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Study the Effects of Mechanical, Chemical, Growth Regulators and Irradiation on the Germination of *Atropa belladonna* In-Vitro Conditions

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Abstract
Atropa belladonna a perennial herb (Family: Solanaceae), endemic to Central and Southern Europe and India and is being cultivated worldwide. The plant species is the source of alkaloid atropine which has been a cornerstone in the study of pharmacology. The active agents in belladonna, Atropine, scopolamine and hyoscyamine, have anti-cholinergic properties and cause a bizarre delirium and hallucinations. The drug atropine is also derived from the plant. The present investigation was carried out to improve germination percentage of Atropa belladonna seeds by different Scarification treatments, both mechanical and chemical methods significantly stimulated seed germination in varying percentage. Gibberellic acid (GA), an environmentally friendly bio-regulator is widely used to enhance the productivity and phenotypic characteristics of the plant. The explants, leaf midrib and petiole region showed organogenesis after 25 days differentiated into callus with MS media constituent with growth hormone supplements (NAA and BA) with different concentration. This study provides an insight into the potential use of X-rays in manipulating growth parameters of Atropa belladonna and enhances understanding of the physiological responses inflicted by irradiation stress and try to confirm this result by RAPD polymerase chain reaction (PCR).

Key words: *Atropa belladonna, Gibberellic acid, Growth hormone, irradiation, RAPD-PCR, Scarification treatment*

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I. Introduction

Atropa belladonna which is commonly known as Deadlynightshade is a perennial herbaceous plant used for commercial production of tropane alkaloids in, which was purified from it in the 1830s, have accepted in the pharmaceutical industry **(Mallinson, 2010).** Seeds usually sown during the first half of March and it take almost 3 months for germination and hence farmers prefer bulbs of *belladonna* for cultivation. Seeds of it are small dark brown in color (**Fatur and Samo, 2020)**. Seed testa has both physical and physiological dormancy **(Valipour*et al.*, 2014).** Dormancy is a mechanism to prevent germination during unsuitable ecological conditions, when the probability of seedling survival is low. To increasing the *belladonna* germination percent, this occurs by using plant regulators and growth hormones. Auxins are compounds widely used in plant tissue culture to stimulate plant cell elongation and division, and callus formation when they interact with cytokinins **(Abdel- Hady, 2008).**

In Europe, through ancient times, plants from deadly nightshades were used to treat various airway diseases e.g. the fumes from the burnt plants were inhaled for relief from bronchoconstriction **(Berdai *et al.*, 2012).** Muscarinic antagonists are known to be used as bronchodilators in asthma treatments **(Dewitt *et al.*, 1997).**Due to their side effects, the anti-cholinergic drugs are not the first line of treatment; instead, β-adrenergic receptor agonists and anti-inflammatory corticosteroids are routinely administered in patients with asthma and chronic obstructive pulmonary diseases **(Donahaye, 2000).**

At least one 19th-century eclectic medicine journal explained how to prepare a *belladonna* tincture for direct administration. In homeopathic practices, it was prescribed by German physician Samuel Hahnemann as a topical medication for inflammation and pain **(Kuhn *et al.*, 2008).**So it has been used for a pain reliever, muscle relaxer, and anti-inflammatory, and to treat menstrual problems, peptic ulcer disease, histaminic reaction, headache and motion sickness. It is used to overcome bronchial spasms, whooping cough **(Maqbool *et al.*, 2014).**It is used for Parkinson's disease, antidote for snakebite, gastric agent **(Kennedy and David, 2014).**

Induced mutations in numerous crop plants have resulted in significant improvements in both qualitative and quantitative characteristics in recent years **(Urbanova and Leubner-Metzger, 2018).** Chemical mutagens and irradiation have been widely used to induce a large number of functional variations *Atropa*. Chemicals induce mainly point mutations, and are thus ideal for producing missense and nonsense mutations,

which would provide a series of change of function mutations. On the other hand, ionizing radiations normally induce chromosomal rearrangements and deletions **(Bhat *et al.*, 2008).**

Ii. Material And Methods:

2.1 Seed germination and seedling growth of mother plant

The seeds of *Atropa belladonna*were collected from Faculty of Agriculture, Cairo University, Egypt. Before germination, surface-sterilized seeds were soaked in 10 mL distilled waterfor one hourand dried at room temperature. For each combination, three replicates of 50 seeds were germinated in covered Petri dishes containing 3 layers of moistened filter paper and placed in germination chambers under a diurnal cycle of 16 h of light, at 30ºC, and 8 h of darkness, at 20ºC, for 14 days **(Wang *et al.*, 2011).**

2.2 *Atropa belladonna* seeds scarification

The seeds were cultivated at MS medium **(Murashige andSkoog, 1962).**The pH of themedium was adjusted to 5.6, then incubated at 25 ºC within controlled growth room, 16h of light and 8h dark photoperiod.

Table 1: The composition of MS medium with mineral and vitamins concentration

Components	Weight (mg/L)	Components	Weight (mg/L)
NH_4NO_3	1650.00	$CoCl_2.6H_2O$	0.025
KNO_3	1900.00	EDTA ferric monosodium salt	7.34
$CaCl_2$	440.00	myo - Inositol	125.0
KH_2PO_4	170.00	Glycine	3.0
$MgSO_4$	370.00	Thiamine HCl	5.0
H_3PO_4	6.20	Pyridoxine HCl	2.5
KI	0.83	Nicotinic Acid	5.0
$MnSO4.H_2O$	22.3		
$Na_2MoO_4.H_2O$	0.25		
$ZnSO_4.7H_2O$	8.60		
$CuSO_4.5H_2O$	0.025		

2.2.1 Mechanical treatment

a) Sand paper process: To remove hard seed coat, dried seeds were taken and placed in metal container with gravels. The container was vigorously shaken with sand paper **(Agboola and Adedire, 1989).** This process harmed the seeds and damaged the cotyledon and when transferred to media they got mouldy.

b) Boiling water treatment: Seeds were transferred to boiling water for 20 minutes to lost seed coat, then allowed to cool by soaking in tap water and when transferred into media they showed very promising results **(Aliero *et al.*, 2001).**

2.2.2Hormonal and Chemicals treatment

Eight groups of *Atropa belladonna*seeds were soaked at different concentrations of hormones and chemicals. Each one contains seven seeds soaked at Gibrellic acid(GA) (40, 60, and 80%) for 24h., NaOH (100%) for 12 h., Ethyl ether (80%) for 9h., Absolute ethanol for 2 min., H_2O_2 (70%) for 5 min and H_2SO_4 (98%) for 2 min.Data on germination was recorded from initiation to completion of germination and based upon this different germination parameters was calculated **(Hu *et al.*, 2002).**

2.3 Effects of growth regulators on organogenesis

The following combinations of medium were used for organogenesis experiments; MS with Napthaleneacetic acid (NAA) (0.5mg/l), NAA (1mg/l) and butanoic acid (BA) (1mg/l).Explants: Leaf parts, lamellae (5mm length), Midrib (7mm length) and petiole (5mm length) areused as explants. Each piece planted on a solid agar medium in six replicates tubes with frequency of 3 explants pre tube for callus induction.Newly developed shoots were sub cultured on same medium after every 3 weeks for further proliferation and maintenance **(Tejavathi and Manjula, 2010).** The fresh weight (mg) of different callus was dried at 70˚c for 48 hours in oven to calculate Callus dry weight (mg).

2.4 Irradiation treatments

Using X-ray devices at the Radiology Department of University of King Saud Hospital handled the machine; the explants were irradiated with different X-ray doses (40, 50, 60,70,80,90 and 100 KeV) for 1, 3 and 6 sec.Seedlings from respective doses were used for analysis of various parameters, numbers of root and vegetative growth, length of vegetative growth and middle leaf **(Muller *et al.*, 2011).**

2.5 Random Amplified polymorphic DNA analysis (RAPD-PCR)

The extraction of total genomic DNA was performed with grinding 500 gm of plant leaves within adequate amount of liquid nitrogen. The procedures of extraction was guided by the Bio-Rad AquaPure Genomic DNA Kit; CA .USA.To determine the DNA concentration and purity by this equation**:**

- Unknown DNA conc. (µg/ml) = Unknown (O.D.) 260nm × 50 µg/ml × Dilution factor
- Purity of DNA = (O.D.) 260nm / (O.D.) 280nm ≈ 1.7 - 2.

RAPD-PCR is an in vitro method for enzymatic amplification of DNA sequences uses primers were listed at **table 2**.The reaction was performed in 25µl reaction volume containing 1x buffer (10mM tris- Hcl PH 8.3, 50mM Kcl, 2m M Mgcl2), 250µM each of dGTP, dATP, dCTP and dTTP, 2.5 units of Taq DNA polymerase, 100 pmol of each primer and DNA template. Components were overlaid with a drop of mineral and DNA amplification started with denaturing the template DNA at 94^{o}C for 5 min followed by 35 cycles. Each cycle consisted of; denaturation at 94^{o}C for 1 min, annealing for at 36^{o}C for 45 sec and extension at 72^{o}Cfor 3 min, and a final extension step at 72^{o}c for 7 min.PCR product was analyzed by agarose gel electrophoresis **(Bashalkhanov and Rajora, 2008).**

Table (2): The nucleotide sequences of the primers

Primers	Nucleotide sequences
RAPD1	**(5'CCTCTGACCC'3)**
RAPD2	**(5'GTTTCGCTCC'3)**
RAPD3	**(5'GCTACCAGCT'3)**
RAPD4	**(5'GGTGAACGCT'3)**
RAPD5	**(5'ACC CGA CCCT'3)**
RAPD6	**(5'CCCG TCA GCA'3)**

III. Results:

3.1 Mechanical and chemicals treatments

The reduced rate of germination of *Atropa belladonna* is not just due to hard seed coat, but may be also of chemical insufficiency. Some seed need hot water to stimulate the growth. Water imbibitions inside seed were important to overcome this entire problem. Mechanical and chemical scarification was carried to break testa so that water imbibes easily and seed germination. Sand paper treatment was not effective and even damaged the seeds. Boiling water treatment responded almost 90% on seed and even seed germination started by the next day of the treatment. Sand plates showed poor growth while the result in MS media was best **table (3).**

Table 3: Mechanical method treatment for germination of *Atropa belladonna*

Treatment	Days Required For Sprouting			Sprouting%		
	MS	MS+ sand	2 MS+ sand	MS	MS+ sand	2 MS+ sand
Sand paper	-	-	-	-	-	-
Boiling water	3 days	3 days	3 days	30%	60%	80%

Gibrellic acid (GA) is essential plant regulators for multiple plant development processes as seed germination, stem elongation and leaf extension. The rate of germination was 90% on GAon 2 MS plus sand plates and 70% on M S blank plates. Chemical Scarification treatment has proved to be more successful than mechanical treatment as it removes the seed coat successfully. Ethanol may involve in modification of membrane thus facilitating water and oxygen. Pre treated ethanol seeds germinated on the 25th day of inoculation only on MS plus sand plate and M S media did not show any change. Using Sodiumhydroxide solution the growth rate was totally stopped in all plates may be because of high salinity **table (4).**

After 4 months of seeding selected mother plants characterized by strong growth, free from diseases and mechanical damage to study genetic stability of *Atropa belladonna* **fig (1).**

Fig (1): Mother plant morphology of *Atropa belladonna*

Table 4: Hormonal and chemicals effects for germination of *Atropa belladonna*

Treatment	Concentration of Solution	Duration of treatment	Days Required For Sprouting			% Of Sprouting		
			MS	MS+ sand	2 MS+ sand	MS	MS+ sand	2 MS+ sand
Gibrellic acid (GA)	40%	24h	25days	20days	20days	70%	70%	90%
Gibrellic acid (GA)	60%	24h	25 days	20days	15days	60%	70%	80%
Gibrellic acid (GA)	80%	24h	25 days	20days	15days	50%	70%	80%
Sodium hydroxide	100%	12 h	-	-	-	-	-	-
ethyl ether	80%	9h	25 days	25 days	25 days	30%	30%	30%
Ethanol	99%	2 min	-	25 days	25 days	-	40%	50%
Hydrogen peroxide	70%	5 min	25days	-	-	20%	-	-
Sulphuric acid	98%	1 min	25days	25 days	20days	15%	20%	20%

3.2 Effects of growth regulators on organogenesis of *Atropa belladonna*

Atropa belladonna fresh explants are surface sterilized and transferred into MS media supplement with definite hormonal NAA and BA with different concentration. Explants showed results exactly after 25days of inoculation and after 6 weeks inoculation the direct organogenesis was observed.The roots differentiated into callus with exposure for long time in same conditional media **table (5) and fig (2).**

Table 5: MS media supplemented with NAA and BA hormones at different concentration

Hormone	concentration(mg/l)	Leaf parts	Length of root in 25 days (cm)	Callus dry weight (mg)
NAA	0.5	Lamellae	2.5 ± 0.2	103.0
		Midrib	0.8 ± 0.3	
		Petiole	-	
NAA	1.0	Lamellae	2.9 ± 0.3	137.0
		Midrib	1.1 ± 0.2	
		Petiole	0.9 ± 0.4	
BA	1.0	Lamellae	1.7 ± 0.1	170.0
		Midrib	0.9 ± 0.1	
		Petiole	0.7 ± 0.1	

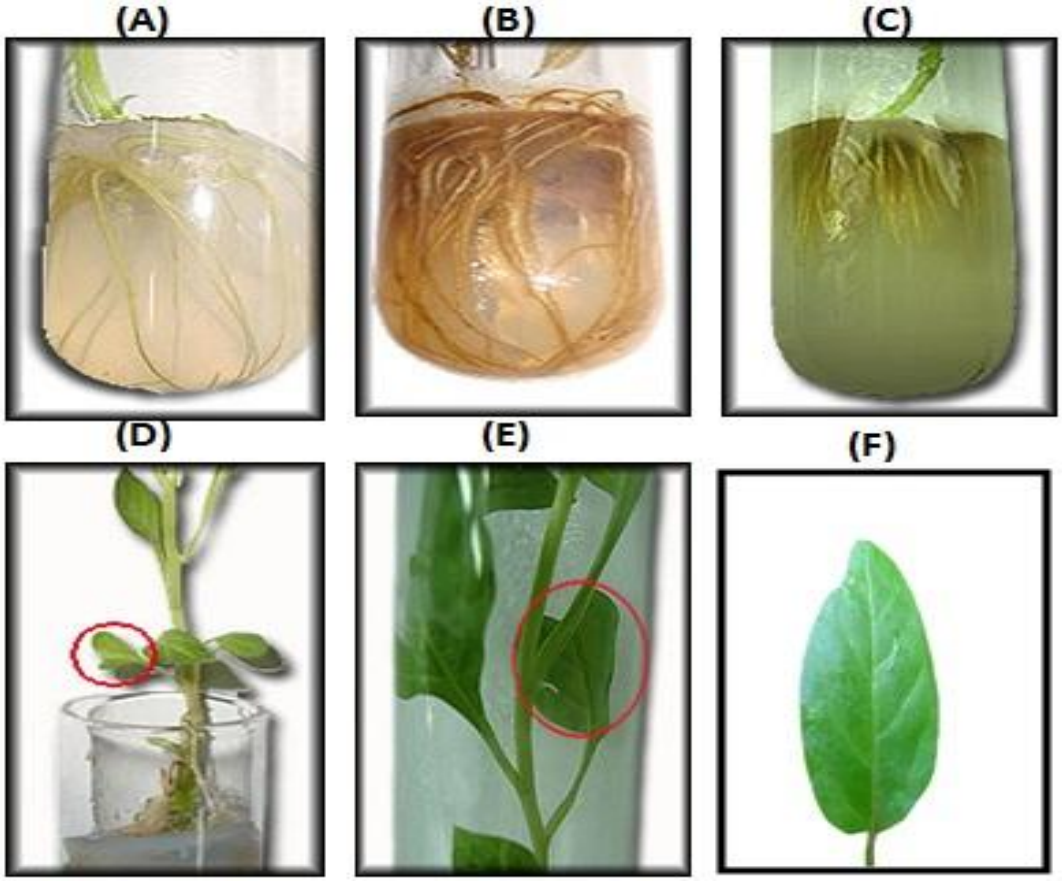

Fig (2): Organogenesis of *Atropa belladonna***(A)** long root appearance on MS media without growth regulators **(B)** Root formation on MS supplemented with 0.5 mg/l of ANN **(C)** Root formation on MS supplemented with 1.0 mg/l of ANN **(D)** shape of leaf in MS media only **(E)** shape of leaf in MS media supplemented with 1.0 mg/l of ANN**(F)** shape of leaf in MS media supplemented with 1.0 mg/l of BA.

3.3 Effect of X-ray exposure dose on Growth of *Atropa belladonna*

The effects of X-rays on plant germination are still not fully understood. Increasing X-ray irradiation doses for about 6 sec were seen to reduce seed germination percentage and root growth of *Atropa belladonna;* on the other hand there is no effect of low doses (40-60 KV).While the exposure to one second at X-ray dose 110 KV leads to stunting of vegetative growths.

The number of vegetative growths decreases with increase the X-ray doses when exposed to it for a one sec only; while the length increase with increasing the doses. The numbers of roots decrease with increase the X-ray doses. The middle leaf length recorded the highest measurement at 100 KV forone sec **fig (3).**

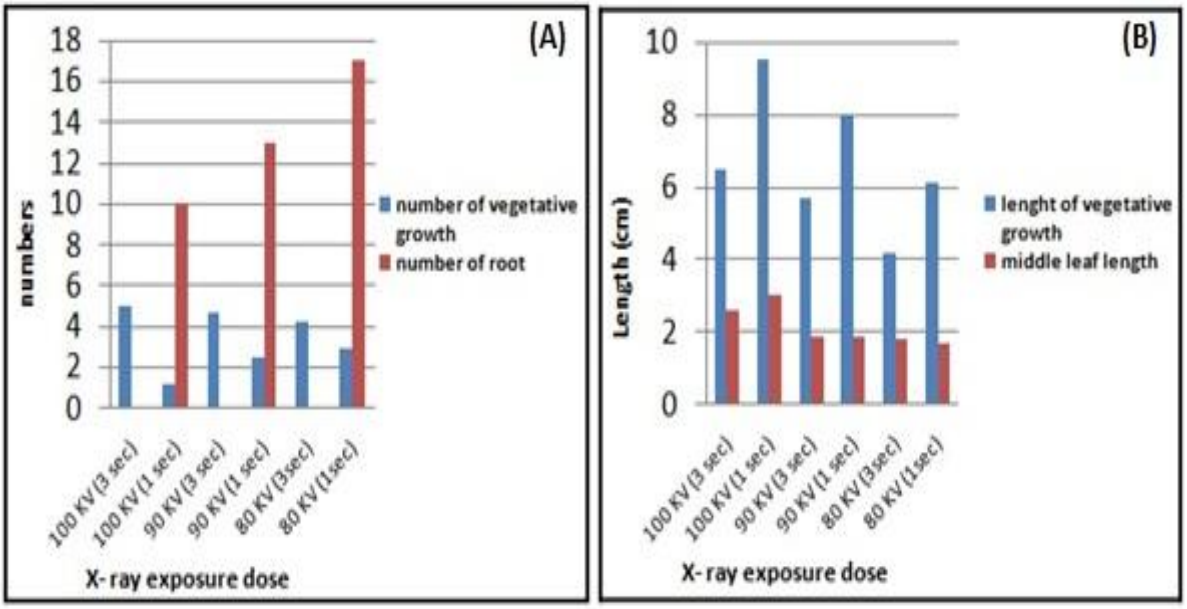

Fig (3): (A) effects of x-rays on numbers of vegetative growth and roots,**(B)** effects of x-rays on length of vegetative growth and middle leaf length.

In general, the different doses of x-rays when exposed to them for 3 seconds led to morphological changes in the shape of the leaf (formation abnormal or heart-shaped leaves) **fig (4).**

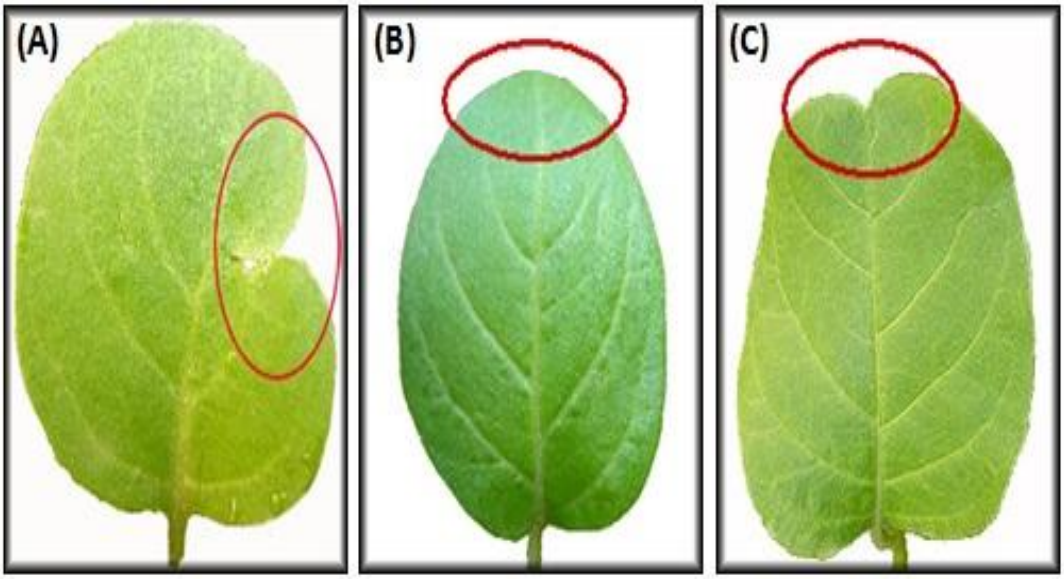

Fig (4): Morphological changes in leaves after exposed to different doses of X-rays (A) Changes in leaf edges and cavities appearance on it, (B) The top of the leaf takes bow shape, (C)heart-shaped leaves.

3.4 RAPD-PCR amplification

A molecular test is a powerful tool to give some explanations for understanding results which consume time, sometimes inconclusive and appears of genes associated with germination after effected by different mutagen as chemical, growth hormones or x-rays. All the primers which used in this study not give satisfying results except primers RAPD2 and RAPD6.

By using RAPD2 primer produces for about 11 bands with molecular weight ranged from 900-200 bp. When comparison all lanes with lane no.1 (mother plant), we notice that all appear to be homologous except in the following bands;Band at 500bp appear in all lanes but band thickness increase in lanes supplemented with growth hormones and lanes which exposure to different doses and time of x-rays. Band at 400 bp is disappearance in lanes 7, 8 and 9 while band at 380bp disappearance in lanes 2, 3, 4 and5. When exposure at

100kV for 3 sec appear clearness band at 330bp on the other hand band at 900 bp appear in all lanes except mother plant **fig (5).**

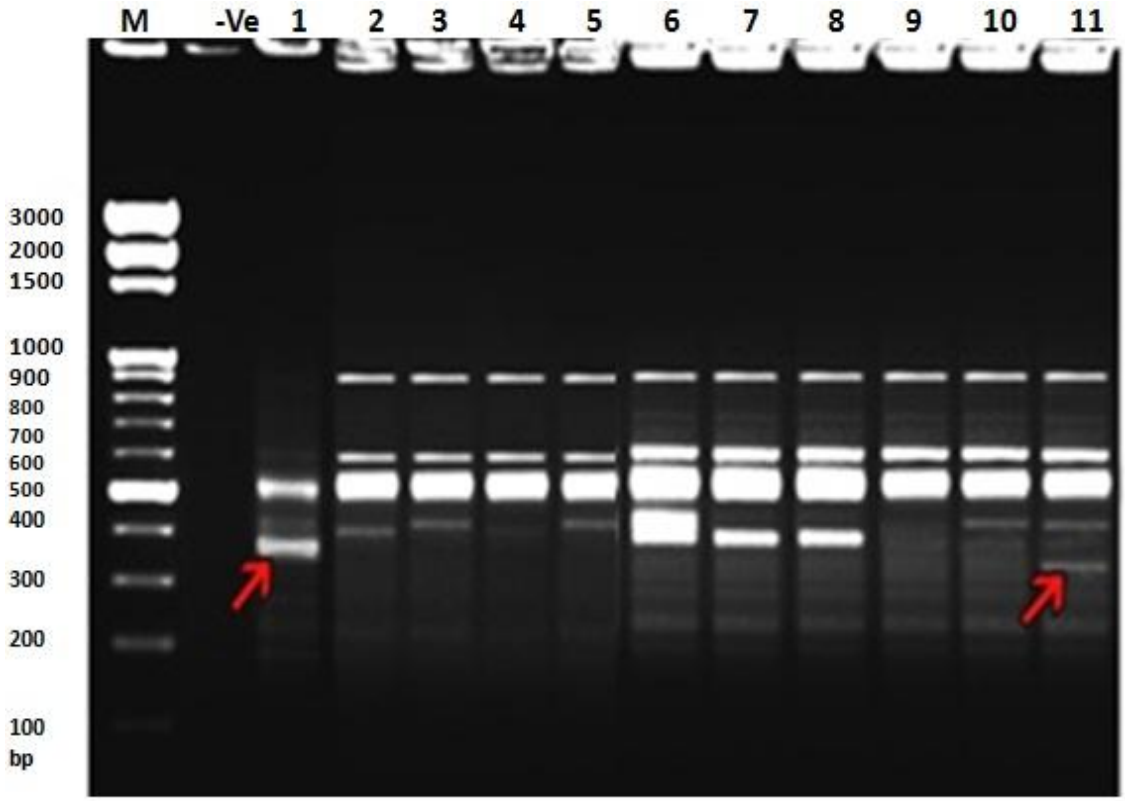

Fig (5): RAPD-PCR product by using RAPD2 primer applied in 11treatmentslanes;**1)**Mother plants,**2)**Plants on MS media without growth regulators, **3)**On MS media plus 0.5 mg/l of ANN,**4)**On MS media plus 1.0 mg/l of ANN, **5)**On MS media plus 1.0mg/l of BA, **6)**KV 80(1sec),**7)** KV 80(3sec),**8)** KV 90(1sec),**9)** KV 100(1sec),**10)** KV 90(3sec),**11)** KV 100(3sec), **M**DNA marker, **-ve** negative results.

By using RAPD6 primer produces for about 16 bands with molecular weight ranged from 1500-200 bp. Bands at 1480, 1240 and 520bp appear in lanes exposure to different doses of x- rays and also band at 520 appear on mother plant. Band at 580 bp appear in mother plant and lanes supplemented media with growth hormones while band at 380 appear lanes 6, 7, 8 and 9 **Fig (6).**

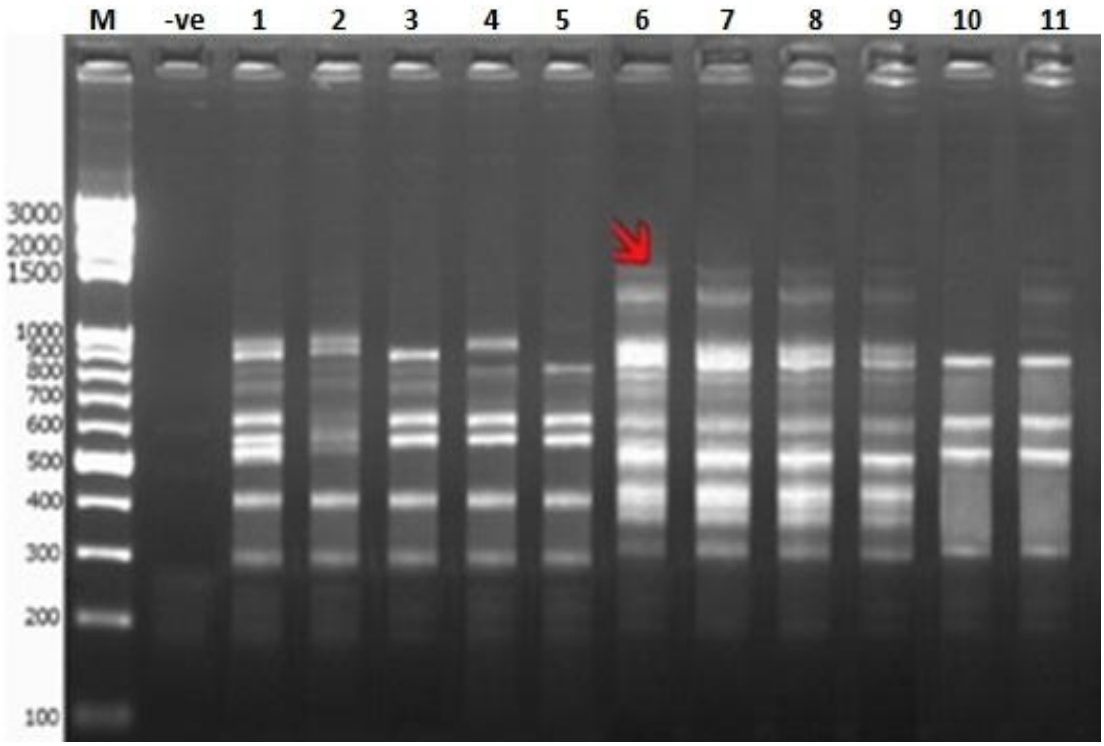

Fig (6): RAPD-PCR product by using RAPD6 primer applied in 11treatmentslanes;**1)**Mother plants, **2)**Plants on MS media without growth regulators, **3)**On MS media plus 0.5 mg/l of ANN, **4)**On MS media plus 1.0 mg/l of ANN, **5)**On MS media plus 1.0mg/l of BA, **6)**KV 80(1sec),**7)** KV 80(3sec),**8)** KV 90(1sec),**9)** KV 100(1sec),**10)** KV 90(3sec),**11)** KV 100(3sec), **M**DNA marker, **-ve** negative results.

IV. Discussion

Germination differs from species to species. Species with small seeds tend to require light for germination more than large seeded species **(Milberg *et al*., 2000)**, so all the experiments were carried out in the presence of light.Seed germination which has hard testa can be increased by treatment with Gibberlic acid (GA) **(Nikolaeva, 1982)**. The results of the experiments showed that suitable concentration of GA could significantly support of seed germination and seedling growth of *Atropa belladonna*. GA are extensively used in agriculture and horticulture as tools for boosting productivity, cost reduction and enhancing ornamental value of multiple plants**(Abdel- Hady, 2008).**

Temperature is the most important factor in regulating the changes in dormancy **(Dubinskaya, 1949)**.Under influences of different temperature both testa and seed function was affected and germination was achieved **(Ruminska, 1978).** High percentage germination in seeds of *Acacia nilotica* with increasing ratio of seed weight to hot water volume was reported **(Duguma *et al*., 1988)**.Acid scarification was first reported by Bonner, 1974 **(Qureshi*et al*., 2016).**Ethanol has been reported to have stimulatory effect on the germination of seeds of many plant species **(Baninasab and Rahemi, 2001).**

Plant growth regulator is an organic substance required by the plant in small amount to regulate the growth of a plant **(Chen *et al*., 2008).** They play roles in promoting cell differentiation, growth and cell division on the other hand higher concentration can cause shoot bud retardation because excess hormone can become toxic to the plant.**Wang *et al.* (2000)** successfully reported rapid *Atropa belladonna* propagation from seedling was successfully reported with BA & NAA. **Taha, (2003)** reported high growth of micro-propagation was recorded with NAA+BA 1mg/l at 24˚c.NAA proved the best for root initiation and hence for Micro-propagation **(Khater *et al*., 2013).**

Exposure to X and gamma rays causes physicochemical effects hence influencing the growth and physiological changes in plants **(Ahloowalia and Maluszynski, 2001).** In this concern, some studies indicate that low ionizing radiation doses caused stimulation of plant growth and productivity**(Aly *et al*., 2018)** owing to modifications in plasma membrane intake, safeguard the membrane, stimulated cell proliferation, cell growth, and enzymatic antioxidants **(Aly *et al*., 2019)** while higher doses result in growth abnormalities, germination retardation or even plants death **(Hong *et al*., 2018).** The elevated doses of radiation that triggered the growth may be due to the effect on the permeability of the plasma membrane, transpiration, and the stomata opening also, it induced the reduction in the plant growth hormones **(Maherchandani, 1975).**

Since mutagenic agents simply increase the rate of spontaneous mutations, therefore, every conceivable change is expected provided the size of population is big and the frequency of induced mutations high. In conclusion we can say the level of dormancy in seeds is determined by several factors such as maternal environment during maturation, chemical composition, hormone concentration, radiation dose and position of the seeds on the plant. Over all view support that, seeds require proper internal environment and adequate external factors for germination. The laboratory report and analysis are only approximation and close approach of situation expected in nature.

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