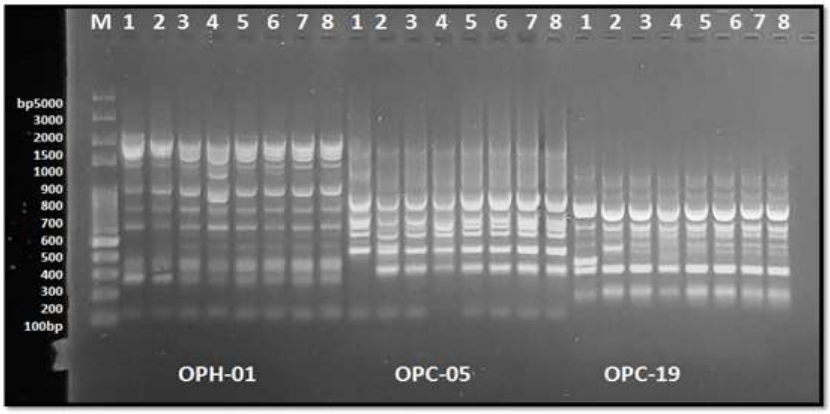

Figure 7. Amplification product of primers OPH-01, OPC-05, and OPC-19, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

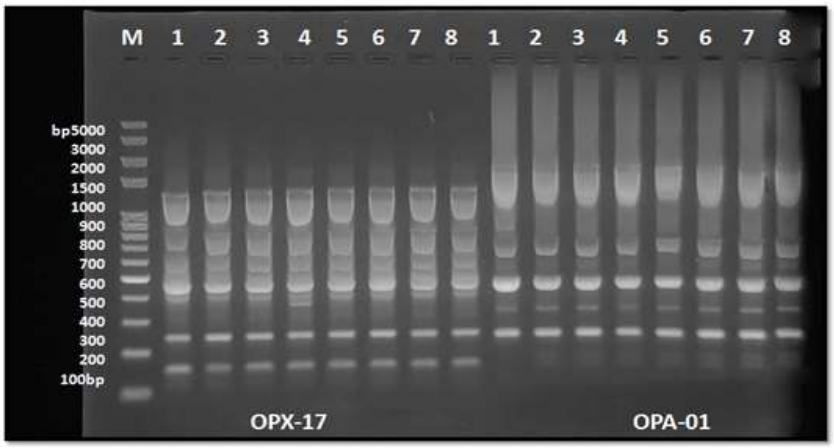

Figure 8. Amplification product of primers OPX-17 and OPA-01, M: DNA ladder, 1) Local variety (untreated), 2) White variety (untreated), 3) 50 Gy, 4) 75 Gy, 5) 0.2% S.A., 6) 0.4% S.A., 7) 0.6% S.A., 8) 0.8% S.A. (S.A: Sodium azide, Gy: Gray).

Table 3. Summarized results of RAPDs amplification product include amplified bands molecular size (bp) with the number of main, amplified, monomorphic, polymorphic, and unique bands, and percentage of primer polymorphism, efficiency, and discriminatory value.

Primers	Molecular size	Main bands	Amplified bands	Mono-morphic bands	Poly-morphic bands	Unique bands	Poly-morphism (%)	Efficiency	Discrimi-natory value (%)
OPA-03	894-115	14	73	5	7	2	50	0.095	10.29
OPA-04	1637-266	13	69	4	7	2	53.8	0.101	10.29
OPA-02	171-1826	16	67	4	7	5	43.7	0.104	10.29
OPA-14	144-1224	7	56	7	0	0	0	0	0
OPB-06	336-1981	3	24	3	0	0	0	0	0
OPA-17	315-938	5	25	2	1	2	20	0.04	1.47
OPB-17	220-2543	15	65	5	6	4	40	0.092	8.82
OPA-10	117-1635	18	62	3	10	5	55.5	0.161	14.7
OPC-08	114-1368	10	47	2	6	2	61	0.127	8.82
OPA-15	266-1974	10	53	6	1	3	10	0.018	1.47
OPC-09	172-1414	14	78	7	4	3	28.5	0.051	5.88
OPH-01	115-1843	14	84	7	4	3	28.5	0.047	5.88
OPC-05	115-1708	12	69	4	5	3	41.6	0.072	7.35
OPC-19	209-1389	15	73	5	5	5	33.3	0.068	7.35
OPX-17	160-1311	10	68	8	1	1	10	0.014	1.47
OPA-01	166-1966	10	67	5	4	1	40	0.059	5.88

Since irradiation and sodium azide ably altered the DNA profile in some treatments, this appeared clearly through the emergence of polymorphic and unique bands. However, its association with mutation is by insertion and deletion mutations, which cause a change in primer annealing sites, and, consequently, change the size of the amplified fragment. Subsequently, it could change the distance between two annealing sites of primer on target DNA, which later affects both amplified and main bands (Al-Rehbawy, 2022; Al-Saadi, 2022).

The highest number of primary and polymorphic bands was 18 and 10, respectively, in primer OPA-10. Recognition of the highest number of annealing sites by primer usually results in a high number of main bands, also established and reported in past studies in maize (Al-Tamimi and Al-Saadi 2018), in wheat (Tahir, 2014; Al-Ghufaili, 2017), and that later enhances the chances to provide polymorphic bands.

In primer OPX-17, the highest number of monomorphic bands was eight, which affects its ability to give the lowest value for polymorphism, efficiency, and discriminatory value since it provided the lowest number of polymorphic and unique bands. Since all the treatments belong to *C. roseus*, the

appearance of monomorphic bands related to the common sequences among the treatments was not affected by chemo physical exposure (Al-Judy, 2004). The plant genome contains conserved sequences retained in all the treatments, and these shared sequences appeared in the form of monomorphic bands (Al-Tamimi, 2020). The higher number of amplified bands was 78 in primer OPC-09, and the increased binding site of the primer consequently increases the number of amplified bands, which results in an increased chance of detecting polymorphism among individuals (Williams *et al.*, 1990; Roy *et al.* 1992). Primer OPA-10 provided the highest number of polymorphic and unique bands and the highest value for discrimination and primer efficiency. Primer ability to give a unique band is related to its ability to recognize a unique annealing site on the genome (Fadoul *et al*., 2013).

Further cloning of these unique bands can get the nucleotide sequences linked to a trait of interest. These genotypes could serve efficiently in crop genetic improvement and breeding programs. Thus, the result of the presented study could help facilitate the improved crop plants in breeding programs (Sharma *et al.,* 2019). Discrimination, in addition to primer efficiency value, produced their higher values, and both criteria aid primer ability to make polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995).

The highest polymorphism was 61% produced by primer OPC-08, and it strongly relates to the primer ability to make the polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995). When primers recognize high annealing sites due to their structure, this increases the possibility of detecting DNA polymorphisms among individuals (Al-Tamimi, 2020). In addition, polymorphism always relates to increasing numbers of polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995). Thus, RAPD could effectively detect DNA alteration after the influence of a mutagenic agent. Irradiation increased the level of DNA breaking its formation, leading to form new variations easily detected by changes in RAPD profiles (Selvi *et al*., 2007).

The lowest values for the studied primers emerged with primer OPB-06 for main and amplified bands, monomorphic bands in primers OPC-08 and OPA-17, polymorphic bands in primers OPA-17, OPA-15, and OPX-17, unique bands in primers OPX-17 and OPA-01, polymorphism and discrimination in primers OPA-15 and OPX-17, with the lowest efficiency in primer OPX-17. Primers that failed to give any polymorphic band, including OPA-14 and OPB-06, failed to provide any value for polymorphism, discrimination, and primer efficiency.

CONCLUSIONS

Both gamma rays and sodium azide irradiations generated genetic variations in *Catharanthus roseus* L. concerning different doses and concentrations of the mutagens. These variations could serve further improvement in *C. roseus* L. through intensive selection.

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