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Somaclonal Variation in Callus Cultures of Rose Periwinkle, *Catharanthus Roseus L.* Under Induced Salt and Osmotic Stresses

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Abstract

The experiment was carried out at the laboratories of the National Commission for Biotechnology in Syria during the period 2020-2022 to detect the somaclonal genetic variation occurring in callus cultures at different ages and to compare them with the *In vitro* growing plants and introduced species of Rose Periwinkle that grew up in the Syrian environment, in addition, exploring the effect of abiotic stresses in causing genetic variations in callus, by the Inter Simple Sequence Repeats technique (ISSR) using 21 primers. Seeds were germinated on an MS nutrient medium devoid of growth regulators; explants were transferred to a propagation medium fortified with Naphthalene Acetic Acid (NAA) (1 mg L^{-1}) and 6-Benzyladenine (BA) (2 mg L^{-1}). Callus was developed from Rose periwinkle *Catharanthus roseus L*. leaves using 5C01 medium fortified with NAA and Kinetin (Kin). After that, callus was exposed to gradually increasing levels of PEG-6000-induced osmotic stress and salt stress (NaCl) with a higher level every 30 days. The molecular study results proved the effectiveness of all the used primers (21 primers) in presenting polymorphism between the studied samples at a rate of 92.16%. The cluster analysis showed separating the analyzed samples into two

main clusters. The first cluster contained the osmotic stressed callus, while the second cluster separated into two sub-clusters that had the rest of the studied samples. It was found that there were genetic variations between *In vitro* plants and the callus. In addition, the stressed callus (salt and osmotic) was compared to the unstressed callus, and this confirmed that callus cultures were an essential source of genetic variations, and the ISSR technique was an effective tool for detecting these variations.

Keywords
Catharanthus roseus L.; callus; salt stress; polyethylene glycol; somaclonal variation; ISSR

1. Introduction

Rose periwinkle*, Catharanthus roseus* L. It is one of the most important medicinal plants recognized in the international pharmacopoeia. This plant belongs to the Apocynaceae family [1], which includes a large number of genera, and the genus *Catharanthus* is the most important, as this genus consists of eight species *C. coriaceus, C. longifolius, C. lanceus, C. ovalis, C. pusillus, C. trichophyllus, C. scitulus*, in addition to *C. roseus*. It is widespread in warm regions of the world, characterized by the ability to withstand high temperatures, and grown as an ornamental plant in gardens and parks due to the variety of flower colors (white, pink, and purple) [2, 3].

Rose periwinkle, Catharanthus roseus L. plant contains more than 130 indole alkaloids [4, 5]. Recent studies have been able to identify the active components of *C. roseus,* such as Vincristine (VCR) and Vinblastine (VLB), which have anti-cell division properties [6, 7]. Due to the low concentration of alkaloids in cultivated plants, the extraction process of (VLB) and (VCR) is usually costly and exhausting [8]. This prompted researchers to work on extracting these medically valuable compounds in larger quantities through modern biotechnology methods, such as tissue culture [9], which is based on the Totipotency theory, which is the ability of any living cell to form a complete plant, and the genetic information required for the manufacture of secondary metabolites is present in the callus cells. By activating these genes, the required secondary metabolites can be produced [10].

The application of biotechnology in the *C. roseus* plant, especially callus cultures, will lead to new genotypes with good characteristics based on the somaclonal genetic variation in *In vitro* cell populations [11]. Moreover, applying these technologies in the presence of the stress factor in the nutrient medium will improve tolerance to abiotic stresses, with an increase in the production efficiency of secondary metabolites. Callus culture technology is considered one of the important technologies as a new source of secondary metabolites [12], and to obtain the necessary somaclonal variation (genetic variation) in breeding programs [13]. The findings of [14] showed that somaclonal variation through *In vitro* regeneration might be used to create new phenotypes from Rose Periwinkle. Many studies, such as [15] showed that somaclonal variation may be exploited for *C. roseus* callus improvement, which is used as an alternative source of bioactive metabolites.

The term somaclonal variation was used as an indicator to denote the changes that occur in regenerative plants from tissue culture and plant cells, and it is known that such changes occur frequently when protoplasts are isolated also in callus culture [16]. Somaclonal variation describes

the genetic and non-genetic changes in plants (the variation in physiological, biochemical, and genetic characteristics among *In vitro* cells). It may appear during or after the *In vitro* culture of plant cells, organs, or calluses [17]. These variations can be detected using one of the molecular markers methods. The Inter Simple Sequence Repeats technique (ISSR) is an effective method for detecting the somaclonal variation expected to occur in calluses in the presence or absence of the stress factor, which is a helpful tool in detecting such variations [18]. It has been used to detect genetic variation in many crops, such as potato, *Solanum tuberosum* [19], and barley, *Hordeum vulgare* [20], as well as to reveal the somaclonal variation among *In vitro* callus cultures in comparison with the mother plant (*In vitro*) of many medicinal plants, such as Golden Henbane, *Hyoscyamus aureus* [21].

This research aimed to study the somaclonal variations in callus cultures of Rose periwinkle, *C. roseus* plant at different ages (14, 21, and 30 days) and compare them with the introduced species and *In vitro* plants. In addition, determine the effect of induced osmotic stress and salt stress on causing genetic changes by using the ISSR technique.

2. Materials and Methods

2.1 Location of Research

This research was conducted at the Biotechnology of Medicinal Plants Department laboratories, the National Commission for Biotechnology, Damascus, Syria, from 2020-2022.

2.2 Plant Material

C. roseus seeds were obtained from the Dutch company Syngenta Flowers (goldsmith seeds) and germinated In vitro. As for the introduced species, the plant was presented to Syria and classified by Damascus University. It is adapted to the Syrian environment and is widespread as an ornamental plant.

2.3 Seeds Germination

The seeds were sterilized with ethanol (from SIGMA) and then with 0.5% sodium hypochlorite NaOCl solution (from DTEC) for 5 minutes, then washed with sterile distilled water three times at a rate of 5 minutes each time. Seeds germinated using pyrex® tubes (25 × 150 mm) in the MS primary culture medium [22] without growth regulators. The boxes were incubated at 24 ± 2°C, 16 hours of light, and 8 hours of darkness alternately until the seed germinated. After 45 days, they were re-planted on MS medium supplemented with two hormones, 6-benzyl adenine (BA) (2 mg L^{-1}) and Naphthalene Acetic Acid (NAA) (1 mg L^{-1}) (from MERCK) [23], to obtain sufficient plant material needed to carry out the callus development experiments.

2.4 Callus Development

Callus was developed from the leaves of *C. roseus* after 90 days on 5C01 nutrient medium [24] by using pyrex® tubes (25 × 150 mm), and fortified with a group of vitamins (from MERCK), such as Myo-inositol (0.08 g), and casein (0.5 g), in addition to some plant growth regulators NAA (1 mg L^{-1}) and Kinetin (Kin) (2 mg L^{-1}). The samples were taken at 14, 21, and 30 days before applying stress factors.

2.5 Stress Applied to Callus Cultures

Calluses were cultured in tubes with the same 5C01 media and supplementary hormones, with the addition of progressively increasing levels of a stress factor (NaCl) (0, 30, 60, 90, and 120 mM) and polyethylene glycol PEG-6000-induced osmotic stress (0, -0.2, -0.4, and -0.6 Mpa). Calluses were transferred to a higher level every 30 days.

2.6 Study of Somaclonal Variations

Somaclonal variations were studied in all samples: the introduced species, the *In vitro* plant, the unstressed callus at ages (14 days, 21 days, and 30 days), the salt-stressed callus, and the osmotic stress callus, the ISSR technique was used. Studying these variations was carried out by the following steps:

2.6.1 DNA Isolation by Cetyltrimethylammonium Bromide (CTAB) Method [25]

Approximately 200 mg of the frozen and ground plant material, in liquid nitrogen, for all the studied samples was placed in a 2 ml Eppendorf tube, then 750 µl of preheated CTAB extraction solution at 65°C was added, and the mixture was stirred well. The extraction solution consisted of the following materials:

(2% w/v) CTAB (Vivantis) + 100 mM Tris-Hcl (pH = 8) (Vivantis) + 1.4 M NaCl (ScP) + 20 mM EDTA (pH = 8) (AVONCHEM) + 0.2% mercaptoethanol (v/v).

The tubes were placed in a water bath at 65°C for an hour. 750 µL of chloroform: isoamyl alcohol (24:1) (from Scharlau) was added to remove proteins and lipids. The tubes were centrifuged with a Heraeus centrifuge at a speed of (12000 rpm) for 10 minutes at a temperature of 4°C. At this stage, the mixture was separated into three phases. Isopropanol from (Merck) was added to the resulting aqueous phase by stirring gently until DNA precipitated. The next day, the tubes were centrifuged with cold washing buffer (70% ethanol stored at -20°C), then the DNA samples were dissolved in 100 µl of TE solution.

2.6.2 Quantitative and Qualitative Determination of DNA

A Hitachi U2900 UV Spectrophotometer was used to quantify DNA and determine its purity at wavelengths of 260 and 280 nm [26], and the DNA concentration was calculated from the following mathematical equation [26]:

$$\text{DNA concentration} = 50\ \mu g/mL \times OD_{260} \times \text{dilution factor}$$

Then, the DNA samples were diluted to obtain a concentration of 50 ng μl^{-1}, and the qualitative assessment was done on an agarose gel (0.8%) (From SIGMA), as the excellent quality DNA appeared in the form of a band. In contrast, the lousy-quality DNA was smeared.

2.6.3 DNA Amplification

The DNA was amplified by polymerase chain reaction (PCR) according to the protocol of Williams *et al* [27], with some modifications so that the final reaction volume was (25 µl), consisting of 2 µl of DNA at a concentration of (50 ng μl^{-1}) per sample, and 12.5 µl Master Mix (Kapa) and 1.25 µl of the primer at a concentration of (10 pmol μl^{-1}), then complete the volume to 25 µl with sterile distilled water. The PCR reaction was performed using the PCR kit (Kapa 2G fast-ready Mix) according to the manufacturer's instructions. The DNA bands were separated by electrophoresis on an agarose gel (2%) in a 1X TBE solution.

2.6.4 ISSR Technique Application

A group of primers (Table 1) from BioNeer Company was used to study the genetic and somaclonal variations occurring in callus cultures. After that the polymorphic ratio of the used primers was calculated, and a dendrogram of the genetic relationship was drawn.

Table 1 Primers tested in the ISSR technique and their Annealing temperatures.

Primers	Sequence 5'-3'	Annealing temperature	Primers	Sequence 5'-3'	Annealing temperature
A1	(CT)8TG	48.7	L12	(CAC)3GC	38
B2	(CT)8AC	47.7	M13	(GAG)3GC	38
C3	(CT)8GC	51.5	N14	(CTC)3GC	38
D4	(CA)6AC	42	P15	(GTG)3GC	38
E5	(CA)6GT	42	Q16	(GT)8C	52.3
F6	(CA)6AG	42	R17	(AC)8G	54.3
G7	(CA)6GG	44	S18	(AGG)6	57.2
H8	(GA)6GG	44	T19	(GA)9T	50
I9	(GT)6GG	44	U20	(GACA)4	47.9
J10	(GA)6CC	44	V21	T(GA)9	51.4
K11	(GT)6CC	44	-	-	-

2.6.5 Electrophoresis, Staining and Imaging

The migration was carried out on a 2% agarose gel (Agarose Ultrapure Fingerprinting USA) in 1X TBE solution and with red-safe dye (from intron). An electric field of 90 volts was used for two hours to separate the DNA bands resulting from the amplification, and a (DNA marker -100 bp from JenaBioscience) to determine the molecular size of the resulting bands. After that, the gel was imaged with the Imag Analyzer (Agle Eye II teratogen).

2.7 Statistical Analysis

The Total Lab Program was used to analyze the results of the molecular study. The results of the amplification process were collected in tables based on comparing the presence or absence of DNA bands between samples. The somaclonal variations between the samples were studied using the Jaccard coefficient, and then the Dendogram of the genetic relationship was drawn by applying the

UPGMA (Unweighted Pair Group Method with Arithmetic Averaging) using the Power Marker Program.

3. Results

3.1 Polymorphism Resulting from the Application of the ISSR Technique

The molecular study included exploiting 21 primers, all of which proved effective in exhibiting polymorphism among the studied samples (Table 2) (Figure 1), and resulted in 141 bands. The number of bands ranged between a minimum 3 bands for primers (I9 and Q16), and 11 bands as a maximum in the primers (T19), with an average of 6.71 bands per primer. The number of polymorphic bands was 128, and ranged between 3 bands for the primers (I9 and Q16) as a minimum and 10 bands for the primers (T19 and A1), with an average of 6.10 for each primer. The standard of polymorphic ratio was 92.16%, the lowest in the primer H8 (57.14%), and the highest in the primers A1, B2, D4, I9, J10, L12, M13, N14, P15, Q16, S18, and U20, reaching 100%.

Table 2 The polymorphism percentage resulting from using ISSR technique.

Primer	T-B	P-B.	Polymorphism %	A	B	C	D	E	F	G	Total bands
A1	10	10	100.00	8	6	7	6	7	6	2	42
B2	6	6	100.00	5	5	4	4	5	3	0	26
C3	8	7	87.50	3	7	7	3	7	5	1	33
D4	6	6	100.00	2	2	4	4	4	4	2	22
E5	8	7	87.50	6	6	5	4	4	4	1	30
F6	9	7	77.78	6	9	9	6	9	7	2	48
G7	9	7	77.78	6	9	9	6	9	7	2	48
H8	7	4	57.14	8	7	7	6	7	8	3	46
I9	3	3	100.00	1	3	0	1	2	2	0	9
J10	4	4	100.00	2	3	3	3	3	3	1	18
K11	10	9	90.00	9	6	9	7	7	7	1	46
L12	8	8	100.00	8	4	5	5	5	3	0	30
M13	4	4	100.00	1	3	2	4	3	3	0	16
N14	4	4	100.00	3	3	3	1	3	2	0	15
P15	6	6	100.00	4	4	5	5	5	4	0	27
Q16	3	3	100.00	2	2	2	3	2	3	0	14
R17	6	5	83.33	5	4	6	5	4	4	1	29
S18	6	6	100.00	1	2	2	1	1	3	3	13
T19	11	10	90.91	5	5	4	6	4	6	3	33
U20	7	7	100.00	4	5	5	3	5	3	3	28
V21	6	5	83.33	4	4	4	4	4	3	4	27
Total sum	141	128	-	93	99	102	87	100	90	29	600
Mean	6.71	6.10	92.16	4.43	4.71	4.86	4.14	4.76	4.29	1.38	-
P-B	-	-	-	91	95	100	85	97	87	22	577
Polymorphism %	-	-	-	97.85	95.96	98.04	97.70	97.00	96.67	75.86	94.15

T-B: Total bands, P-B: Polymorphic bands, A: introduced species, B: *in vitro* plants, C: 14-day-old callus, D: 21-day-old callus, E: 30-day-old callus, F: salt stressed callus, G: induced osmotic stressed callus.

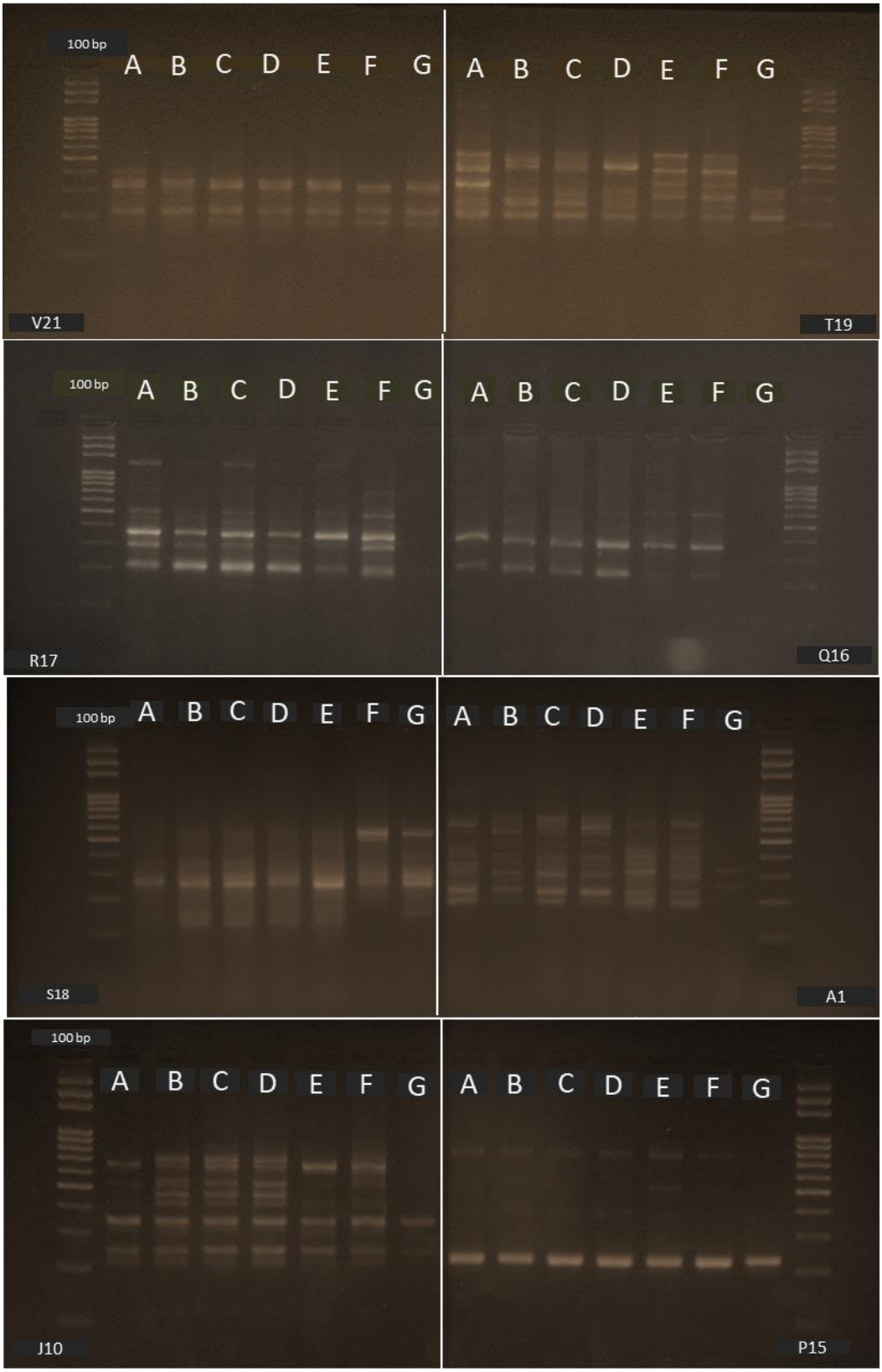

Figure 1 Migration images of ISSR-PCR for studied samples (Primers: V21, t19, R17, Q16, S18, A1, J10, P15). A: introduced species, B: *in vitro* plants, C: 14-day-old callus, D: 21-day-old callus, E: 30-day-old callus, F: salt stressed callus, G: induced osmotic stressed callus.

The total number of bands was (93, 99, 102, 87, 100, 90, and 29 bands). The number of polymorphic bands was (91, 95, 100, 85, 97, 87, and 22 bands) for introduced species, *In vitro* plant, 14-day-old callus, 21-day-old callus, 30-day-old callus, salt-stressed calluses, and osmotically stressed calluses, respectively. Moreover, it was noted that the highest polymorphism percentage was in the 14-day-old callus (98.04), followed directly by the introduced species (97.85%). In comparison, the polymorphism percentage in salt-stressed calluses was approximately 96.67%, and in osmotic-stressed calluses, 75.86%, it was about 95.96% for *In vitro* plants.

When applying the Percent Disagreement Values (PDV) (Table 3), where the high values indicated the presence of genetic variation and this genetic variation increased with the increase of these values, the lowest value of Jaccard coefficient was 0.1121 for 14-day-old calluses and 21-day-old calluses, and this indicated that they were the most similar genetically, while the highest value was approximately 0.8364 for the *In vitro* plant and the osmotic stressed callus, which stated that they were the farthest genetically.

Table 3 Percent Disagreement Values based on the Jaccard coefficient.

	A	B	C	D	E	F	G
A	0						
B	0.359	0					
C	0.3043	0.1892	0				
D	0.3362	0.1402	0.1121	0			
E	0.3636	0.4878	0.3983	0.4153	0		
F	0.4359	0.5118	0.464	0.4426	0.3143	0	
G	0.8155	0.8364	0.8198	0.8165	0.7789	0.7857	0

A: introduced species, B*: in vitro* plants, C: 14-day-old callus, D: 21-day-old callus, E: 30-day-old callus, F: salt stressed callus, G: induced osmotic stressed callus

3.2 Cluster Analysis of Cellular Lines of C. Roseus Callus, the Introduced Species Plant and the In Vitro Plant:

Cluster analysis (Figure 2) showed the separation of the studied samples according to the degree of genetic similarity, as the models were separated into two main clusters. The first cluster contained the osmotic-stressed callus in a separate collection and was genetically the farthest from the other samples. In contrast, the second cluster was separated into two sub-clusters. Under the first sub-cluster, the salt-stressed callus and the 30-day-old callus were included, while the second sub-cluster was divided into two parts. The first part contained the introduced species, while the second part contained the *In vitro* mother plant in the glass, the 14-day-old callus, and the 21-day-old callus, as these treatments were genetically closest.

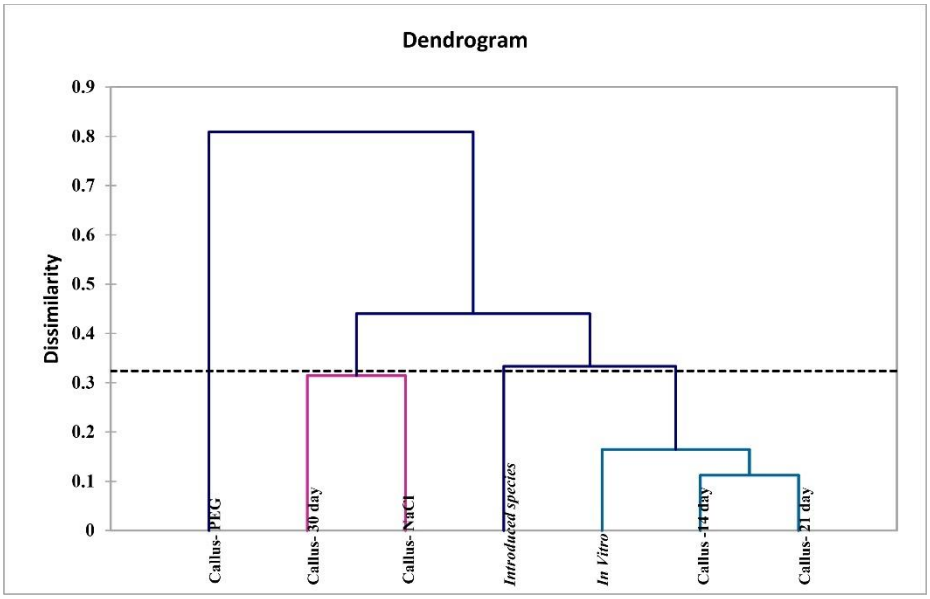

Figure 2 The cluster analysis of the studied samples.

4. Discussion

The somaclonal variation primarily results from mutations that take place during tissue culture. Variable stress conditions such as culture media (hormonal imbalance), tissue exposure to chemicals during surface sterilization, and wounding can lead to mutations. Oxidative stress was discovered to be the primary source of somaclonal variation in tissue culture plants in many instances [28].

Plant growth regulators are considered one of the main contributors to somaclonal variations. According to studies, tissue culture conditions with an imbalanced hormone ratio can increase polyploid plants. In contrast, their absence or insufficient use might lead to the production of typical plants [29, 30]. In addition to the hormone concentration, the type of hormone utilized in culture media can also result in somaclonal variation [31]. For instance, increasing the rate of DNA methylation by adding auxins such as 2,4-Dichlorophenoxyacetic acid (2, 4D) and NAA to the growth conditions of callus or cell suspension [32].

Depending on the source, explants have different levels of genetic stability [33]. Meristematic or undifferentiated tissues like cambium and procambium explants minimize variations. However, highly differentiated tissues, such as stems, roots, and leaves, result in more variations [34].

It was noted that there were genetic variations between the introduced species*, In vitro* growing plants, and the resulting callus. The callus cells lost the differentiation and returned to the meristematic division state, thus becoming morphologically, physiologically, and genetically different from the *In vitro* growing plants [35]. These results were consistent with the results of Ikeuchi *et al*. [35], which confirmed that the types of callus cultures that belong to the same plant were genetically different. These differences are because the process of callus development includes massive changes in gene expression due to changes in the level of cell differentiation [36]. This confirms that the interaction between environment and genotype limits the characteristic of genetic variations in callus cultures. These differences are attributed to a group of mechanisms, such as changes in the methylation of DNA, the release of the genetic material outside the cell membrane or what is known as Exocytosis, the exchange of the genetic material between cells, and the loss of a large amount of DNA especially in polyploid cells [37].

Through cluster analysis, it was noted that there were genetic variations between the Rose Periwinkle *C. roseus* introduced species, which is widely cultivated and adapted to the Syrian environment, and the *In vitro* plant, whose source was the seeds imported from Syngenta Flowers Company, and this confirmed the need to use seeds from a reliable source to ensure the genetic purity of the variety on which studies were required. Moreover, it was noted that there were genetic variations between the *In vitro* and the resulting callus, in addition to the occurrence of genetic variations during the growth stages of the callus. This confirmed the importance of callus cultures as an essential source of genetic variations and the effectiveness of the ISSR technique in detecting these variations. Furthermore, callus culture technology is helpful in cellular selection processes to reach cell strains that can be highly productive of biologically active substances. These results were consistent with the results of [38] in their study on genetic variations induced by tissue culture of *Gentiana* spp., and the effects of [21] in their reflections on the Golden Henbane, *Hyoscyamus aureus* who, showed that genetic variations occurred between the *In vitro* plant and the resulting callus, where the callus separated by a distance of 0.33 from the *In vitro* plant. [39] Stated that abiotic stresses affected morphological, physiological, chemical, and molecular traits and negatively affected growth and productivity. In addition to many studies that proved the damage caused by free radicals resulting from abiotic stresses at the cellular level [40-42], free radicals attacked all cell components, such as proteins, enzymes, and nucleic acids, especially when the enzymatic defense systems were ineffective, and caused mutations at the DNA level, represented by a change in the chromosome number, aneuploidy as a result of a break in the DNA chain, in addition to deletions and replacements in the nitrogenous bases involved in the structure of DNA [43]. This study also agreed with the results of [44], who showed the effect of the salt medium in causing genetic variations in callus developed from the leaves of corn, *Zea mays* L.

5. Conclusions

Callus culture technology was an essential source of genetic variations that can be exploited in selecting cell lines with specific and targeted traits, and the ISSR technique was effective in detecting these genetic variations that appeared between the callus and the *In vitro* plant. Moreover, instability was observed in the genetic structure of the callus during its growth period, as the genetic structure of the callus changed between the ages of 14 days and 30 days, and the addition of the stress factor to the culture medium resulted in genetic variations between the unstressed callus and the salt and osmotic stressed callus. On the other hand, cluster analysis and the dendrogram of genetic relationship showed the separation of the studied samples into two main clusters, and the PEG-induced osmotic stress was the most isolated from other calluses, as it lacked most of the DNA bands that appeared in the different samples.

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Author Contributions

All authors participate in the research concept and design. The first draft of this manuscript, material preparation, and data collection were performed by the first author; data analysis and manuscript proofreading were performed by the second author.

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Competing Interests

The authors have declared that no competing interests exist.

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