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Using RAPD-PCR to Determine the Genetic Stability of *Dianthus Chinesis* in Vitro Treated with the Chemical Mutagenic Ethyl Methyl Sulfonate EMS

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Abstract

This study was conducted in the Molecular Biology Lab. and the Central Lab. at the University of Tikrit on 1/29/2023 for the purpose of determining the genetic stability of the Dianthus chinesis after cultivating it in vitro using 3 RAPD primers, which are OPA-02, OPD-10, OPD- 18, As 9 samples were used in the study, the first is a field sample of this plant that was not treated with EMS (was considered as a control sample) and the remaining samples (4 callus samples and 4 tissue branches samples) were treated with concentrations of (0.0, 0.2, 0.4, 0.6)% of EMS, after a month, DNA was extracted, RAPD reactions were performed for the primers used ,as it was shown through the results that there was a genetic variation in the genetic material of the Dianthus chinesis in the recognition sites of the used primers, as it was not only affected by EMS, but by its cultivation in vitro.

Introduction

Carnation is a perennial herbaceous plant that belongs to the Caryophyllaceae family, the genus *Dianthus*. The islands of the Mediterranean are its original home. It is considered one of the economically important cut flowers in the world because of its distinctive scent and beautiful colors. As for its medicinal importance, it is characterized by its pungent aromatic scent that stimulates the appetite. It has a digestive and gas repellent effect, in addition to being used as an anti-nausea, vomiting and analgesic for toothache, and its essential oil is used as an anti-fungal and anti-bacterial (Khudair & Khazal, 2013).

Tissue culture technology provided a great opportunity to evaluate the techniques of inducing genetic variations and the stability of the resulting genotypes, which led to improving the characteristics of the resulting plants according to the desired variation (Desta & Ortiz, 2014). Therefore, mutagens are considered one of the most important tools used in the creation of genetic variations, which come after the technology of genetic engineering, through which a gene or several specific genes can be inserted into the host genome for the purpose of giving it one or several desirable characteristics (Wang et al., 2017).

The chemical mutagen EMS is considered one of the most widely used mutagens because of its point effect and its ability to cause genetic variations in a relatively short time. Some also considered it an effective tool in regulating gene expression and studying phenotypic and histological characteristics (Nguyen et al., 2020).

The Random Amplified Polymorphic DNA (RAPD) technique is characterized by not requiring a large amount of genetic material, its speed and simplicity, in addition to the fact that these primers allow covering different regions of the genomes of the studied cultivars. This technique was also used for the purpose of studying the relationship between Populations, whether they are of the same species or of species close to each other, were used to investigate hybrids as well as their use in determining genetic identity, and this technique helped in

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drawing genetic maps because it was used in determining genetic relationships between plants belonging to one species, as it was done through This technique classifies many taxa of plant species (Al-Abras et al., 2012; Hussain, 2011)

Methods

This study was conducted in the molecular biology lab. in the College of Science and the central lab. in the University of Tikrit on 1/29/2023, as 3 RAPD primers were used in this study, and all of them were productive, as bands of varying molecular sizes appeared on different sites and other bands were absent. Through the appearance or absence of these bundles, the genetic variation of the samples under study was determined.

Sample Preparation: Four weeks after the treatment of callus and branches with concentrations of EMS, nine samples were used for molecular analysis, as follows; (1) A field sample was considered as a control sample; (2) A sample of tissue branches not treated with the EMS; (3) A sample of tissue branches that was treated with a concentration of 0.2% of EMS; (4) A sample of tissue branches that was treated with a concentration of 0.4% of EMS; (5) A sample of tissue branches that was treated with a concentration of 0.6% of EMS; (6) Callus sample not treated with EMS; (7) A callus sample was treated with a concentration of 0.2% of EMS; (8) A callus sample was treated with a concentration of 0.4% of EMS; (9) A callus sample was treated with a concentration of 0.6% of EMS.

The numbering of the samples above was adopted in the results of the RAPD.

DNA Extraction

The first method; (1) 1 gm of young leaves or callus is taken and placed in an earthenware mortar; (2) Adding liquid nitrogen with rapid manual crushing, and repeating the process until powder is obtained (to ensure breaking down the cell components), then the powder is placed in a 2 ml Eppendorf tube; (3) Add 750 µl of Lysis buffer to the sample in an Eppendorf tube and shake the sample with the solution in a Vortex vibrator; (4) Transfer the tubes of the Eppendorf to the water bath at a temperature of 60°C for (30) minutes, with stirring every 5 minutes; (5) Remove the mixture from the water bath and leave it to cool to add 200 microliters of protein lysis solution to it. Shake the tubes well and transfer them to the cooling device for a period of 5 minutes. Then, the tubes are placed in the centrifuge at 13000 RPM for 4 minutes; (6) Transfer 700 µL of the clear to a new Eppendorf tube and add 500 µL of Binding buffer; (7) Transfer 750 µL of the mixture to a column and put it in a centrifuge at 10,000 RPM for one minute and repeat this process twice; (8) Discard the liquid in the tube, and add 500 µL of wash solution (1) to the column, then centrifuged at 10,000 RPM for one minute; (9) Adding 500 microliters of wash solution (1) to the column, then centrifuged at 13000 RPM for one minute. The liquid in the tube is discarded, then the separation column is left in the centrifuge for drying; (10) Add 100 microliters of DNA elution buffer and leave the tube for 5 minutes at the lab temperature without moving it, then centrifuge at 13000 RPM for two minutes and neglect the separation column and leave the elution buffer containing DNA for 24 hours at the lab temperature and Then it is kept in the refrigerator until it is used in conducting experiments.

The Second Method: This method is similar to the first method, except that 800µL of CTAB is added instead of Lysis buffer, then 600µL of chloroform is added, and the mixture is centrifuged at 14000 RPM for 5 minutes, after which the filtrate is taken and 800µL of the binding buffer is added to it, and the rest of the steps are completed in the same the first way.

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Detecting DNA in the sample

Using electrophoresis

The presence of DNA is detected after extracting it from the sample and its molecular size is estimated by electrophoresis, depending on the method (Maniatis et al., 2001).

First, 1% agarose gel is prepared, which is dissolved in SB concentration 1X using a microwave (or any heat source) until it boils (or becomes clear) and is left in the lab atmosphere until its temperature ranges (50-60)°C, to which 3 microliters of dye (Red Safe) are added.

The gel solution is poured into a tray of the relay device after placing the comb for this basin, depending on the number of wells required at one end of the basin, and the pouring is done quietly and from one of the angles on the opposite side of the comb in order to avoid the formation of bubbles, which if formed must be removed using The pipette, and then the gel is left until it solidifies.

After the gel hardens, the comb is lifted and the tray is placed in the electrophoresis basin containing an appropriate amount of 1X SB solution to immerse the gel pits.

Mix 8 µL of DNA sample with 2 µL of blue loading dye using a micropipette to be loaded into the gel pits.

Operating the electrophoresis device by means of passing electric current with a voltage difference of 3volts/cm, taking into account the adjustment of the electrodes, so that the side containing the samples from the migration gel is at the negative pole, so that the samples migrate towards the positive pole, and the total time that this process takes in the two stages is 30 -35 minutes.

After the migration is over, the presence of DNA in the gel is checked by exposing the gel to ultraviolet light using a UV-Trans illuminator at a wavelength of 260 nm to be able to see the DNA bands.

As for the PCR electrophoresis, an agarose gel with a concentration of 1.5% is prepared, weighing 1.5 g of agarose powder and dissolved in 100 ml of SB solution concentration 1X using Microwave, the solution is left until its temperature reaches (50-60) °C and poured as in the above method.

5µL of Volumetric Marker (100bp-3000bp) was taken in the first well of the gel and then the resulting samples from RAPD-PCR were loaded in the other wells in the same way. Migrating gels are imaged with a Trans illuminator.

Using Nano drop

The measurement process was carried out using this device by measuring the absorbance of the ultraviolet spectrum at the wavelength of 260 nanometers, by means of a spectrophoto meter, as 1 µl of the dissolving solution containing DNA were taken, and then it was placed in the device and the measurement order was given to the device (These steps were taken after filtering the Nano drop on the solubilization solution). Concentrations are measured in this device in ng/ml, and the purity is estimated by dividing the absorbance reading at the wavelength of 260 nm by its reading at the wavelength of 280nm (Maniatis et al., 2001).

RAPD-PCR reactions

These reactions were made based on was mentioned by (Williams et al., 1993).Materials, solutions and the processing company Premix.

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Primers: 3 primers were used, and all of them were produced. These primers were supplied by U.S.A. Operon technologies, as shown in Table (1).

Table 1. RAPD primer sequences

No.	Primers	Seq. 5`→3`
P1	OP A-02	CAGGCCCTTC
P2	OP D-10	TGGACCGTGC
P3	OP D-18	CCTTGACGCA

Nuclease-free water.

Template DNA: extracted from the sample.

The RAPD-PCR main reaction mixture was prepared by mixing the Premix with 2µL of primer in addition to 2µL of DNA for the field sample and for the offshoot samples. As for the callus samples, 5µL was added with 16µL of distilled deionized water, then these tubes (containing the mixture) were placed in the thermo cycler device, the program mentioned in Table (2) is applied, and after the reaction is finished, the tubes are removed from the device and the samples are transported, or they are preserved by freezing until they are electrophoresis.

Table 2. RAPD–PCR program

Stage	Temperature (°C)	Cycles	Time
Pre denaturation	94	1	4min
Denaturation	93	40	45sec
Annealing	36		1 min
Elongation	72		1.5min

Statistical Analysis

After taking the results of the doubling of the RAPD primers and converting them into tables depending on the presence or absence of DNA bundles of the studied cultivars, as the presence of the bundle is symbolized by the number (1) and its absence by the number (0).

Estimating the discriminatory ability and efficiency of RAPD primers: The efficiency of each initiator was estimated according to the following equation (Khudair & Khazal, 2013). Efficiency = (number of bands per primer/total number of bands multiplied by all primers)*100

The discriminatory ability was found using the following equation:

Discriminatory power = (number of differential bands in each primer/total number of differential bands in all primers) *100. Contrast ratio = (number of different bands per primer / total number of bands) *100

Results and Discussion

DNA Extraction and Detection

Using electrophoresis: DNA was detected by electrophoresis, as only the DNA bundles extracted from the field sample and tissue branch samples appeared, and the DNA bundles extracted from callus did not appear due to its small quantity, so it was detected using the Nano drop device, Figure (1).

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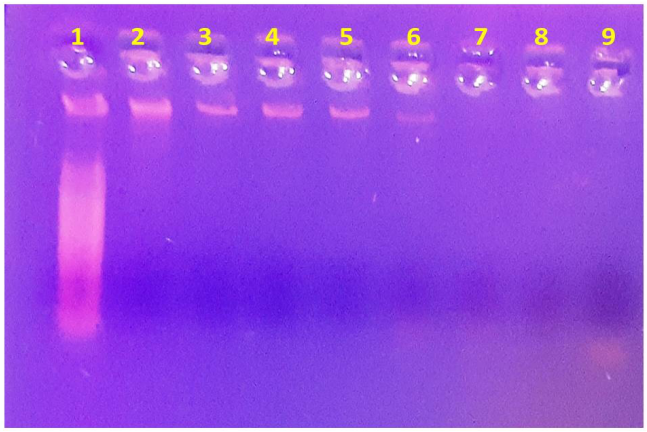

Figure 1. Results of electrophoresis of extracted DNA on agarose gel

Using Nano drop: DNA concentration and purity were estimated using this device and for callus samples only, as shown in Table (3).

Table 3. DNA concentration and purity of callus samples

Sample No.	EMS conc.	DNA Conc. (ng/µL)	Purity (260/280)
.6	0.0%	8.7	7.56
.7	0.2%	9.8	3.04
.8	0.4%	28.0	2.14
.9	0.6%	6.5	1.87

Results of the Primers used:

P1 (OP A-02)

This primer gave 12 recognition sites, 11 which are distinct , one site is general and produced 34 bands, 31 which were distinct and the sizes of the bands ranged between 300-1700 bp. The contrast percentage of this primer was 91%, in which callus samples excelled (which It represents the samples from 6-9) in its contrast to the samples of the branches (which represent the samples from 2-5), as the callus formed 50% of the total variance percentage, while the branches formed 41% of this percentage and the efficiency of this primer was estimated at 8.1 and its discriminating ability 9.2.

The branches samples, concentration of 0.0% (not treated with EMS) showed two unique bands and two absent bands, while the concentration of 0.6% did not show any unique bands, only 3 absent bands. As for the callus samples, two unique bands appeared in a concentration of 0.6% and 3 unique bands in a concentration of 0.6%, 0.4% and no other unique bands appeared at a concentration of 0.0% and a concentration of 0.2%, but only bands were absent, as 3 bands were absent at a concentration of 0.0% and two bands at a concentration of 0.6%, and thus the callus sample shows a concentration of 0.6% with the presence of unique bands and their absence for the site The identification of this primer is similar to the two samples of the branches, concentration 0.0% and 0.2%, in addition to the similarity of the callus sample, concentration of 0.0%, with the sample of the branches, concentration of 0.6%.

The absence of bands and their appearance from a concentration of 0.0% for callus and branches samples (not treated with EMS) indicates that the recognition site of this primer in callus and branches samples was not only affected by EMS, but was also affected by tissue culture, with regard to branch samples, all concentrations of the substance. The EMS was

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similar in its effect on the samples (no concentration of the EMS was superior to the rest of the concentrations, but they were all with the same effect). On the other hand, the concentration of 0.4% was the most effective in callus samples because it produced the highest number of divergent bands (6 bands), as shown in the tables (4), (7), (8), and Figure (2).

Table 4. The bands resulting from electrophoresis on 1.5% agarose gel for P1 with 9 samples studied in the presence of a Marker.

S / Bp	1	2	3	4	5	6	7	8	9
1700	0	0	0	0	0	0	0	1	0
1600	0	0	0	0	0	0	0	1	0
1500	0	0	0	0	0	0	0	0	1
1250	1	1	1	1	0	0	0	1	1
900	0	0	1	0	0	0	0	0	1
750	1	1	1	1	1	1	1	1	1
700	1	0	0	0	0	1	0	0	0
600	0	1	0	0	0	0	0	0	0
550	1	0	0	0	0	0	0	0	0
500	0	1	1	1	0	0	0	0	0
450	1	1	1	1	1	0	0	0	1
400	1	0	0	0	0	1	1	0	0
300	0	0	0	0	0	0	0	1	0
Marker	Control	Branches samples				Callus samples			

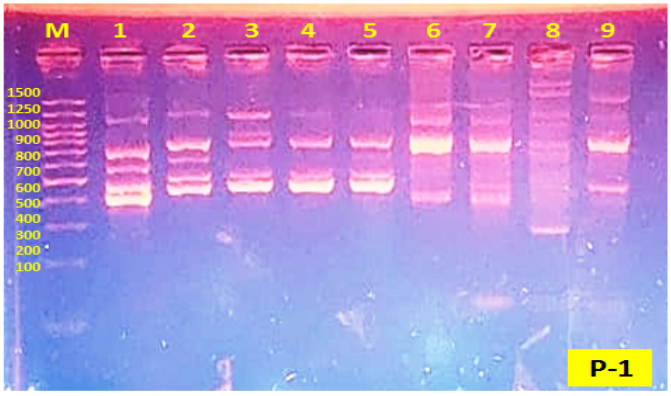

Figure 2. Bands resulting from electrophoresis of P1 with the study samples on the gel in the presence of Marker

P2 (OP D-10)

The number of recognition sites produced by this primer is 13 sites, they were all differentiated, as the total of the divergent bundles of this primer was 54, including 26 bundles for the callus (represented by samples from 6 to 9), which was close to the total of the divergent bundles produced by the branches (represented by the samples from 2-5) as it reached 28 differentiated bands. The efficiency of this primer was estimated at 5.9, while the discriminating power was 16.1.

As for the branches samples, unique band appeared in a concentration of 0.6% and two bands in the other concentrations and 6 bands were absent in each of the concentrations of 0.0% and a concentration of 0.4%. As for callus samples, one unique band appeared in a concentration

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of 0.0% only at 1250bp and did not unique band appear in the rest of the concentrations, but it was noticed that many bands were absent, as 5 bands were absent at a concentration of 0.0%. This primer was affected by tissue culture, when comparing the concentration of 0.0% of the samples of branches and callus with samples treated with the mutagen, it is noted that the mutagen had no significant effect because the numbers of divergent bands resulting from all samples were close, as shown in Tables (5), (7), (8), and Figure (3).

Table 5. Bands produced by electrophoresis on 1.5% agarose gel for P2 with 9 samples studied in the presence of a Marker

S / Bp	1	2	3	4	5	6	7	8	9
1700	1	0	1	0	0	0	0	1	0
1600	1	1	1	1	1	1	0	0	0
1550	0	0	1	0	0	0	0	0	0
1500	0	0	1	0	0	0	0	0	0
1450	0	0	0	1	0	0	0	0	0
1250	0	0	1	0	0	1	0	0	0
1100	1	0	0	0	1	0	0	0	0
1000	1	0	0	0	0	0	1	0	0
800	0	0	0	0	1	0	0	0	0
750	0	1	0	0	0	0	0	0	0
700	1	0	0	0	0	0	0	0	0
500	0	1	0	1	0	0	0	0	0
450	1	0	0	0	0	1	0	0	0
Marker	Control	Branches samples				Callus samples			

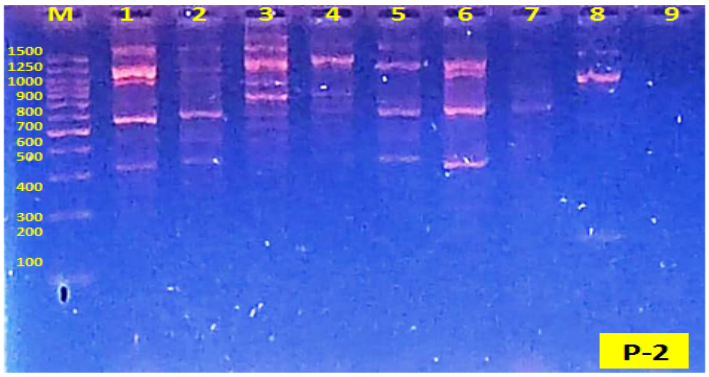

Figure 3. Bands resulting from electrophoresis of the P2 with the study samples on the gel with the presence of Marker

P3 (OP D-18)

16 recognition sites were obtained from this primer and they were all differentiated. As for the divergent bands of this primer, 66 differentiated bands were produced, 33 of which were for branch samples and the other 33 were for callus samples, with a contrast rate of 90%. As for the total number of bands that were produced by This primer consists of 73 bands whose sizes ranged between 100-2800bp. The efficiency of this initiator was 17.2 and its discriminatory power was 19.6.

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The unique bands of the branch samples, two unique bands appeared for each of the concentrations (0.2, 0.4, 0.6)%, and no unique bands appeared in the 0.0% concentration. As for the absent bands, 9 bands were absent in the concentration of 0.0% and 6 bands in the concentration of 0.6%. Evidence of the EMS caused the recognition site of this primer to appear in the samples of tissue branches. As for the callus samples, one unique band appeared in the concentration of 0.0% and two bands for each of the concentration (0.2, 0.4)%, and no band appeared in the concentration of 0.6%. As for the absent bands, the results showed the absence of 3 bands at a concentration of 0.0%. The number of absent bands increased to 10 bands at a concentration of (0.4, 0.6)%, which indicates that the effect of the EMS on the callus reversed its effect on the branches, as it caused the absence of bands in the callus. All the concentrations of EMS were similar in their effect on the samples of the branches, as they produced close numbers of divergent bands. As for the callus samples, the treatment with a concentration of 0.4% of the mutagen was superior to the rest of the concentrations, as it produced 12 differentiated bands, as shown in Tables (6)(7)(8) and Figure (4).

When the multiply bands of the study samples resulting from the RAPD-PCR reaction are compared with the bands of the control sample, the absence of bands is noticed in some samples (these bands are known as absent bands). This is consistent with the characteristics of RAPD, the reason for this is the absence of binding sites complementary to the primer on DNA the sample, and this may be caused in some samples by the activity of enzymes and DNA inhibitors, on the contrary, it may be observed that bands appear in the samples, although they are not present in the control sample. These bands are known as unique bands. The reason for the appearance of these bands is that the size of the multiplied pieces depends on the distance between the primer binding sites on the DNA strand. The presence of genetic stability of Dianthus chinesis due to the occurrence of variations in the recognition sites of the primers used in the study.

Table 6. Bands resulting from electrophoresis on 1.5% agarose gel for the P3 with 9 samples studied in the presence of Marker

S / Bp	1	2	3	4	5	6	7	8	9
2800	0	0	1	1	1	0	1	1	0
2000	0	0	0	0	1	0	1	1	0
1600	1	0	1	1	0	0	1	1	0
1500	1	0	1	1	1	1	1	0	1
1250	1	0	1	1	1	1	1	1	1
1000	1	1	0	0	1	1	1	0	0
900	1	1	1	1	1	1	1	0	0
800	1	1	1	1	1	1	1	0	0
750	1	1	1	1	1	1	1	0	0
700	1	0	0	0	1	1	0	0	0
600	1	1	0	1	0	1	1	1	1
400	1	0	0	0	0	1	0	0	0
350	0	0	1	1	0	1	0	0	0
300	1	0	0	0	0	0	0	0	0
200	1	0	0	0	0	1	0	0	0
100	1	0	0	0	0	0	0	0	0
Marker	Control	Branch samples				Callus sample			

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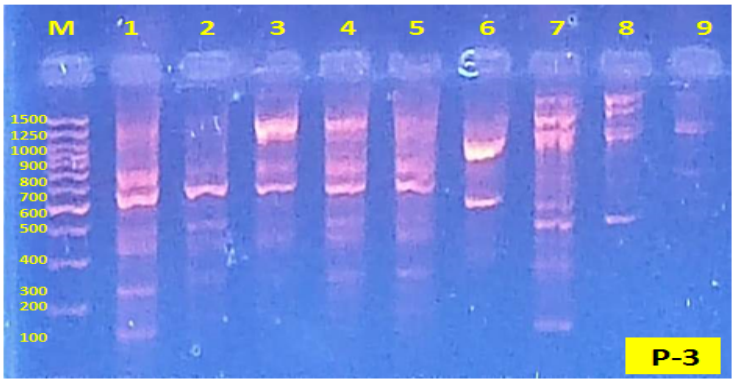

Figure 4. Bands resulting from electrophoresis of P3 with the study samples on the gel in the presence of the Marker

Table 7. Results of the Primers used in the study

Primer	Resulting sites	Public sites	Variance sites	Total bands	Public bands	Variance bands	Unique bands	Absence bands	Variance ratio%		
									Total	Branches	callus
P1	12	1	11	34	8	31	10	21	91	41	50
P2	13	0	13	25	0	54	8	46	100	50	50
P3	16	0	16	73	0	66	11	55	90	45	45

Table 8. The variance bands and the discriminatory ability of the primers used in the study and their efficiency

Primers	Base pair	Branch samples(EMS%)								callus samples(EMS%)								Efficiency	Discriminatory ability
		0.0		0.2		0.4		0.6		0.0		0.2		0.4		0.6			
		Unique	Absent	Unique	Absent	Unique	Absent	Unique	Absent	Unique	Absent	Unique	Absent	Unique	Absent	Unique	Absent		
P1	1700-300	2	2	2	2	1	2	0	3	0	3	0	4	3	3	2	2	8.1	9.2
P2	1700-450	2	6	2	4	2	6	1	5	1	5	0	6	0	7	0	7	5.9	16.1
P3	2800-100	0	9	2	7	2	5	2	6	1	3	2	5	2	10	0	10	17.2	19.6

Conclusion

The analysis revealed genetic variations among the samples of Dianthus chinesis, indicating that both EMS treatment and in vitro cultivation exerted an influence. The RAPD primers exhibited variability in terms of their efficiency and discriminatory capacity. The research specifically drew attention to the observation that EMS has an impact on the recognition sites of primers, leading to the occurrence of genetic variants. The examination of the findings from each primer revealed the presence of distinctive and absent bands. It was observed that the concentrations of EMS had an impact on the presence or absence of these bands. Distinct patterns were observed in callus samples in comparison to tissue branch samples, underscoring the necessity of employing tissue culture techniques to ensure genetic stability.

Based on the available evidence, it can be observed that electromagnetic field stimulation (EMS) treatment and in vitro culture exert an influence on the genetic stability of Dianthus

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chinesis. This study emphasizes the importance of considering the impact of tissue culture on genetic stability and offers valuable insights into the genetic variations induced by carcinogenic treatments. Further research and investigation into the various variants of Dianthus chinesis could potentially yield methods to augment its qualities for both horticultural and medicinal purposes.

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