UNDER PEER REVIEW

1 *Original Research Article*

2 **MOLECULAR AND AGRO-MORPHOLOGICAL GENETIC DIVERSITY**
3 **ASSESSMENT OF *GLORIOSA SUPERBA* MUTANTS**

4

5 **ABSTRACT**

6 The analysis of variance indicated significant differences among the treatments for most
7 of the traits. Estimates of phenotypic coefficient of variation for the selected traits in VM_2
8 generation were in general slightly higher than the genotypic coefficient of variation indicating
9 the influence of environmental factors on these traits. High heritability and genetic advance as
10 per cent of mean was recorded for most of the characters under study indicating better scope for
11 further selection. Differential patterns resulted in ISSR analysis indicating the polymorphism
12 created by induced mutagenesis, creating scope for selection of desirable mutants in *G. superba.*

13 **KEYWORDS**

14 Genetic Variation, Heritability, Molecular Markers, Polymorphism, Mutants

15 **1. INTRODUCTION**

16 One of the very important exported medicinal plants of India that has become endangered
17 within a very short span of the last 50 years is *Gloriosa superba L.* Seeds and tubers contain
18 valuable alkaloids, *viz.*, colchicine and colchicoside, which are used to treat gout and
19 rheumatism. Due to the action of colchicoside on spindle fiber formation during cell division, the
20 plant has been identified as a potential anti-cancerous drug [27]. Though *G. superba* has an
21 extensive natural distribution, the species has become endangered due to over exploitation of its
22 tubers and low percent germination. Hence there is an urgent need to explore the possibilities for
23 developing variability in this species with high seed yield and improved colchicine content

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24 through breeding techniques. New cultivars of *Gloriosa superba* are developed by radiation-
25 induced mutation. Since the effect of mutation in gloriosa is clearly visible, selection for changed
26 plant stature, high seed yield, increased active principle content is possible in the M1 generation
27 itself because of vegetatively propagating nature. Novelty visible in any form is of high value
28 and hence mutation breeding played a key role in the improvement of in general and gloriosa in
29 particular. The aim of the present study was to generate information on character association,
30 direct and indirect influence of characters on seed yield in the induced mutants of Glory lily and
31 to assess the genetic variation among mutants by ISSR profile.

32 **2. MATERIALS AND METHODS**

33 **2.1. Genetic characterization of mutants**

34 Tubers of *Gloriosa superba* collected from Mulanur, Tamil Nadu, were subjected to three doses
35 of gamma irradiation (0.50, 1.00, 1.50 kR), ethyl methyl sulphonate (1.0, 1.5 and 2.0%) and
36 diethyl sulphonate (1.0, 1.5 and 2.0%). The experiment was conducted at the Department of
37 Medicinal and Aromatic Crops, Horticultural College and Research Institute, Coimbatore during
38 the first week of August, 2010 ($VM_{1,}$ first generation of vegetative mutant) and first week of
39 August, 2011 ($VM_{2,}$ second generation of vegetative mutant). In the VM_2 generation, 18
40 biometrical and yield parameters recorded were subjected to analysis of phenotypic and
41 genotypic co-efficient of variability [8], heritability [4], genetic advance and correlation co-
42 efficient [14] and path coefficients [12].

43 **2.2. Molecular profiling of mutants through ISSR analysis**

44 **2.2.1. DNA extraction**

45 DNA from the five mutants of *G. superba* in VM_2 generation was extracted by following the
46 protocol of [32], with a slight modification. Mercaptoethanol (1 per cent) and polyvinyl

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47 pyrrolidone (PVP) 0.2 per cent were added to the extraction buffer to remove the phenolics.
48 Three grams of young leaf tissue was ground with liquid nitrogen and to this powder, 15 ml of
49 preheated CTAB buffer (65°C) was added. It was then incubated at 65°C in a water bath for one
50 hour. After bringing the tubes to room temperature, equal volume (15 ml) of chloroform: isoamyl
51 alcohol (24:1) was added and the contents were mixed well for 10 minutes to form an emulsion.
52 It was then centrifuged at 10,000 rpm for 15 minutes at 15°C. The supernatant was transferred to
53 a fresh tube and the chloroform: isoamyl alcohol step was again repeated.

54 The aqueous phase was transferred to a new tube and equal volume of ice cold isopropanol was
55 added and incubated in a freezer for overnight. The contents were then centrifuged at 10,000 rpm
56 for 20 minutes at 16°C. The pellet was then saved by discarding the solution. The pellet was
57 washed with 70 per cent ethanol by centrifuging the contents at 10,000 rpm for 10 minutes. The
58 alcohol was discarded and the pellet was air dried. The pellet was then dissolved in 3 ml of
59 double distilled water. Then 1 µl of RNase was added and incubated at 37°C for 30 minutes.
60 DNA was precipitated by adding 50 µl of 3M sodium acetate and 7.5 ml of 100 per cent ethanol
61 and the contents were again centrifuged at 10,000 rpm for 10 minutes. Supernatant was
62 discarded. The pellet was washed with 70 per cent ethanol and air dried. It was finally dissolved
63 in TE buffer (150 µl) and stored at - 20°C.

64 **2.2.2. DNA quality and quantity check**

65 To check the quality and quantity of the extracted genomic DNA, gel electrophoresis was carried out
66 on 0.8 per cent agarose gel. DNA concentration for PCR amplification was estimated by
67 comparing the band intensity of a sample with the band intensities of known dilutions that gave

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68 good amplifications. Based on the band intensity, the DNA was further diluted to the required
69 concentration (25-50 ng) using double distilled water [10].

70 **2.3.1. ISSR analysis**

71 PCR was performed by means of 12 ISSR primers (as described by University of British
72 Columbia, Canada) synthesized at Sigma - Aldrich (USA), Bangalore, that were selected out of
73 45 ISSR primers tested (Table 1). The primer selection was based on the degree of
74 polymorphism and the distinctness of the bands they produced when tested on a sample set.

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

83 **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

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9	UBC-828	**TGTGTGTGTGTGTGTGA**	53.2
10	UBC-846	**CACACACACACACACART**	53.7
11	UBC-848	**CACACACACACACACARG**	55.5
12	UBC-849	**GTGTGTGTGTGTGTGTYA**	50.5

84

85 **2.3.2. Separation of amplified fragments using Polyacrylamide gel electrophoresis**

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

89 **2.3.3. Assembling and pouring the gel**

90 Spacers (0.5 mm thickness) were placed along the side edges of the bind silane treated surface of
91 the glass plate. The repel silane treated glass plate (notched plate) was kept on the bind silane
92 treated surface so that treated surfaces face each other (in a sandwich like fusion).The spacers
93 were fitted well against each other so that there is no gap. By using cellotape, all the edges were
94 tightly sealed so that there is no gap for bottom or sides. For casting each gel, 250 ml of gel
95 solution was required. Acrylamide solution mix, 10X TBE buffer and distilled water were mixed
96 well and finally 10 per cent APS and TEMED were added. The contents were mixed gently by
97 swirling and bubbles were avoided. The assembly was kept on a bench top so that it makes 45
98 degree angle with the bench top. The solution was carefully poured into the space between the
99 glass plates starting at the lower corner. After filling, the comb (0.5 mm thickness) was inserted
100 straight across the top moving not more than 5 mm of notched plate. The gel was left for 20-40
101 minutes for polymerization to proceed [6].

102 **2.3.4. Electrophoresis**

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

110 **2.3.5. Visualization of bands**

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

116 **2.3.6. Steps followed for silver staining**

117 Improved staining method was followed for staining. This method was a combination of
118 different steps proposed by [7]. After electrophoresis, gels were washed in 1000 ml cold (10-
119 12°C) fixing solution (10 per cent absolute ethanol, 0.5 per cent acetic acid) for 5 minutes.
120 Washed gels were soaked for 6-7 minutes at room temperature (22-24°C) in a 1000 ml solution
121 of 0.15 per cent Silver Nitrate, 1.5 ml 37 per cent Formaldehyde. Gels were rinsed quickly (10-
122 15 sec) once with 1000 ml double distilled water. They were then developed by soaking them at
123 room temperature (22-24°C) in a 1000 ml developing solution (1.5 per cent Sodium Hydroxide, 2
124 ml of 37 per cent Formaldehyde) until the bands appeared with a sufficient intensity and finally

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125 impregnating the gel in a 2000 ml stop solution (10 per cent absolute ethanol, 0.5 per cent acetic
126 acid) for 2 minutes. All steps were done in plastic containers. The gel plates were agitated in a
127 shaker throughout the staining process. The fix stop, developer and silver nitrate solutions were
128 prepared in advance but Formaldehyde was added just before use.

129 **3. RESULT AND DISCUSSION**

130

131 Induction of mutation in polygenic quantitative traits can be well detected by the estimation of
132 variance, genetic advance and other genetic parameters of mutants. It is evident from the current
133 study that the analysis of variance indicated significant differences among the treatments for
134 most of the traits. Estimates of phenotypic coefficient of variation (PCV) for the selected traits
135 in VM_2 generation were in general slightly higher than the genotypic coefficient of variation
136 (GCV) indicating the influence of environmental factors on these traits (**Table 2**).
137 The mutants exhibited a GCV range from 1.24 % (number of leaves / plant) to 55.06 % (number
138 of secondary branches per plant). The PCV was highest for number of secondary branches/plant
139 (102.02 %) while it was lowest (1.76%) for number of leaves / plant. Among the characters
140 studied, higher heritability was noticed for stem girth (89.74 %) followed by fresh pod weight
141 (82.39 %). The genetic advance as percentage of mean was highest for stem girth (69.30)
142 followed by number of secondary branches / plant (61.22), while it was the lowest for plant
143 height (1.73).
144 In VM_2 generation, PCV was higher than the GCV, thus, revealing a strong association at
145 phenotypic level between the characters. This might be due to the masking effect of environment
146 in modifying the total expression of the phenotypes and hence genotypic expression was
147 reduced. In VM_2 generation, the traits *viz.,* stem girth, number of primary branches / plant,

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148 number of secondary branches / plant, fresh seed weight / pod, number of seed / pod, 100 fresh
149 seed weight, tuber length, tuber weight and dry seed yield / plant recorded high PCV and GCV
150 emphasizing these characters to be potentially variable. It was also observed that the differences
151 between PCV and GCV were meagre revealing the fact that these traits were less influenced by
152 the environment. High values of GCV suggested better improvement for selection of traits. How-
153 ever, [29] observed highest PCV for plant height, number of laterals plant^{-1}, number of leaves
154 plant^{-1}, number of tuber plant^{-1}, tuber length, tuber girth in *Coleus forskohlii.*
155 High genetic advance as % of mean was recorded for stem girth, number of primary branches /
156 plant, number of secondary branches / plant, fresh pod weight, fresh seed weight / pod, number
157 of seed / pod, 100 fresh seed weight, tuber length, tuber weight and dry seed yield / plant. This
158 indicates that selection can be relied upon for improvement of these parameters among the progenies.
159 High genetic advance was governed by additive genes and paves the way for improvement of those
160 characters in individual plant selection [19].

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

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

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162 High heritability was recorded for stem girth, fresh pod weight, fresh seed weight / pod, number
163 of seeds / pod, 100 fresh seed weight, 100 dry seed weight, dry seed yield / plant, tuber length
164 and tuber weight. It indicates that selection of such characters is easy because of the close
165 correspondence between the phenotype and genotype due to relatively smaller contribution of the
166 environment to genotype. [22] also reported high heritability for plant height and moderate for
167 seeds per inflorescence in case of *Dianthus caryophyllus.*
168

169 ### 3.1. Association analysis

170 The highest and positive significant correlation for dry seed yield / plant (g) was observed with
171 number of seeds / pod (0.928) closely followed by number of leaves / plant (0.537) and dry pod
172 weight (0.454) which was further followed by fresh seed weight / pod (0.366), fresh pod weight
173 (0.298), plant height (0.282) and number of secondary branches / plant (0.236) (**Table 3**).
174 Positive significance of intercorrelation (Residual effect-0.3465) with plant height was observed
175 for the traits *viz.*, number of leaves / plant (0.471), number of seeds / pod (0.270), tuber length
176 (0.379) and tuber weight (0.309). Similarly number of leaves / plant showed positive significance
177 with number of flowers / plant (0.487), fresh pod weight (0.260), dry pod weight (0.378),
178 number of seeds / pod (0.549), fresh seed weight (0.323), tuber girth (0.222) and tuber weight
179 (0.216). Number of primary branches / plant showed positive and significant correlation with
180 number of secondary branches / plant (0.350) and plant girth (0.421) while number of secondary
181 branches / plant exhibited positive and significant correlation with plant girth (0.274), number of
182 flowers / plant (0.253), number of seeds / pod (0.239), 100 fresh seed weight (0.219) and tuber
183 girth (0.230).

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184 A positive and significant correlation was exerted by stem girth (0.219) and number of flowers / 185 plant (0.235) with dry pod weight and fresh 100 seed weight respectively. On the other hand, 186 fresh pod weight exhibited significance in the positive direction with dry pod weight (0.353), 187 number of seeds / pod (0.263), fresh seed weight / pod (0.270) and 100 fresh seed weight 188 (0.202). Dry pod weight exerted a positive and significant correlation with number of seeds / pod 189 (0.448) and fresh seed weight / pod (0.316). Similarly, number of seeds / pod, fresh seed weight / 190 pod and 100 fresh seed weight exhibited a positive and significant correlation with fresh seed 191 weight (0.382), 100 fresh seed weight (0.275) and 100 dry seed weight (0.399) respectively. 192 Significant correlation in the negative direction was observed for pod girth with 100 dry seed 193 weight and tuber girth.

194 Positive correlation of number of seeds / pod with dry seed yield / plant was reported by [18] in 195 chick pea and [23] in *Phaseolus vulgaris.* Positive association of number of branches with dry 196 seed yield was reported by [18] in chick pea and [9] in *Cajanus cajan*. Positive correlation of 197 number of leaves / plant with seed yield / plant was reported by [11], while [30] reported positive 198 correlation of pod weight with seed yield in long bean.

199 This analysis revealed that selecting plants with more plant height, number of secondary 200 branches / plant, number of leaves / plant, number of seeds / pod, dry pod weight, fresh seed 201 weight / pod and fresh pod weight were desirable for future crop improvement programme. The 202 positive inter correlation among the yield components also indicated the possibility of simultaneous 203 improvement of seed yield.

204

205 **3.2.Path analysis**

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206 Correlation coefficient between any two characters would not give a complete picture for a
207 parameter like yield which is controlled by several other traits, either directly or indirectly. In
208 such situations, path coefficient analysis furnishes a means of measuring the direct effect of each
209 trait as well as the indirect effect *via* other characters on yield. So information on the direct and
210 indirect effect on yield is important which is explicable by path analysis as proposed by [33] and
211 illustrated by [12]. The interrelationships of the component characters on yield provide the likely
212 consequences of their selection for simultaneous improvement of desirable characters with yield.

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

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

219 Since the correlation of these characters with yield is positive, preference should be given to
220 these characters in the selection programme to isolate superior mutants with genetic potential for
221 improving yield [31]. Direct effect of number of branches with seed yield / plant was reported by
222 [25]. The number of seeds / pod had high direct effect on dry seed yield / plant. Indirect positive
223 effects of dry pod weight, fresh seed weight / pod on dry seed yield / plant was recorded by [18],
224 [23] and [9]. Direct effect of pod weight with seed yield / plant was reported by [30].

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

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Table 3. Effect of mutagens on simple correlation coefficient 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	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	No. of leaves / plant	X_8	Pod girth (cm)	X_{14}	100 dry seed weight (g)
X_3	No. of primary branches / plant	X_9	Fresh pod weight (g)	X_{15}	Tuber length (cm)
X_4	No. 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)

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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

236

237 * Significant at 5% level; ** Significant at 1% level
238 Residual effect: 0.3465
239

X_1	Plant height (cm)	X_7	Pod length (cm)	X_{13}	100 fresh seed weight (g)
X_2	No. of leaves / plant	X_8	Pod girth (cm)	X_{14}	100 dry seed weight (g)
X_3	No. of primary branches / plant	X_9	Fresh pod weight (g)	X_{15}	Tuber length (cm)
X_4	No. 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)

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244 **3.3.Molecular characterization of mutants**

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

251 **3.3.1.Marker polymorphism**

252 In the present investigation, six samples were used to study the genetic diversity using 12
253 ISSR primers. The PCR amplification using these 12 primers yielded 444 reproducible amplified
254 bands. The number of amplified bands varied from 12 (UBC 824) to 73 (UBC 807). Out of 444
255 bands, 116 were found to be polymorphic. Average number of bands and polymorphic bands per
256 primer were 37 and 9.67 respectively (**Table 5**). The Polymorphic Information Content (PIC)
257 value as a relative measure of polymorphism level ranged between 0.764 (UBC 810) to 0.947
258 (UBC 807). The higher PIC value indicated the informativeness of the primer. Among the
259 primers used in the study, five primers *viz*., UBC 846, UBC 821, UBC 827, UBC 848 and UBC
260 828 exhibited the PIC value from 0.926 to 0.912. These primers can provide the basis for Gloriosa
261 DNA profile system. Such high level of polymorphism is comparable to the results of some similar
262 molecular researches on medicinal plants of Lamiaceae family [17, 1, 2] . [35,24] observed similar
263 reports in thyme and patchouli respectively.

264 **Table 5. Percentage of polymorphism and Polymorphic Information Content (PIC) value**
265 **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

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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	-

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268 **3.3.2.Similarity index**

269 The similarity matrix was computed using ISSR markers based on Jaccard's coefficient
270 using NTSYS-Pc programme. The similarity coefficients based on 12 ISSR markers ranged from
271 0.503 to 0.780 (**Table 6**). The maximum similarity (0.780) was observed between the T_{10}-1 and
272 control followed by T_{10}-1 and T_2-2 (0.765). Low similarity was observed between control and
273 T_7-3 (0.503). The similarity coefficients ranged from 0.243 to 0.629 with a mean similarity index
274 of 0.436 was observed by [16] in chrysanthemum. and [3] in citrus.

275 The molecular data were analyzed using Sequential Hierarchial and Nested (SAHN) clustering
276 methods of the NTSYS-pc program version 2.02 [21] based on Jaccard's similarity coefficient
277 with an Unweighted Pair Group Method with Arithmetic average (UPGMA). The similarity
278 coefficients based on 12 ISSR markers ranged from 0.503 to 0.780. The control and T7-3 were

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279 identified as diverse genotypes; T_{10}-1 and control followed by T_{10}-1 and T_2-2 were identified as
280 close genotypes.

281 **Table 6. Jaccard's similarity coefficients for five *Gloriosa superba* mutants based on ISSR**
282 **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

283

284 T-Treatment; P-Plant number

285

286 **3.3.3.Clustering**

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

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

291 Cluster I : Control, T_{10}-2, T_2-2

Cluster II : T_8-2, T_{10}-4

Cluster III : T_7-3

292 Based on this similarity index, dendrogram was constructed and grouped into three clusters at
293 0.65 coefficients. The cluster I was found to have three mutants while the cluster II comprised of
294 two mutants. Cluster III was solitary with single mutant. The control, mutant T_{10}-1, T_2-2 was

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295 observed to have close similarity and same for the mutants T_8-2, T_{10}-4. Mutants from diverse
296 cluster may be intercrossed to generate higher variability.
297 It is directly revealed that DNA changes had happened to these mutants and the dendrogram,
298 showing the formation of three main groups ofmutants, indicated that the effects of different
299 mutagen dosages on tubers are far from each other. This result was in accordance with studies in
300 lily [34], banana [15], *Jatropha curcas* L. [13], sugar beet [26].
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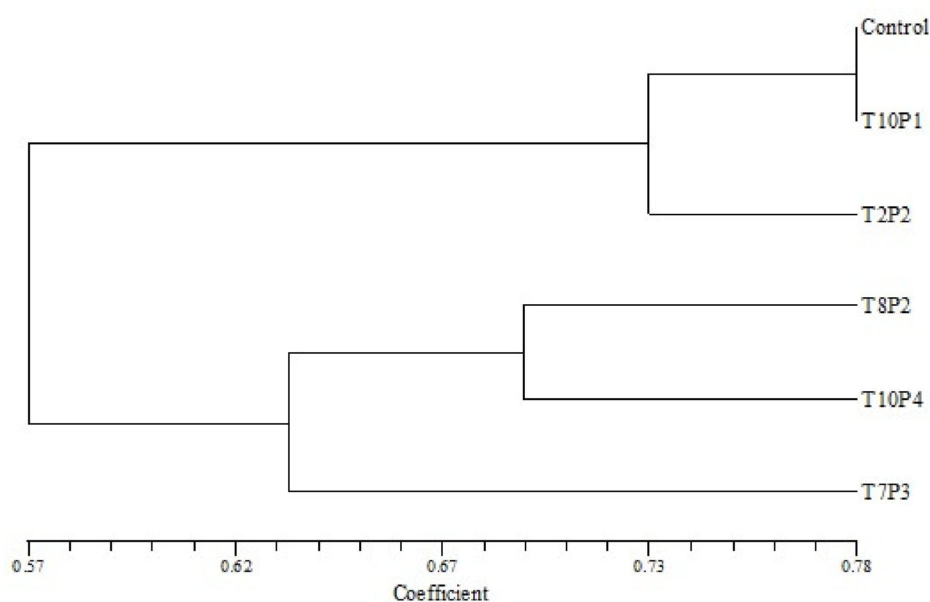
302
303
304 Fig. 1. Dendrogram of Gloriosa superba mutants using UPGMA based on Jaccard's coefficient
305



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

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312 **4.Conclusion**

313 The path analysis of component characters on dry seed yield per plant of *G. superba* in
314 VM_2 generation exerted positive direct effect through the characters *viz*., number of leaves per
315 plant, dry pod weight, number of seeds per pod, fresh seed weight per pod. The ISSR analysis of
316 the mutants revealed that polymorphism created by induced mutagenesis can be used to select
317 desirable mutants in *G. superba*.

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