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HIGH ALKALOIDS PROMISING INDUCED MUTANTS BY GAMMA RAYS AND THEIR MOLECULAR MARKERS IN *ATROPA BELLADONNA* L.

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ABSTRACT: This investigation was carried out to induce gamma rays mutants in *Atropa belladonna* L. possessing high alkaloids contents. The used gamma rays doses were 50, 80, 110 and 150 Gy. The mutants had apparent morphological changes in plant height, no. of leaves and flowers as well as larg leaf area. Three promising high alkaloids mutants were selected from M_2, M-11-1, M-11-2 and M-15-1. These promising mutants seemed to be a very important for their high alkaloids content, they possessed twice values than the control .These high alkaloids mutants, possessed stable morphological criteria at M_3 generation. Molecular studies on these mutants were done for identification of them by ISSR technique confirming the difference between these mutants and control. The three mutants were distiguished by unique molecular markers, i.e. Mutant M-11-1 distinguished by three molecular markers with molecular sizes 1397, 1149 and 874 bp (base pair). Mutant M-11-2 distinguished by four molecular markers with molecular sizes 1537, 1075, 839 and 510 bp. Mutant M-15-1 distinguished by three molecular markers with molecular sizes 1749, 817and 756 bp. These findings drew the attention to the importance of genetic variation between these mutants and mother genotype, as well as, it considered a primary study to finger printing them.

Key words: *Atropa belladonna* L., gamma rays, molecular markers, total alkaloids.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of world's population, or thousands of years. Even today, the World Health Organization estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines. *Atropa belladonna* L. is valued for the use of alkaloids in the treatment of Parkinson's disease, anti-inflammatory properties for relief of bronchial asthma and motion sickness, ability to counteract toxic agents and for dilation of the pupils in optometry (Grieve *et al.*, 1995).

Irradiated *Atropa belladonna* L . seeds with various doses of gamma rays can be produced altered plant phenotypes having different alkaloidal content at 1st and 2nd mutagenic generation,M_1 and M_2, (Ghiorghita *et al.*, 1982). Irradiation can also increase in the alkaloids percentage in the different organs of plant, particularly the leaves (Abo Elseud, 1983., El-Kholy, 1987., and Habba ,1989).

Inter simple sequence repeat (ISSR) markers have recently become widely used for identification between genotypes on molecular basis. The ISSR primers were described as highly conserved in most of the studied plant genomes (Blair *et al.*, 1999). Jain *et al.*, (1999) evaluated the genetic diversity and generated genome fingerprinting of genus Pandorea. Therefore, the present study is aimed to induce some *Atropa belladonna* L. mutations possessing high alkaloids contents and identification as well as characterzation of such mutants on the molecular basis using ISSR markers.

MATERIALS AND METHODS

The present study was carried out during three successive seasons, 2003 -2006 at the Greenhouse of Botany Department, National Research Center, Giza, Egypt

Atropa belladonna L. seeds were kindly obtained from the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University. Seeds of *Atropa belladonna* L. were irradiated by 0, 50, 80, 110 and 150 Gy. γ – rays at National Center For Radiation Research and Technology, Cairo, Egypt. Irradiated seeds were sown directly in multi pot transplant trays, filled with a mixture of peat-moss and sand, and arranged in a

complete randomized block experimental design with three replications.

Random samples from generation M_1 plants were taken to study the effect of gamma irradiation on the following morphological characters, plant height (cm) at 90 days old, No. of leaves per plant, Leaf diameter and Leaf area (cm^2).

Total alkaloids were estimated in dry leaves of twenty five M_1 plants from irradiated treatments 110 and 150 Gy only at flowering stage according to Cordel (1981) and adapted by mahmoud (2004). Total alkaloids were determined as mg/g of dry leaves weight and mg/g of total dry plant leaves weight.

Seeds of these and control plants along with control seeds were grown to obtain M_2 plants. The studied morphological characters and alkaloids content were also estimated in leaves at M_2 plants. Only three plants were selscted having highr alkaloids content than control plants. Seeds of three plants were sown to obtain M_3 plants. Progenies of these plants, M_3 plants, were subjected to study the same morphological characters in addition to number of branches and flowers per plant.

Total Alkaloids

The leaves of selected M_1 and M_2 plants at flowering stage were shade dried and ground . Extraction and isolation of tropane alkaloids from the leaves of *Atropa belladonna* L. was done according to Cordel (1981) and Mahmoud (2004).

Molecular Genetic Studies

Genomic DNA extraction

Total DNA was extracted from young leaves of three high alkaloid M_2 plants and control plants following Dellaporta procedure (Dellaporta and Hicks, 1983).

ISSR –PCR analysis

The ISSR markers are generated from single – primer PCR reactions where the primer is designed from di – or tri nucleotides repeat motifs with a 5' or 3' anchoring sequence of one to three nucleotides (Zietkiewicz *et al.*, 1994) and (Wolfe *et al.*, 1995). The following three ISSR primers were used in the present study according to Sharama *et al.*, (1995),IS- 1: 17899A with sequence 5´ (CA)$_6$ AG 3´

IS- 2: 17898 with sequence 5´ (CA) $_6$ GT 3´ IS- 3: HB8 with sequence 5´ (GA)$_6$ GG 3´

Amplification was carried out in 25 µl reaction volume containing 2.5 µl PCR buffer (10x), 1.5 µl $MgCl_2$, 2 µl dNTPs, 2 µl primers, 0.2 µl Taq DNA polymerase, 2 µl Template DNA and 14.8 µl ddH_2O.

PCR amplification was performed in a Hybrid Cycler programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 30 sec, an annealing step at 42°C for 45 sec, and an elongation step at 72°C for 1 min and 30 sec. The primer extension segment was extended to 10 min at 72°C in the final cycle. Agarose gel (1.2 %) electrophoresis was used for separating the PCR products. Gels were photographed and scanned with Bio-Rad video densitometer Model 620, at a wavelength of 577.

Statistical Analysis

Data were statistically analyzed. Means, and standard errors, and L.S.D. were estimated according to Sokal and Rohlf (1995).

RESULTS AND DISCUSSION

Highly significant differences were recorded between the effect of different doses of gamma rays and control at M_1 generation Table 1.Positive effects of γ – rays doses were recorded on plant height, No. of leaves/plant, length and width of leaf and leaf area till150 Gy dose which possessed negative effect on most studied characters espicially leaf area.

These results confirmed with the principle concept of γ – rays effects as ionizing radiation, which low doses stimulate metabolism and subsequently increasing of almost performence of organism, while high doses induce sever damage espicially to enzymes as protein molecules and subsequently decrease of performence characters of any organism. Therefore, the doses from 50 to 110 Gy had stimulation effects, while the 150 Gy dose had an inhibition effect.

These results are in great agreement with researcheres Helmy (1984), Soliman (1984), El-Kholy (1987), Habba (1989), Soliman *et al.*, (2003) and Abla Nassar *et al.*, (2004).

Total alkaloids of the three mutant plants that selected as well control plants at M_1 and M_2 generations are shown in Table 2.

Table1. Mean performance for morphological criteria in M_1 generation under different doses of γ – ray at 90 days from sowing

Treatments	Characters				
	Plant height (cm)	NO. of leaves	Leaf diameter (cm)		Leaf area (cm^2)
			Length	Width	
Control	24.167	17.333	7.533	6.067	34.437
50 Gy	31.333	18.000	8.700	7.033	45.933
80 Gy	43.767	33.333	7.167	5.667	30.590
110 Gy	54.000	20.333	15.200	9.067	106.423
150 Gy	34.50	24.000	7.167	4.267	22.840
L.S.D. 5%	2.311	6.159	4.643	1.462	43.168
1%	3.363	8.961	6.755	2.127	62.805

Table 2. The average mean of total alkaloids of selected mutants at M_1 and M_2 generation

Treatment	Total alkaloid (mg/g)		Total alkaloid (mg / plant)	
	M_1	M_2	M_1	M_2
Control	1.96	2.03	48.84	53.41
M-11-1	4.80	4.01	128.54	109.67
M-11-2	4.28	3.43	113.56	96.33
M-15-1	3.31	3.79	89.24	108.05

The mutants had higher total alkaloids than the control, they possessed twice values than the control, especially M-11-1, which had 4.01 mg/g and 109.67 mg/plant at M_2 generation comparing with 2.03 mg/g and 53.41 mg/plant for the control.

These mutants might require more detail studies on the kinds of alkaloids and their fractionations as well as testing their stability upto M_5 generation. These results are in the same trend with several invistigators on *Atropa belladonna* L. Helmy (1984), Habba (1989) and Mahmoud (2004).

The above results cleared the importance of high alkaloid mutants in the genetic improvement of alkaloids content in *Atropa belladonna* L. Therefore, molecular studies on these mutants should be done for identification and confirm differences between these mutants and control on the basis molecular genetic marker.

The mean performance of morphological criteria of selected mutants at M_2 and M_3 generations was shown in Fig. 1 and Table 3. Fig.1 appeared more difference between the selected mutants as well as between the control at flowering stage for the studied morphological characters. The average means and their satandard errors Table 3 assured such detected differences in most characters. M-11-1 mutant might consider as a promising mutants for large leaves, leaf area at M_2 and M_3 generation exceeding the other mutants and mother plant. M-11-2 and M-15-1 possessed highly number of leaves, the later one was more stable for number of flowers at M_2 and M_3 generations.

Molecular Genetic Markers

In this study ISSR markers were employed to distinguish among three mutants M-11-1, M-11-2 M-15-1 and control. Three ISSR primers were used. The results showed that the total number of bands were 15, 3 and 3 respectively as shown in Fig.2 and Table 4. From these results the total bands amplified by the three primers were 21 bands varied from 3 to 15. A total of 21 bands were polymorphic across the entire sample, so, the percentage of polymorphic was 100 %.

Furthermore, primer IS -1 gave seven molecular markers, defined as five positive molecular markers, which found in M-11-1, M-11-2 and M-15-1 in comparable with control. Molecular sizes of positive molecular markers were 1749, 1537, 817, 756 and 530 bp

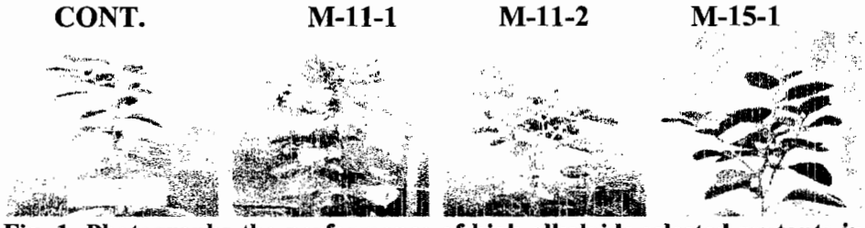


Fig. 1. Photographs the performance of high alkaloids selected mutants in *Atropa belladonna* L M_2 generation at flowering stage

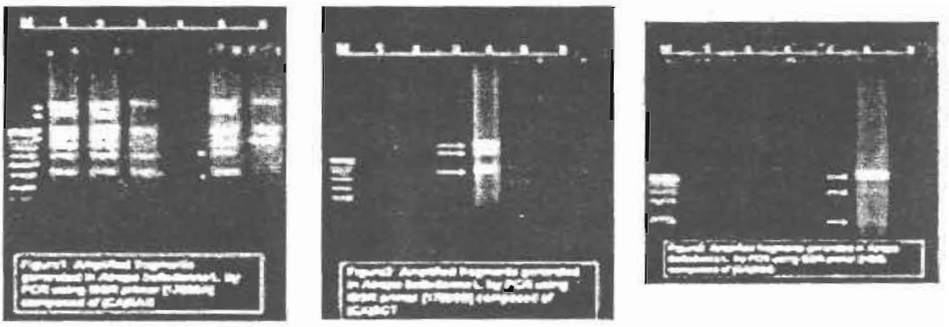
Table 3. Average mean ± standard error of selected mutants of *Atropa belladonna* L. at M_2 and M_3 generations for studied morphological criteria

Genotypes	Plant height	No. branches	No. leaves	No. flowers	Length of leaf	Width of leaf	Leaf area
			M_2 Generation				
Cont.	57.17	3.33	53.67	7.33	7.03	5.83	30.85
M-11-1	32.00	2.33	31.00	35.00	14.47	7.03	76.40
M-11-2	58.05	2.25	60.18	11.63	9.34	7.83	54.85
M-15-1	21.00	0.67	17.67	18.00	10.01	5.39	40.37
LSD 5%	5.717	2.877	4.048	24.177	1.594	1.522	18.397
1%	9.481	0.771	6.132	40.098	2.643	2.524	30.512
			M_3 Generation				
Cont.	73.25± 1.25	3.25± 0.49	51.75± 1.18	10.25± 0.63	8.85± 0.12	6.28± 0.16	41.65± 0.99
M-11-1	53.00± 0.41	2.25± 0.48	29.00± 0.41	10.00± 0.41	22.06± 0.44	10.46± 0.2	173.17± 6.04
M-11-2	77.50± 1.19	4.25± 0.25	63.25± 1.97	10.50± 0.87	11.23± 0.17	8.15± 0.7	68.64± 0.75
M-15-1	58.75± 1.75	4.00± 00	67.25±2.2	19.50± 0.5	6.88±0.36	5.18± 0.18	26.82± 2.3
LSD 5%	4.307	2.497	4.469	1.964	0.888	0.542	8.797
1%	6.188	3.588	6.412	2.821	1.275	0.779	12.64

Table 5. On the other hand, there were two negative molecular markers, which found only in control as compared with the three mutants, with molecular sizes 1441 and 978 bp. Moreover, primer IS -2 gave three positive molecular markers found only in M-11-1, with molecular sizes 1397, 1149 and 874 bp. Also, primer IS -3 gave three positive molecular markers found only in M-11-2, with molecular sizes 1075, 839 and 510 bp.

Moreover, there were ten molecular markers distinguished and identified in these mutants (M-11-1, M-11-2 and M-15-1). By applying this technique (ISSR), these markers could confirm the difference between these mutants:

Mutant M-11-1 distinguished by three molecular markers with molecular sizes 1397, 1149 and 874 bp. Mutant M-11-2 distinguished by four molecular markers with molecular sizes 1537, 1075, 839 and 510 bp. Mutant M-15-1 distinguished by three molecular markers with molecular sizes 1749, 817and 756 bp.



IS 01 (99 A) **IS 02 (98 B)** **IS 03 (HB 8)**

Fig. 2. ISSR-based PCR fragments of three primers IS-01, IS-02, IS-03 M= DNA standard marker, 1= Control, 4= M-11-1, 5=M-11-2 , 6= M.15 -1

Table 4. Summary of the results obtained in the construction of molecular identification profiles of mutants *Atropa belladonna* L. plants

Primers	Amplified bands			Polymorphic %
	Monomorphic	Polymorphic	Total	
1	0	15	15	100
2	0	3	3	100
3	0	3	3	100
Total	0	21	21	

Table 5. ISSR- markers for selected mutants of *Atropa belladonna L.* by three primers

Primer	M.S (bp)	Genotype				Marker type
		Cont.	M-11-1	M-11-2	M-15-1	
IS- 1	1749	-	-	-	+	Positive
	1537	-	-	+	-	Positive
	1441	+	-	-	-	Negative
	978	+	-	-	-	Negative
	817	-	-	-	+	Positive
	756	-	-	-	+	Positive
	530	-	+	+	+	Positive
IS- 2	1397	-	+	-	-	Positive
	1149	-	+	-	-	Positive
	874	-	+	-	-	Positive
IS- 3	1075	-	-	+	-	Positive
	839		-	+	-	Positive
	510		-	+	-	Positive

\+ Present - Absent

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طفرات مبشرة مستحدثة عالية المحتوي من القلويدات باستخدام أشعة جاما والواسمات الجزيئية لها في نبات البلادونا

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أجري هذا البحث بهدف إستحداث طفرات في نبات البلادونا بهدف زيادة المحتوي الكلي للقلويدات وتمييزها علي المستوي الجزيئي. استخدمت أشعة جاما بجرعات ٥٠، ٨٠، ١١٠، ١٥٠ جراي.احتوت الطفرات على تغيرات مورفولوجية في (طول النبات- عدد الأوراق- التزهير- مساحة الورقة وأبعاد الورقة). بعد دراسة المحتوي الكلي للقلويدات تم إنتخاب ثلاث طفرات فقط من الجيل الطفرى الثانى ذات محتوي عالي من القلويدات وهي (M-11-1، M-11-2،M-15-1) حيث وجد أن هذه الطفرات الثلاثة تحتوي علي ضعف المحتوي الكلي للقلويدات عن النبات الأم الأصلى. هذه الطفرات ذات المحتوي العالي من القلويدات تم زراعتها في الجيل الطفري الثالث حيث وجد ثبات هذه الطفرات في الصفات المورفولوجية مقارنة بالجيل الطفري الثاني.

درس الإختلاف بين الطفرات الثلاثة والنبات الأم علي المستوي الجزيئي لمحاولة تمييزهم علي المستوي الجزيئي بإستخدام تكنيك ISSR. وتم تمييز هذه الطفرات بمعلمات جزيئية خاصة به وأمكن تمييز الطفرة (M-11-1 من الممكن تمييزه بثلاث معلمات جزيئية ذات أوزان جزيئية 1397، 1149، 874 bp، وتميز M-11-2 بأربع معلمات جزيئية ذات أوزان جزيئية 1537،1075 ، 839، 510 bp، أما الطفرة M-15- أمكن تميزها بثلاث معلمات جزيئية ذات أوزان جزيئية1749،817 ،756 bp)٠ هذه النتائج تؤكد وجود فروق حقيقية بين هذه الطفرات الثلاثة والنبات الأم، حيث يمكن إعتبار ذلك دراسة مبدئية لتحديد البصمة الوراثية لهم.