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Molecular and Agro-Morphological Genetic Diversity Assessment of *Gloriosa superba* Mutants

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Authors' contributions

This work was carried out in collaboration between both authors. Author AS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript under the guidance of author RK. Both authors read and approved the final manuscript.

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ABSTRACT

Glory lily (*Gloriosa superba* L.), a high value medicinal crop cultivated in Tamil Nadu for its valuable seeds and tubers. This crop belongs to the family Colchicaceae. The active principle Colchicine and Colchicoside present in seeds and tubers cures gout and rheumatism. The genetic variability also is low owing to the continued vegetative propagation through tubers. There is an urgent need to explore the possibilities for developing variability in this species with high seed yield and improved colchicine content through induced mutations. Mutation breeding was effected involving physical and chemical mutagens *viz.,* gamma rays, Ethyl methyl sulfonate (EMS) and Diethyl Sulphate (DES). On analysing the variance, significant differences were observed among the treatments for most of the traits. Phenotypic coefficient of variation was in general slightly higher than the genotypic coefficient of variation for the selected traits in VM_2 generation indicating the influence of environmental factors on these traits. High heritability and genetic advance as per cent of mean was recorded for most of the characters under study indicating better scope for further selection. Differential patterns resulted in ISSR analysis indicating the polymorphism created by induced mutagenesis, creating scope for selection of desirable mutants in *G. superba.*

Keywords: Genetic variation; heritability; molecular markers; polymorphism; mutants.

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1. INTRODUCTION

Gloriosa superba L. is one of the very important, export oriented medicinal plants of India that has become endangered within a very short span of last 50 years. This climber belonging to the family Colchicaceae, is a major high value medicinal crop cultivated in Tamil Nadu. *Gloriosa* derives its name from the word 'gloriosus', which means handsome and *superba* from the word 'superb' means splendid or majestic. Seeds and tubers contain valuable alkaloids, *viz*., colchicine and colchicoside, which cures gout and rheumatism. This plant has been identified as a potential anti-cancerous drug due to the action of colchicoside on spindle fiber formation during cell division [1].

Though *G. superba* has an extensive natural distribution, the species has become endangered due to over exploitation of its tubers and low percent germination. Hence there is an urgent need to explore the possibilities for developing variability in this species with high seed yield and improved colchicine content through breeding techniques. New cultivars of *Gloriosa superba* are developed by radiation-induced mutation. Since the effect of mutation in gloriosa is clearly visible, selection for changed plant stature, high seed yield, increased active principle content is possible in the M1 generation itself because of vegetatively propagating nature. Novelty visible in any form is of high value and hence mutation breeding played a key role in the improvement of in general and gloriosa in particular.

Usefulness and reliability of any genetic marker is dependent on its heritability and the level of polymorphism. The more polymorphic and heritable the trait, the greater is its potential value for germplasm characterization. DNA markers are considered the best tools for determining genetic relationships or diversity, as they are unlimited in number. They show high polymorphism and independent of environment interaction *i.e*., highly heritable.

Most of the medicinal plants provide new genetic systems to the biologists and are amenable to be characterized by RAPD and ISSR markers. ISSR markers rely on a primer containing simple repeat sequences as primer for PCR amplification to generate reproducible fingerprints. The primers may be the unanchored or anchored generally at the 5' end by selective nucleotides to prevent internal priming and to amplify only a subset of the targeted inter-repeat regions. These markers have proved quite useful for genetic diversity analysis in medicinal plants.

This study aims to generate information on character association, direct and indirect influence of characters on seed yield in the induced mutants of Glory lily and to assess the genetic variation among mutants.

2. MATERIALS AND METHODS

2.1 Genetic Characterization of Mutants

Gloriosa superba tubers collected from Mulanur of Tamil Nadu were subjected to three doses of gamma irradiation (0.50, 1.00, 1.50 kR), ethyl methyl sulphonate (1.0, 1.5 and 2.0%) and diethyl sulphonate (1.0, 1.5 and 2.0%). The experiment was conducted during the first week of August, 2010 (VM_1, first generation of vegetative mutant) and first week of August, 2011 (VM_2, second generation of vegetative mutant) at the Department of Medicinal and Aromatic Crops, Horticultural College and Research Institute, Coimbatore. 18 plant characters *viz*., plant height, stem girth, no. of leaves/plant, no. of primary branches/plant, no. of secondary branches/plant, no. of flowers/plant, pod length, pod girth, fresh pod weight, dry pod weight, fresh seed weight/pod, no. of seed/pod, 100 fresh seed weight, 100 dry seed weight, dry seed yield/plant, tuber length, tuber girth, tuber weight were the observation recorded in the VM_2 generation. They were subjected to analysis of phenotypic and genotypic co-efficient of variability [2], heritability [3], genetic advance and correlation co-efficient [4] and path coefficients [5].

2.1.1 Genetic parameters

In VM_2 generations, the genotypic co-efficient of variation (GCV) and phenotypic co-efficient of variation (PCV) were estimated from the genotypic and phenotypic variances as suggested by [2].

2.1.1.1 Phenotypic and genotypic coefficient of variation

$$GCV = \frac{\sqrt{\text{Genotypic variance}}}{\text{Mean}} \times 100$$

$$PCV = \frac{\sqrt{\text{Phenotypic variance}}}{\text{Mean}} \times 100$$

The PCV and GCV were classified as,

Less than 10 % = Low
10-20 % = Moderate
More than 20 % = High

2.1.1.2 Heritability

As suggested by [3], the heritability (h^2) estimates were worked out and expressed as percentage

$$\text{Heritability} = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times 100$$

The heritability per cent was categorized as,

0 - 30 % = Low
31 – 60 % = Medium
Above 60 % = High

2.1.1.3 Genetic advance

Genetic advance (GA) was calculated by

$GA = k \times h^2 \times P$

Where,

k = Selection differential which is equal to 2.06 at 5 per cent selection intensity
h^2 = Heritability
P = Phenotypic standard deviation

For comparison, the genetic advance (GA) was expressed as percentage of mean. Genetic advance (GA) as percentage of mean was calculated and categorized as suggested by [4]

Genetic advance as per cent of mean = $\frac{GA}{\text{mean}} \times 100$

Less than 10 % = Low
10- 20 % = Moderate
More than 20 % = High

2.1.1.4 Association analysis

The idea on genetic variability existing among different parameters is important in crop improvement. Correlation coefficient analysis elaborates the degree and extent of relationship among important plant characters and it provides basic criteria for selection and leads to directional model based on yield and its components in the field experiments. Path coefficient analysis is an efficient statistical technique specially designed to quantify the interrelationship of different components and their direct and indirect effects on yield.

2.1.1.5 Correlation coefficient

The coefficients of simple correlation were estimated for the different generations using the following formula:

$$r_{xy} = \frac{Cov_{(xy)}}{\sqrt{Var_{(x)} Var_{(y)}}}$$

where,

r_{xy} = simple correlation co-efficient between x and y
$Cov_{(xy)}$ = covariance between the character x and y
$Var_{(x)}$ and $Var_{(y)}$ = variance of characters x and y

2.1.1.6 Path analysis

According to [5] path coefficient analysis was carried out by partitioning the genotypic correlation into direct and indirect effects.

Path coefficients	Category
More than 1.00	Very high
0.30 to 0.99	High
0.20 to 0.29	Moderate
0.10 to 0.19	Low
0.0 to 0.09	Negligible

2.2 Molecular Profiling of Mutants through ISSR Analysis

2.2.1 DNA extraction

DNA from the five mutants of *G. superba* in VM_2 generation was extracted by following the protocol of [6], with a slight modification. Mercaptoethanol (1 per cent) and polyvinyl pyrrolidone (PVP) 0.2 per cent were added to the extraction buffer to remove the phenolics. Three grams of young leaf tissue was ground with liquid nitrogen and to this powder, 15 ml of preheated CTAB buffer (65°C) was added. It was then incubated at 65°C in a water bath for one hour. After bringing the tubes to room temperature, equal volume (15 ml) of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed well for 10 minutes to form an emulsion. It was then centrifuged at 10,000 rpm for 15 minutes at 15°C. The supernatant was transferred to a fresh tube and the chloroform: isoamyl alcohol step was again repeated.

The aqueous phase was transferred to a new tube and equal volume of ice cold isopropanol was added and incubated in a freezer for overnight. The contents were then centrifuged at

10,000 rpm for 20 minutes at 16°C. The pellet was then saved by discarding the solution. The pellet was washed with 70 per cent ethanol by centrifuging the contents at 10,000 rpm for 10 minutes. The alcohol was discarded and the pellet was air dried. The pellet was then dissolved in 3 ml of double distilled water. Then 1 µl of RNase was added and incubated at 37°C for 30 minutes. DNA was precipitated by adding 50 µl of 3M sodium acetate and 7.5 ml of 100 per cent ethanol and the contents were again centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded. The pellet was washed with 70 per cent ethanol and air dried. It was finally dissolved in TE buffer (150 µl) and stored at - 20°C.

2.2.2 DNA quality and quantity check

To check the quality and quantity of the extracted genomic DNA, gel electrophoresis was carried out on 0.8 per cent agarose gel. For PCR amplification, DNA concentration was estimated by comparing the band intensity of a sample with known dilutions that gave good amplifications. Based on the band intensity, the DNA was further diluted to the required concentration (25-50 ng) using double distilled water [7].

2.2.3 ISSR analysis

Out of 45 ISSR primers tested, 12 ISSR primers (as described by University of British Columbia, Canada) synthesized at Sigma - Aldrich (USA), Bangalore, were selected (Table 1) based on the degree of polymorphism and the distinctness of the bands they produce when tested on a sample set. PCR was performed by means of the selected 12 ISSR primers.

Amplification reactions were in volumes of 10 µl containing 20 ng of genomic DNA, 1.0 µl of Taq buffer (including 15 mM $MgCl_2$), 1.0 µl of dNTPs (10 mM each of dATP, dTTP, dGTP and dCTP), 1 µl of primer, 4.9 µl of double distilled water, 0.1 µl of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplifications were performed in Bio-Rad (MyCycler thermal cycler) programmed for an initial denaturation at 94°C for 3 minutes, 40 cycles of 30 seconds denaturation at 94°C, 30 seconds at specific annealing temperature for each primer and 1 minutes extension at 72°C and a final extension of 10 minutes at 72°C and then at 4°C till storage.

2.2.4 Separation of amplified fragments using polyacrylamide gel electrophoresis

Six per cent polyacrylamide gels were used for better separation and visualization of PCR amplified microsatellite products. Both the glass plates were cleaned with warm water, detergent and finally rinsed with deionized water.

2.2.5 Assembling and pouring the gel

Spacers (0.5 mm thickness) were placed along the side edges of the bind silane treated surface of the glass plate. The repel silane treated glass plate (notched plate) was kept on the bind silane treated surface so that treated surfaces face each other (in a sandwich like fusion).The spacers were fitted well against each other so that there is no gap. By using cellotape, all the edges were tightly sealed so that there is no gap for bottom or sides. For casting each gel, 250 ml of gel solution was required. Acrylamide solution mix, 10X TBE buffer and distilled water were mixed well and finally 10 per cent APS and TEMED were added. The contents were mixed gently by swirling and bubbles were avoided. The assembly was kept on a bench top so that between the glass plates starting at the lower

Table 1. List of primers used for ISSR analysis

S. no.	Primer	Nucleotide sequence (5'- 3')	Annealing temperature (T_a)
1	UBC-807	AGAGAGAGAGAGAGAGT	42.5
2	UBC-810	GAGAGAGAGAGAGAGAT	42.9
3	UBC-820	GTGTGTGTGTGTGTGTC	50.3
4	UBC-821	GTGTGTGTGTGTGTGTT	49.9
5	UBC-824	CTCTCTCTCTCTCTCTG	49.0
6	UBC-825	ACACACACACACACACT	49.2
7	UBC-826	ACACACACACACACACC	53.3
8	UBC-827	ACACACACACACACACG	54.9
9	UBC-828	TGTGTGTGTGTGTGTGA	53.2
10	UBC-846	CACACACACACACACART	53.7
11	UBC-848	CACACACACACACACARG	55.5
12	UBC-849	GTGTGTGTGTGTGTGTYA	50.5

it makes 45 degree angle with the bench top. The solution was carefully poured into the space corner. After filling, the comb (0.5 mm thickness) was inserted straight across the top moving not more than 5 mm of notched plate. The gel was left for 20-40 minutes for polymerization to proceed [8].

2.2.6 Electrophoresis

After the polymerization process, the cello tape around the assembly was removed and it was placed in the unit. Then 0.5X TBE buffer was filled over the upper and lower tanks. The comb was removed carefully and excess polyacrylamide gel was removed with a plastic spatula. An amount of 2 µl of PCR products were loaded in to the wells along with 1 kb ladder. The assembly with buffer tank was connected to the power pack and the PCR products were allowed to run through the gel at 150 V for 3 hours (DNA is negatively charged and run from black to red).

2.2.7 Visualization of bands

After electrophoresis, remove the assembly from the buffer tank. The glass plates were separated using plastic wedge at the right corner. The gel was bound to the bind silane plate. DNA fragments were separated and detected using 20 minutes silver staining protocol. The same solutions can be used four times over a period of 48 hours except for developer, which should be freshly prepared during the staining process.

2.2.8 Steps followed for silver staining

Improved staining method was followed for staining. This method was a combination of different steps proposed by [9]. After electrophoresis, gels were washed in 1000 ml cold (10-12°C) fixing solution (10 per cent absolute ethanol, 0.5 per cent acetic acid) for 5 minutes. Washed gels were soaked for 6-7 minutes at room temperature (22-24°C) in a 1000 ml solution of 0.15 per cent Silver Nitrate, 1.5 ml 37 per cent Formaldehyde. Gels were rinsed quickly (10-15 sec) once with 1000 ml double distilled water. They were then developed by soaking them at room temperature (22-24°C) in a 1000 ml developing solution (1.5 per cent Sodium Hydroxide, 2 ml of 37 per cent Formaldehyde) until the bands appeared with a sufficient intensity and finally impregnating the gel in a 2000 ml stop solution (10 per cent absolute ethanol, 0.5 per cent acetic acid) for 2 minutes. All steps were done in plastic containers. The gel plates were agitated in a shaker throughout the staining process. The fix stop, developer and silver nitrate solutions were prepared in advance but Formaldehyde was added just before use.

3. RESULTS AND DISCUSSION

The estimation of variance, genetic advance and other genetic parameters of mutants detects the induction of mutation in polygenic quantitative traits. On analysing the variance, significant differences were observed among the treatments for most of the traits. Phenotypic coefficient of variation was in general slightly higher than the genotypic coefficient of variation for the selected traits in VM_2 generation indicating the influence of environmental factors on these traits (Table 2).

The GCV ranged from 1.24% (number of leaves / plant) to 55.06% (number of secondary branches per plant) in the mutants. The PCV was lowest (1.76%) for number of leaves / plant while it was highest for number of secondary branches/plant (102.02%). The genetic advance as percentage of mean was lowest for plant height (1.73), while it was highest for stem girth (69.30) followed by number of secondary branches / plant (61.22). Higher heritability was noticed for stem girth (89.74%) followed by fresh pod weight (82.39%) for the characters studied.

In VM_2 generation, a strong association at phenotypic level between the characters results in higher PCV than the GCV. Genotypic expression was reduced which might be due to the masking effect of environment in modifying the total expression of the phenotypes. In VM_2 generation, high PCV and GCV was recorded for the traits *viz.,* stem girth, number of primary branches / plant, number of secondary branches / plant, fresh seed weight / pod, number of seed / pod, 100 fresh seed weight, tuber length, tuber weight and dry seed yield / plant, emphasizing these characters to be potentially variable. The differences between PCV and GCV were meagre revealing the fact that these traits were less influenced by the environment. High values of GCV suggested better improvement for selection of traits. However, [10] observed highest PCV for plant height, number of laterals plant^{-1}, number of leaves plant^{-1}, number of tuber plant^{-1}, tuber length, tuber girth in *Coleus forskohlii.*

High genetic advance as % of mean was observed for the traits *viz.,* stem girth, number of primary branches / plant, number of secondary branches / plant, fresh pod weight, fresh seed

weight / pod, number of seed / pod, 100 fresh seed weight, tuber length, tuber weight and dry seed yield / plant. The selection can be relied upon for improvement of these parameters among the progenies. Additive genes governs high genetic advance and paves the way for improvement of those characters in individual plant selection [11].

The plant characters *viz.,* stem girth, fresh pod weight, fresh seed weight / pod, number of seeds / pod, 100 fresh seed weight, 100 dry seed weight, dry seed yield / plant, tuber length and tuber weight recorded high heritability. This shows that selection of such characters is easy because of the close correspondence between the genotype and phenotype due to relatively smaller contribution of the environment to genotype. Similar reports on high heritability for moderate for seeds per inflorescence and plant height in case of *Dianthus caryophyllus* [12].

3.1 Association Analysis

The positive and highest significant correlation for dry seed yield / plant (g) was observed with number of seeds / pod (0.928) closely followed by number of leaves / plant (0.537) and dry pod weight (0.454) which was further followed by fresh seed weight / pod (0.366), fresh pod weight (0.298), plant height (0.282) and number of secondary branches / plant (0.236) (Table 3).

Plant height showed positive significance of intercorrelation (Residual effect-0.3465) for the traits *viz.*, number of leaves / plant (0.471), number of seeds / pod (0.270), tuber length (0.379) and tuber weight (0.309). Similarly positive significance with number of leaves / plant was observed with number of flowers / plant (0.487), fresh pod weight (0.260), dry pod weight (0.378), number of seeds / pod (0.549), fresh seed weight (0.323), tuber girth (0.222) and tuber weight (0.216). Positive and significant correlation for number of primary branches / plant was observed with number of secondary branches / plant (0.350) and plant girth (0.421) while number of secondary branches / plant exhibited positive and significant correlation with plant girth (0.274), number of flowers / plant (0.253), number of seeds / pod (0.239), 100 fresh seed weight (0.219) and tuber girth (0.230).

Fresh pod weight exhibited significance in the positive direction with dry pod weight (0.353), number of seeds / pod (0.263), fresh seed weight / pod (0.270) and 100 fresh seed weight (0.202). On the other hand, a positive and significant correlation was exerted by stem girth (0.219) and number of flowers / plant (0.235) with dry pod weight and fresh 100 seed weight respectively. Positive and significant correlation for dry pod weight was exerted with number of seeds / pod (0.448) and fresh seed weight / pod (0.316). Similarly, number of seeds / pod, fresh seed weight / pod and 100 fresh seed weight exhibited a positive and significant correlation with fresh seed weight (0.382), 100 fresh seed weight (0.275) and 100 dry seed weight (0.399) respectively. Pod girth exerted a significant

Table 2. Estimates of variability, heritability and genetic advance of glory lily derived from large sized tuber in VM_2 generation

Characters	Mean	PCV (%)	GCV (%)	h^2 (%)	GA	GAM
Stem girth (cm)	1.66	37.48	35.51	89.74	1.15	69.30
Plant height (cm)	139.15	2.76	1.52	30.29	2.40	1.73
No. of primary branches/plant	3.26	49.62	33.19	44.73	1.49	45.72
No. of secondary branches/plant	3.45	102.02	55.06	29.13	2.12	61.22
No. of leaves/plant	194.23	1.76	1.24	49.70	3.50	1.80
No. of flowers/plant	40.46	4.91	2.45	24.96	1.30	2.52
Pod length (cm)	7.83	9.14	6.37	48.66	3.71	9.16
Pod girth (cm)	7.48	9.14	7.88	74.44	5.33	14.01
Fresh pod weight (g)	11.65	18.26	16.57	82.39	2.43	30.99
Dry pod weight (g)	8.22	12.85	9.40	53.54	1.06	14.17
Fresh seed weight/pod (g)	6.19	27.42	21.90	63.78	4.20	36.02
No. of seed/pod	52.65	31.57	27.89	78.03	4.17	50.74
100 fresh seed weight (g)	10.05	35.48	30.84	75.54	3.42	55.21
100 dry seed weight (g)	3.19	3.56	3.06	73.80	2.85	5.41
Dry seed yield/plant (g)	52.95	23.24	19.08	67.42	3.25	32.28
Tuber length (cm)	10.61	26.07	22.87	76.96	1.32	41.32
Tuber girth (cm)	5.58	3.86	2.85	54.65	2.30	4.34
Tuber weight (g)	57.52	21.12	18.76	78.90	3.64	34.33

correlation in the negative direction for with 100 dry seed weight and tuber girth.

Positive correlation of number of seeds / pod with dry seed yield / plant was reported by [13] in *Phaseolus vulgaris* and [14] in chick pea. [14] and [15] reported positive association of number of branches with dry seed yield in chick pea and *Cajanus cajan* respectively. [16] reported positive correlation of pod weight with seed yield in long bean while positive correlation of number of leaves / plant with seed yield / plant was reported by [17].

This analysis revealed that for future crop improvement programme, selecting plants with more plant height, number of secondary branches / plant, number of leaves / plant, number of seeds / pod, dry pod weight, fresh seed weight / pod and fresh pod weight were desirable. The positive inter correlation among the yield components indicated the possibility of simultaneous improvement of seed yield.

3.2 Path Analysis

Path coefficient analysis furnishes a means of measuring the direct effect of each trait as well as the indirect effect *via* other characters on yield. So information on the direct and indirect effect on yield is important which is explicable by path analysis as proposed by [18] and illustrated by [19]. The interrelationships of the characters on yield provide the likely consequences of their selection for simultaneous improvement of desirable characters with yield.

Path coefficient analysis revealed that plant height (0.282), number of leaves / plant (0.537), number of secondary branches / plant (0.236), fresh pod weight (0.298), dry pod weight (0.454), number of seeds / pod (0.928) and fresh seed weight / pod (0.366) for the dry seed yield / plant (Table 4) observed significant direct effects.

The number of seeds / pod exhibited indirect effect *via* plant height, fresh pod weight, number of leaves / plant, dry pod weight and fresh seed weight / pod.

Preference should be given to these characters in the selection programme to isolate superior mutants with genetic potential for improving yield, as the correlation of these characters with yield is positive [20]. The number of seeds / pod had high direct effect on dry seed yield / plant. Indirect positive effects of dry pod weight, fresh seed weight / pod on dry seed yield / plant was recorded by [14,13,15]. Direct effect of number of branches with seed yield / plant was reported by [21]. [16] reported direct effect of pod weight with seed yield / plant.

The direct and indirect effect of the path analysis revealed that important selection indices for yield improvement are the plant height, number of leaves /plant, number of seeds / pod, fresh pod weight, dry pod weight and fresh seed weight / pod.

3.3 Molecular Characterization of Mutants

In the mutation experiment carried out in *G. superba*, the mutants were characterized by ISSR. Each mutant was scored for the presence (1) and absence (0) of bands. Genetic distance was calculated on the basis of Jaccard's co-efficient method. A dendrogram was constructed using the TREE procedure by the Numerical Taxonomy and Multivariate Analysis System (NTSYS) based on Jaccard's similarity co-efficient using Unweighted Pair Group with Arithmetic Mean method (UPGMA).

3.3.1 Marker polymorphism

In the present investigation, six samples were used to study the genetic diversity using 12 ISSR primers. The PCR amplification using these 12 primers yielded 444 reproducible amplified bands. The number of amplified bands varied from 12 (UBC 824) to 73 (UBC 807). Out of 444 bands, 116 were found to be polymorphic. Average number of bands and polymorphic bands per primer were 37 and 9.67 respectively (Table 5). As a relative measure of polymorphism level, Polymorphic Information Content (PIC) value ranged between 0.764 (UBC 810) to 0.947 (UBC 807). The informativeness of the primer was indicated by the higher PIC value. Five primers *viz*., UBC 846, UBC 821, UBC 827, UBC 848 and UBC 828 exhibited the PIC value from 0.926 to 0.912 among the primers used in the study. These primers can provide the basis for Gloriosa DNA profile system. Such high level of polymorphism is comparable to the results of some similar molecular researches on medicinal plants of Lamiaceae family [22,23,24]. [25,26] observed similar reports in thyme and patchouli respectively.

Table 3. Effect of mutagens on simple correlation coefficient of glory lily derived from large sized tubers in VM_2 generation

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}
X_1	1.000																	
X_2	0.471**	1.000																
X_3	0.074	0.081	1.000															
X_4	-0.002	0.251*	0.350**	1.000														
X_5	-0.014	0.147	0.421**	0.274**	1.000													
X_6	-0.059	0.487**	0.111	0.253*	0.158	1.000												
X_7	0.115	-0.042	0.037	0.100	0.184	-0.095	1.000											
X_8	-0.097	-0.104	0.187	-0.009	0.155	-0.012	0.099	1.000										
X_9	0.155	0.260**	0.039	-0.027	0.144	0.092	0.084	-0.144	1.000									
X_{10}	0.170	0.378**	0.096	0.019	0.219*	0.068	0.186	-0.033	0.353**	1.000								
X_{11}	0.270**	0.549**	0.063	0.219*	0.128	0.173	0.071	-0.053	0.263**	0.448**	1.000							
X_{12}	0.198	0.323**	-0.039	0.019	0.024	-0.087	0.107	0.095	0.270**	0.316**	0.382**	1.000						
X_{13}	0.047	-0.040	-0.011	-0.239*	-0.183	-0.235*	0.046	0.121	0.202*	0.162	0.061	0.275**	1.000					
X_{14}	0.191	0.020	-0.088	-0.112	-0.101	-0.165	0.147	-0.027	0.043	0.059	0.123	-0.005	0.399**	1.000				
X_{15}	-0.379**	-0.164	-0.104	-0.079	0.106	-0.116	-0.085	0.077	-0.110	-0.077	0.017	0.042	-0.056	-0.063	1.000			
X_{16}	0.109	0.222*	-0.024	0.230*	-0.040	0.146	0.030	-0.225*	-0.013	0.050	0.218*	0.121	-0.111	0.015	-0.150	1.000		
X_{17}	-0.309**	-0.216*	0.166	0.086	0.125	-0.190	0.035	-0.089	0.041	0.048	-0.122	0.007	-0.004	-0.086	0.116	-0.094	1.000	
X_{18}	0.282**	0.537**	0.065	0.236*	0.113	0.125	0.071	-0.105	0.298**	0.454**	0.928**	0.366**	0.087	0.142	0.039	0.168	-0.091	1.000

** Significant at 5% level; ** Significant at 1% level*

X_1	*Plant height (cm)*	X_7	*Pod length (cm)*	X_{13}	*100 fresh seed weight (g)*
X_2	*Number of leaves / plant*	X_8	*Pod girth (cm)*	X_{14}	*100 dry seed weight (g)*
X_3	*Number of primary branches / plant*	X_9	*Fresh pod weight (g)*	X_{15}	*Tuber length (cm)*
X_4	*Number of secondary branches / plant*	X_{10}	*Dry pod weight (g)*	X_{16}	*Tuber girth (cm)*
X_5	*Stem girth (cm)*	X_{11}	*No. of seed/pod*	X_{17}	*Tuber weight (g)*
X_6	*No. of flowers / plant*	X_{12}	*Fresh seed weight / pod (g)*	X_{18}	*Dry seed yield/plant (g)*

Table 4. Effect of mutagens on path analysis in VM_2 generation of glory lily derived from large sized tubers

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}
X_1	0.028	0.023	0.001	-0.001	0.001	0.002	0.001	0.005	0.006	0.006	0.235*	-0.003	0.001	0.002	-0.020	-0.005	-0.001	0.282**
X_2	0.013	0.050	0.001	0.019	-0.004	-0.023	-0.000	0.006	0.011	0.013	0.477*	-0.005	-0.001	0.001	-0.009	-0.112	-0.000	0.537**
X_3	0.002	0.00	0.004	0.026	-0.011	-0.005	0.000	-0.010	0.001	0.003	0.055	0.001	-0.001	-0.001	-0.005	0.001	0.001	0.065
X_4	-0.001	0.012	0.001	0.06	-0.001	-0.012	0.000	0.001	-0.001	0.001	0.190	-0.001	-0.001	-0.001	-0.004	-0.011	0.001	0.236*
X_5	-0.001	0.007	0.001	0.020	-0.027	-0.007	0.001	-0.008	0.006	0.007	0.111	-0.001	-0.005	-0.001	0.005	0.002	0.001	0.113
X_6	-0.001	0.024	0.001	0.019	-0.004	-0.048	-0.000	0.001	0.004	0.002	0.150	0.001	-0.007	-0.002	-0.006	-0.007	-0.001	0.125
X_7	0.003	-0.002	0.001	0.007	-0.005	0.004	0.001	-0.005	0.003	0.006	0.062	-0.001	0.001	0.002	-0.004	-0.001	0.001	0.071
X_8	-0.002	-0.005	0.001	-0.001	-0.004	0.001	0.000	-0.056	-0.006	-0.001	-0.046	-0.001	0.003	-0.001	0.004	0.011	-0.001	-0.105
X_9	0.004	0.013	0.001	-0.002	-0.003	-0.004	0.000	0.008	0.044	0.012	0.229*	-0.004	0.006	0.001	-0.006	0.001	0.001	0.298**
X_{10}	0.004	0.019	0.001	0.001	-0.006	-0.003	0.001	0.001	0.015	0.035	0.390**	-0.004	0.004	0.001	-0.004	-0.002	0.001	0.454**
X_{11}	0.007	0.027	0.001	0.016	-0.003	-0.008	0.000	0.003	0.011	0.016	0.870**	-0.005	0.001	0.001	0.001	-0.011	-0.001	0.928**
X_{12}	0.005	0.016	-0.001	0.001	-0.001	0.004	0.001	-0.005	0.011	0.011	0.332**	-0.015	0.008	-0.001	0.002	-0.006	0.000	0.366**
X_{13}	0.001	-0.002	-0.001	-0.018	0.005	0.011	0.000	-0.006	0.008	0.005	0.053	-0.004	0.030	0.005	-0.003	0.001	-0.000	0.087
X_{14}	0.005	0.001	-0.001	-0.008	0.002	0.008	0.000	0.001	0.001	0.002	0.107	0.001	0.012	0.013	-0.003	-0.001	-0.001	0.142
X_{15}	-0.010	-0.008	-0.001	-0.006	-0.002	0.005	-0.000	-0.004	-0.004	-0.002	0.015	-0.001	-0.001	-0.001	0.054	0.007	0.001	0.039
X_{16}	0.003	0.011	-0.001	0.017	0.001	-0.007	0.000	0.012	-0.001	0.001	0.189	-0.001	-0.001	0.001	-0.008	-0.050	-0.001	0.168
X_{17}	-0.008	-0.010	0.001	0.006	-0.003	0.009	0.000	0.005	0.001	0.001	-0.106	-0.001	-0.001	-0.001	0.006	0.004	0.003	-0.091

** Significant at 5% level; ** Significant at 1% level, Residual effect: 0.3465*

X_1	*Plant height (cm)*	X_7	*Pod length (cm)*	X_{13}	*100 fresh seed weight (g)*
X_2	*Number of leaves / plant*	X_8	*Pod girth (cm)*	X_{14}	*100 dry seed weight (g)*
X_3	*Number of primary branches / plant*	X_9	*Fresh pod weight (g)*	X_{15}	*Tuber length (cm)*
X_4	*Number of secondary branches / plant*	X_{10}	*Dry pod weight (g)*	X_{16}	*Tuber girth (cm)*
X_5	*Stem girth (cm)*	X_{11}	*No. of seed/pod*	X_{17}	*Tuber weight (g)*
X_6	*No. of flowers / plant*	X_{12}	*Fresh seed weight / pod (g)*	X_{18}	*Dry seed yield/plant (g)*

3.3.2 Similarity index

The similarity matrix was computed using ISSR markers based on Jaccard's coefficient using NTSYS-Pc programme. The similarity coefficients based on 12 ISSR markers ranged from 0.503 to 0.780 (Table 6). The maximum similarity (0.780) was observed between the T_{10}-1 and control followed by T_{10}-1 and T_2-2 (0.765). Low similarity was observed between control and T_7-3 (0.503). The similarity coefficients ranged from 0.243 to 0.629 with a mean similarity index of 0.436 was observed by [27] in chrysanthemum and [28] in citrus.

Based on Jaccard's similarity coefficient with an Unweighted Pair Group Method with Arithmetic average (UPGMA), the molecular data were analyzed using Sequential Hierarchial and Nested (SAHN) clustering methods of the NTSYS-pc program version 2.02 [29]. The similarity coefficients based on 12 ISSR markers ranged from 0.503 to 0.780. The control and T7-3 were identified as diverse genotypes; T_{10}-1 and control followed by T_{10}-1 and T_2-2 were identified as close genotypes.

3.3.3 Clustering

A dendrogram was constructed for 6 samples using Jaccard's similarity index values using the NTSYS- pc ver 2.02. In Sequential Agglomerative Hierarchical Non overlapping (SAHN), UPGMA were used to generate dendrogram (Fig. 1).

Based on the Dendrogram, the 6 samples formed four clusters at similarity index of 0.65.

Cluster I : Control, T_{10}-2, T_2-2
Cluster II : T_8-2, T_{10}-4
Cluster III : T_7-3

Based on this similarity index, dendrogram was constructed and grouped into three clusters at 0.65 coefficients. The cluster I was found to have three mutants while the cluster II comprised of two mutants. Cluster III was solitary with single mutant. The control, mutant T_{10}-1, T_2-2 was observed to have close similarity and same for the mutants T_8-2, T_{10}-4. Mutants from diverse cluster may be intercrossed to generate higher variability.

Table 5. Percentage of polymorphism and Polymorphic Information Content (PIC) value for ISSR primer

Primers name and sequence		Total number of bands	Number of polymorphic bands	PIC value
UBC 807	AGAGAGAGAGAGAGAGT	73	14	0.947
UBC 810	GAGAGAGAGAGAGAGAT	21	3	0.764
UBC 820	GTGTGTGTGTGTGTGTC	17	8	0.822
UBC 821	GTGTGTGTGTGTGTGTT	53	17	0.924
UBC 824	CTCTCTCTCTCTCTCTG	12	5	0.767
UBC 825	ACACACACACACACACT	22	6	0.804
UBC 826	ACACACACACACACACC	31	14	0.908
UBC 827	ACACACACACACACACG	36	14	0.918
UBC 828	TGTGTGTGTGTGTGTGA	32	14	0.912
UBC 846	CACACACACACACACART	58	12	0.926
UBC 848	CACACACACACACACARG	59	6	0.917
UBC 849	GTGTGTGTGTGTGTGTYA	30	3	0.826
Total bands		444	116	-
Average number of bands per primer		37	9.67	-

Table 6. Jaccard's similarity coefficients for five *Gloriosa superba* mutants based on ISSR markers

	Control	T_8P_2	$T_{10}P_1$	$T_{10}P_4$	T_7P_3	T_2P_2
Control	1.000					
T_8P_2	0.531	1.000				
$T_{10}P_1$	0.780	0.638	1.000			
$T_{10}P_4$	0.553	0.695	0.631	1.000		
T_7P_3	0.503	0.645	0.567	0.624	1.000	
T_2P_2	0.687	0.588	0.765	0.539	0.560	1.000

T-Treatment; P-Plant number

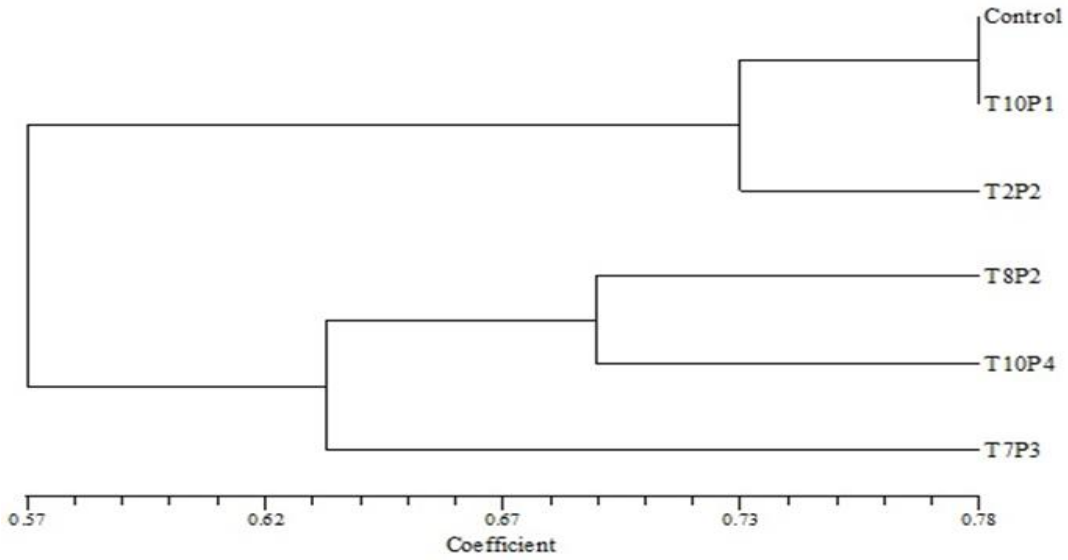

Fig. 1. Dendrogram of *Gloriosa superba* mutants using UPGMA based on Jaccard's coefficient

It is directly revealed that DNA changes had happened to these mutants and the dendrogram, showing the formation of three main groups of mutants, indicated that the effects of different mutagen dosages on tubers are far from each other. This result was in accordance with studies in lily [30], banana [31], *Jatropha curcas* L. [32], sugar beet [33].

Thus, the ISSR analysis of the mutants revealed that polymorphism created by induced mutagenesis can be used to select desirable mutants in *G. superba*.

4. CONCLUSION

The path analysis of component characters *viz*., number of leaves per plant, dry pod weight, number of seeds per pod, fresh seed weight per pod exerted positive direct effect on dry seed yield per plant of *G. superba* in VM_2 generation. The ISSR analysis of the mutants revealed that polymorphism created by induced mutagenesis can be used to select desirable mutants in *G. superba*. High heritability and genetic advance as per cent of mean was recorded for most of the characters under study indicating better scope for further selection. Differential patterns resulted in ISSR analysis indicating the polymorphism created by induced mutagenesis, creating scope for selection of desirable mutants in *G. superba*. Mutants with high yielding characters have to advance to VM_3 generation to assess the stability and quality parameters.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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