ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **30**(2): 209-218, 2020 (December)

PTC&B

Chemical Mutagens Affecting *in vitro* Behavior of *Gardenia jasminoides*

Eman Tawfik* and Mohamed Fathy[1]

Department of Botany and Microbiology, Faculty of Science, Helwan University, Egypt

Key words: Chemical mutagens, *Gardenia jasminoides*, Guanidine hydrochloride, Photosynthetic pigmentations

Abstract

The chemical mutagens, namely sodium azide and guanidine hydrochloride (30, 40 and 50 mM) for each were applied for the *in vitro* culture of *Gardenia jasminoides* Ellis. Eight morphological traits were evaluated to calculate the result of different concentrations of mutagens compared to control. In sodium azide, all morphological traits decreased compared to control, except the number of branches and leaves and leaf length, which increases compared to control. Unlike guanidine hydrochloride, all the morphological traits are enhanced in mutants rather than control. The polymorphism percentage resulted from genetic variation varied (57.71% in the case of sodium azide and 67.59% in guanidine hydrochloride). Hence, guanidine hydrochloride is a more powerful mutagen and causes more genetic variations and instability than sodium azide, as was reflected in morphological parameters (i.e., fresh weight, shoot length, root length, number of lateral roots, number of leaves, numbers of branches, leaf length, and leaf width) and physiological traits (i.e., chlorophyll pigmentation and carotenoids).

Introduction

Gardenia jasminoides (Ellis) is an ornamental plant with fragrant white flowers and is commonly known as gardenia or cape jasmine. It is an evergreen plant and belongs to the family Rubiaceae. It is also known as palm lily and cappage palm. It grows in warm-temperate, humid and tropical regions. It is usually cultivated indoors or outdoors in gardens. It prefers bright indirect sunlight or partial shade rather than direct sunlight. It is preferred to be cultured in quite acidic medium or soil (4.5 - 5.5) to obtain all nutrients. It is also considered a medicinal plant as it has numerous biological activities in both *in*

*Author for correspondence: <emantawfik@science.helwan.edu.eg>. [1]Department of Horticulture, Faculty of Agriculture, Ain Shams University, Egypt.

DOI: https://doi.org/10.3329/ptcb.v30i2.50691

vitro and *in vivo* conditions. It has antidiabetic, antioxidant, antidepression, anti-inflammatory properties. It is also used to treat wounds, fever, hepatitis, influenza (Soliman et al. 2013 and Xiao et al. 2017 and Kesavan et al. 2018).

An induced chemical mutation is a method to make genetic variation leading to new varieties with better characteristics. Induced mutations are also used to provide a novel source of resistance to biotic and abiotic stress factors whereby a new resistant variety can be developed (El-Mokadem and Mostafa 2014, Suprasanna et al. 2015).

Sodium azide (NaN_3) is one of the most robust chemical mutagens used in plants. Its application on the plant is easy and inexpensive and creates mutations to improve their traits. The efficiency of the mutant relies on several circumstances, like the concentration of azide and the treatment duration. It creates point mutation and damages the chromosomes and produces tolerance in the plants for numerous adverse conditions (El-Mokadem and Mostafa 2014).

Guanidine hydrochlorides are a group of chemical mutagens capable of causing alkylation for nitrogenous bases of DNA structure. The guanidine hydrochlorides act as energy transfer inhibitors. There are three groups of guanidine hydrochlorides: Phenylethylbi-guanidide causes only partial inhibition even at relatively high concentrations. Decamethylenedi-guanidide inhibits about 70% of the malate respiration, 55% of the succinate respiration, and 35% of the ascorbate-tetramethyl-p-phenylenediamine respiration. Finally, octyl-guanidine hydrochloride inhibits all three phosphorylation sites and the cyanide-insensitive respiration, but to differing extents and at different concentrations (Wilson and Bonner 1970).

This work aimed at studying the effect of sodium azide and guanidine hydrochloride as examples of two chemical mutagens on the *in vitro* growth of *Gardenia jasminoides*. Also, it aimed to produce genetic variation in the morphological and chlorophyll content. RAPD-PCR is used as a molecular marker to detect variations.

Materials and Methods

*Gardenia jasminoides (*Ellis) plants were collected from the gardens of the Faculty of Agriculture. Two different mutagens used were sodium azide and guanidine hydrochloride. The nodal explants were sterilized and cultured on MS. Then the plantlets were regenerated twice on ¾ MS growth regulator-free media for multiplication. The plantlets were cut into nodal explants and immersed in different concentrations of mutagens for 3 hrs. The explants were then washed in sterile distilled water, transferred to new MS jars, and incubated for 5 weeks in 16 hrs light/8 hrs dark incubators at 25^{o}C.

Eight selected morphological parameters such as fresh weight, shoot length, roots length, number of lateral roots, number of leaves, numbers of branches, leaf length, and leaf width.were used to compare the mutants with the control plants.

Chlorophylls were estimated for both mutants and the control plants. Chlorophyll a, b and carotenoids were determined in the fresh leaves of the plantlets, according to Metzner et al. (1965). Fresh leaves of known weight were homogenized in 85% acetone. After centrifugation the supernatant containing the pigments was made up to a definite volume with 85% acetone. The extract was measured against a blank of pure 85% aqueous acetone at three wavelengths of 452, 645 and 664 nm using a colorimeter. The concentration of chlorophyll a, b and carotenoids were calculated as µg/ml using the following equations:

Chlorophyll a = 10. 3 E664 - 0.918 E645 (1)

Chlorophyll b = 19. 7 E645 - 3.87 E664 (2)

Carotenoids = 4. 3 E452 (0. 0265 Chl. a + 0. 426 Chl. b) (3)

Then, the fractions were calculated as mg/g fresh weight:

$$\frac{\text{Fraction} \times \text{dilution}}{1000}\ \text{mg/g} \qquad (4)$$

The total genomic DNA of *G. jasminoides* (different treatments) was isolated using CTAB method, according to Doyle and Doyle (1990) and modified by Edwards et al. (1991). A 0.5 g of leaves were ground with 700 µl of 2% CTAB buffer and incubated at 65ºC for 30 min with vortex every 10 min. The Eppendorf tubes were centrifuged at 12,000 rpm for 10 min and the supernatant was transferred into new tubes. Equal volume of chloroform: isoamyl alcohol mixture (24 : 1) and the supernatant was added to each other and set for 2 min at room temperature then centrifuged at 12,000 rpm for 10 min at 4ºC. The upper aqueous layer was transferred into new tubes and 800 µl of absolute ice-cold ethanol was added and left for about 2 hrs at –20ºC, then centrifuged to precipitate DNA pellets and then washed with 70% ice-cold ethanol. Finally, the pellets were resuspended in 50 µl of TE buffer and kept at –20ºC until the RAPD-PCR reaction was applied.

Seven RAPD decamer primers were used in this bioassay. Only 6 of them gave reproducible clear bands (Table 1). These primers were listed in Table 3. RAPD-PCR reaction was carried out in Biometra thermocycler. The reaction mixture was carried out in a total volume of 25 µl containing 12.5 µl Taq master mix (COSMO PCR RED M. Mix, W1020300x), 2 µl of genomic DNA, 1 µl for each primer (Willowfort) and 9.5 µl ddH_2O. The reaction program was 35 cycles of the following steps: Denaturation for 30 sec at 94ºC, annealing 30 sec at different degrees for each primer as shown in Table 3 and extension for 1 min at 72ºC. This was followed by one step of the final extension at 72ºC for 10 min then cooling at 4ºC. The amplified PCR product was run on 1.2% agarose gel compared to (New England Biolab, #N3232S) ladder.

The gel electrophoresis images were analyzed using Bio-Rad Quantity one (4.6.2) software. The presence of a band was scored as 1, whereas the band's absence was coded

as 0. A pairwise similarity matrix was generated using Jaccard's similarity coefficient. Using the unweighted pair group method with the arithmetic averaging algorithm (UPGMA), cluster analysis was performed to develop a dendrogram (Shuaib et al. 2007).

Table 1. List of primers, their nucleotide sequences used in the RAPD analysis of *Gardenia jasminoides.*

No.	Primer	Sequence	GC%	Tm
1	OPA-03	5′-AGTCAGCCAC-3′	60	32
2	OPA-04	5′-AATCGGGCTG-3′	70	34
3	OPB-17	5′-AGGGAACGAG-3′	60	33.1
4	OPB-18	5′-CCACAGCAGT-3′	60	32.6
5	OPD-07	5′-TTGGCACGGG-3′	70	34.8
6	OPM-07	5′-CCGTGACTCA-3′	60	33.2

Data collected were subjected to analysis of variance test in SPSS 21. Mean average, standard deviations and correlations were estimated. Significant means were separated using Tukey test multivariate analysis. Tukey homogeneous subsets were applied for the different 25 treatments using one-way ANOVA for the two factors using multivariate analysis.

Results and Discussion

The variations in morphological parameters are shown in Fig. 1 and Table 2) for *Gardenia jasminoides*. There were significant differences due to the applications of treatments. Sodium azide stimulated a number of branches, number of leaves and leaf length significantly as against guanidine hydrochloride, which caused stimulation in all growth and morphological parameters except root length.

For photosynthetic pigmentation of *G. jasminoides*, chlorophyll content was estimated for the response of these plants to the different concentrations of mutagens (Table 3). It was noticed that chlorophyll a, b and carotenoids are inhibited using both the mutagens. All the mutant's pigmentation decreased significantly compared to control individuals. The p value of these morphological parameters and chlorophyll pigmentations showed a significant difference in various mutagens concentrations.

Gardenia jasminoides being an ornamental and medicinal plant it can be mutated to enhance their decorative characters and increase their therapeutic value.

Malaysian Nuclear Agency (Nuclear Malaysia) has started research on improvement of ornamental plants' through induced mutations. Nuclear Malaysia has produced more than 20 new varieties of ornamental and landscaping plants. These new varieties have been transferred to various end-users, private nurseries and government agencies

(Ahmad et al. 2012). Sodium azide was used in many studies to induce mutation, as found by El-Nashar (2006) on *Amaranthus caudatus*, Al-Gawwad and Makka (2009) on *Mirabilis jalapa* and Mostafa (2011) on *Helianthus annuus*. Al-Qurainy (2009) used sodium azide mutagen with *Eruca sativa* and an estimated 9 morphological parameters and photosynthetic pigments. He proved that sodium azide causes significant differences. Also, El-Nashar and Asrar (2016) used chemical mutagens to assess the variation in *Calendula officinalis* as ornamental and medicinal plants. They estimated many morphological and physiological parameters and found significant differences in mutants compared to control.

Fig. 1. Morphology of *Gardenia jasminoides* in response to different concentrations of mutagens. A. Effect of sodium azide and B. Effect of guanidine hydrochloride.

For molecular marker analysis, the total genomic DNA was extracted and RAPD-PCR was performed to estimate the genetic variation resulted from mutagen treatments. The results of selected oligonucleotides decamers were visualized on an agarose gel (Fig. 2a, b) for sodium azide and guanidine hydrochloride, respectively. The polymorphism data resulted from these primers are illustrated in the Table (4). The total polymorphism percentage of *G. jasminoides* affected by sodium azide was 57.71 as against 67.59 in guanidine hydrochloride treatment. Tables 5 and 6 showed the similarity matrix of the different concentrations of sodium azide and guanidine hydrochloride, respectively. The dendrogram showed the genetic effect of sodium azide on *G. jasminoides* (Fig. 3). It illustrated that control and 30 mM treatment were closely related to each other, where 40 and 50 mM treatments were related. The genetic effect of guanidine hydrochloride is shown in Fig. 4. It illustrated that 50 mM is separated away, where 30 and 40 mM are genetically similar and quite related to control.

Table 2. Morphological means estimation of *Gardenia jasminoides* in response to different concentrations of mutagens.

Mutagen	Sodium azide				Guanidine hydrochloride			
Conc. (mM)	0	30	40	50	0	30	40	50
Fresh wt. (g)	0.1134	0.1546	0.1600	0.1268	0.1277	0.1174	0.1448	0.1089
Shoot length (cm)	1.4750	1.9500	1.8000	1.2750	1.5333	1.6000	2.1000	1.7500
Root length (cm)	3.2500	2.3000	2.3250	1.5500	3.3667	3.0333	2.3000	2.8667
No. of roots	3.0000	1.3333	2.3333	1.3333	2.5000	2.5000	1.2500	1.0000
No. of leaves	6.0000	7.6667	6.6667	7.3333	6.0000	4.6667	7.6667	5.6667
No. of branches	1.0000	1.6667	1.3333	1.6667	1.0000	1.6667	1.0000	1.0000
Leaf length (cm)	1.8750	2.1833	1.9417	1.7667	1.8214	1.9357	2.6071	2.4000
Leaf width (cm)	0.9000	0.9083	0.7917	0.8083	0.8571	0.9357	1.1571	0.8643
p value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F value	11.374	41.894	44.527	23.532	9.606	5.825	23.837	55.326

Table 3. Chlorophyll content of *Gardenia jasminoides* in response to different concentrations of mutagens.

Mutagen	Sodium azide				Guanidine hydrochloride			
Conc. (mM)	0	30	40	50	0	30	40	50
Chl. a	0.920519	0.617812	0.706645	0.709272	0.91927	0.48513	0.52971	0.54479
Chl. b	0.807074	0.617927	0.524632	0.509406	0.80674	0.37548	0.42432	0.43233
Cart.	0.531713	0.391106	0.345430	0.315179	0.53131	0.24691	0.28064	0.27576
p value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F value	14427.43	15382.69	13312.98	4964.66	24012.76	23474.59	35459.79	9554.068

Many molecular markers were used to assess the genetic variation in plants in response to mutation. In this study, RAPD-PCR was the molecular marker used to detect the genetic polymorphism within *G. jasminoides* due to the action of both sodium azide and guanidine hydrochloride. Li et al. (2009) performed a study to evaluate the genetic differences and mutual relationship of *G. jasminoides* at DNA level among different cultivars using ISSR molecular marker and obtained a polymorphism percentage of 55.17%. Wannajindaporn et al. (2014) used tissue culture technique to study the effect of sodium azide on *Dendrobium* ornamental plant and used ISSR molecular markers to estimate the variation in mutants compared to control. This agrees with many workers which proved the genetic variation in different plants due to chemical mutagens treatments. Kishk et al. (2016) used ISSR molecular marker to estimate both diethyl sulphate and sodium azide mutagen on banana plant to against salt stress.

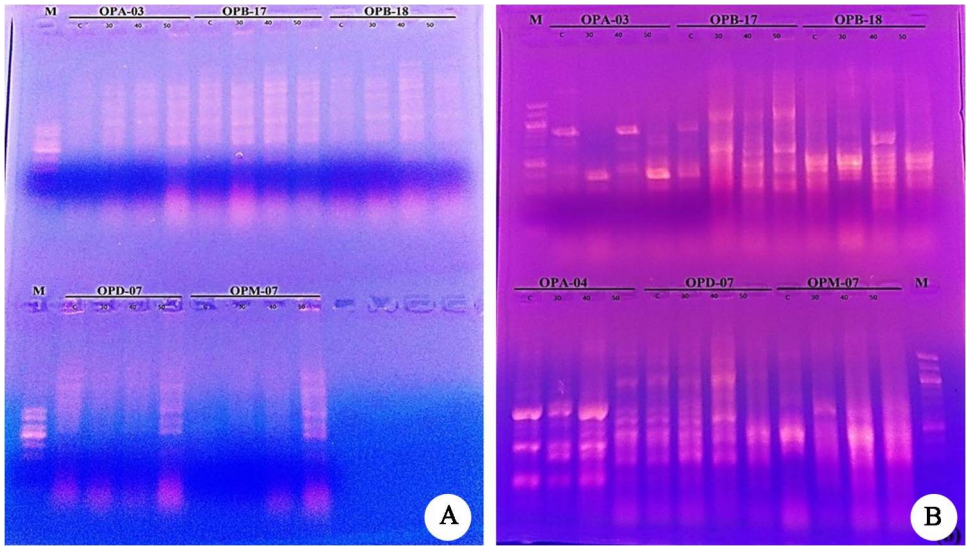

Fig. 2. Agarose gel electrophoresis of *Gardenia* with mutagen. A. Sodium azide and B. Guanidine hydrochloride.

Table 4. List of primers, their nucleotide sequences and polymorphism percentage used in the RAPD analysis of *Gardenia jasminoides* with both mutagens.

No.	Primers	Sodium azide		Guanidine hydrochloride	
		Total bands	Polymorphism (%)	Total bands	Polymorphism (%)
1	OPA-03	5	60	7	71.42
2	OPA-04	-	-	12	41.67
3	OPB-17	7	42.85	8	100
4	OPB-18	8	25	6	50
5	OPD-07	4	75	10	80
6	OPM-07	7	85.71	8	62.5
Total			57.71		67.59

Table 5. Similarity matrix resulted from all RAPD primers for *Gardenia* treated with sodium azide

	Control	30 mM	40 mM	50 mM
Control	100	68.70	56.26	55.96
30 mM	68.70	100	61.98	55.00
40 mM	56.26	61.98	100	71.84
50 mM	55.96	55.00	71.84	100

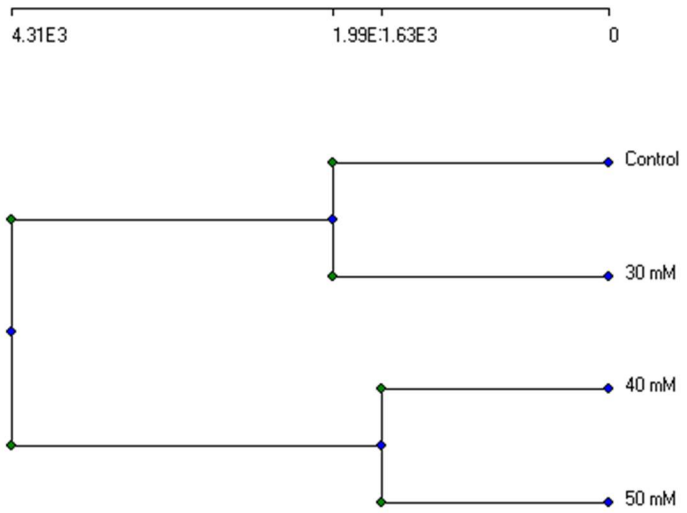

Fig. 3. Dendrogram resulted from all RAPD primers for *Gardenia* treated with sodium azide.

Table 6. Similarity matrix resulted from all RAPD primers for *Gardenia* treated with guanidine hydrochloride.

	Control	30 mM	40 mM	50 mM
Control	100	52.63	57.25	38.07
30 mM	52.63	100	59.67	41.70
40 mM	57.25	59.67	100	48.48
50 mM	38.07	41.70	48.48	100

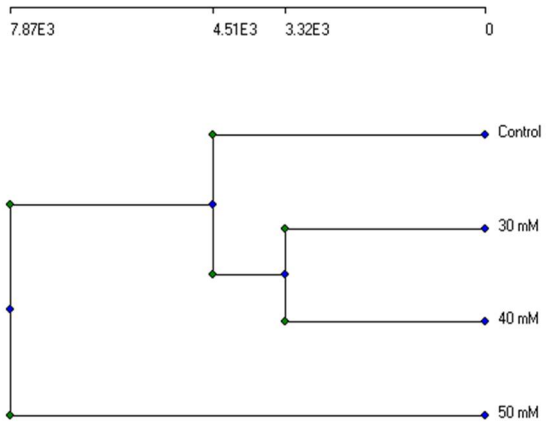

Fig. 4. Dendrogram resulted from all RAPD primers for *Gardenia* treated with guanidine hydrochloride.

Acknowledgment

The authors would like to thank the Agricultural Center for Genetic Engineering and Biotechnology ACGEB, Faculty of Agriculture, Ain Shams University, for the facilitiesof working in tissue culture laboratory

References

Ahmad Z, Hassan AB, Salleh S, Ariffin SN, Shamsudin S, Basiran MN, Tan SG, Zainudin NA and **Yusoh OM** (2012) Improvement of Malaysian ornamental plants through induced mutation. Pertanika J. Trop. Agric. **35**(3): 631-636.

Al Gawwad A and **Makka AH** (2009) Effect of chemical mutagens on *Mirabilis jalapa*. M.Sc. Thesis, Floriculture, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

Al-Qurainy F (2009) Effects of sodium azide on growth and yield traits of *Eruca sativa* (L.). World Appl. Sci. J. **7**(2): 220-226.

Doyle JJ and **Doyle JL** (1990) Isolation of plant DNA from fresh tissue. Focus **12**: 13-15.

Edwards K, Johnstone C and **Thompson C** (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acid Res. **19**: 349. doi: 10.1093/nar/19.6.1349

El-Mokadem HE and **Mostafa GG** (2014) Induction of mutations in *Browallia speciosa* using sodium azide and identification of the genetic variation by peroxidase isozyme. Afr. J. Biotechnol. **13**(1): 106-111.

El-Nashar YIA (2006) Effect of chemical mutagens (sodium azide and diethyl sulphate) on growth, flowering and induced variability in *Amaranthus caudatus* L. and *A. hypochondriacus* L. Ph.D. Thesis, Floriculture, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

El-Nashar YI and **Asrar AA** (2016) Phenotypic and biochemical profile changes in calendula (*Calendula officinalis* L.) plants treated with two chemical mutagenesis. Genet. Mol. Res. **15**(2): gmr.15028071.

Kesavan K, Gnanasekaran J, Gurunagarajan S and **Nayagam AAJ** (2018) Microscopic, physico-chemical and phytochemical analysis of *Gardenia jasminoides* (Ellis). Int. J. Pharm. Pharm. Sci. **10**(1): 97-102.

Kishk AD, Abul-Soad AA, Abbas MS, El-Shabrawi HM, Gaber EI and **Noor El-Deen TM** (2016) Effect of diethyl sulphate and sodium azide on tolerance of *ex vitro* banana to salt stress. International Journal of Chem. Tech. Res. **9**(12): 81-99.

Li L, Yi W, A-Man Z, Pei-Lin Z and **Shi-Liang Z** (2009) Genetic relationship of *Gardenia jasminoides* among plantations revealed by ISSR. Chin. Tradit. Herbal Drugs **40**(1): 116-120.

Metzner H, Rau H and **Senger H** (1965) Untersuchungenzur synchronisie-rbarkeiteinzelner pigmentmangel mutanten von chlorella. Planta **65**: 186-194.

Mostafa GG (2011) Effect of sodium azide on the growth and variability induction in *Helianthus annuus* L. Int. J. Plant Breed. Genet. **5**(1): 76-85.

Shuaib M, Zeb A, Ali Z, Ali W, Ahmad T and **Khan I** (2007) Characterization of wheat varieties by seed storage protein electrophoresis. African J. Biotech. **6**: 497-500.

Soliman TMA, El-Keltawi NE, Khan MA, Nan M and **Zhao LJ** (2013) plant growth and flowering of cape jasmine (*Gardenia Jasminoides*, Ellis) in various substrates amended with sulphur. Glob. J. Plant Ecophysiol. **3**(2): 36-43.

Suprasanna P, Mirajkar SJ and **Bhagwat SG** (2015) Induced mutations and crop improvement. Plant Biotechnol. **1**: 593-617.

Wannajindaporn A, Poolsawat O, Chaowiset W and **Tantasawat PA** (2014) Evaluation of genetic variability in *in vitro* sodium azide-induced Dendrobium 'Earsakul' mutants. Genet. Mol. Res. **13**(3): 5333-5342.

Wilson SB and **Bonner WD** (1970) Effects of guanidine inhibitors on mung bean mitochondria. Plant Physiol. **46**: 21-24.

Xiao W, Li S, Wang S and **Ho CT** (2017) Chemistry and bioactivity of *Gardenia jasminoides*. Review Article. J Food Drug Anal. **25**: 43-61.

(Manuscript reeived on 16 August, 2020; revised on 21 September, 2020)