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Propagation and Chemical Mutgenic Induction of *Eustoma Grandiflorum* Plant Using Tissue Culture Technique

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ABSTRACT

Mutagenesis is one of the powerful tools for breeding biotechnology. Chemical mutagenesis is an easy tool, which doesn't require special tools and provide high mutation rate. Effects of different cytokines (BA, 2ip and Kim at 0.4 mg/l) on in vitro shootlet proliferation of Eustoma grandiflorum were studied. Different concentrations of colchicine (30, 60, 120 and 240 mg/l) and sodium azide (5, 10, 15 and 20 mg/l) were added to free-hormone MS medium. In vitro shootlets, of Eustoma grandiflorum, were culture for 7, 14 and 28 days. After each treatment period, the shootlets were transferred to fresh MS medium free (without the chemical mutagenesis). At acclimatization stage, most of morphological parameters and anthocyanin pigment contents in flower recorded decline by most treatments of sodium azide. All colchicine treatments reached to morphological stage and formed bud initiation and death before flowering. Contrary, highest survival % of acclimatized plants (70%) and highest values of number of branches, branches length (cm), leaf area (cm2), most of floral parameters, photosynthetic pigments, carotenoids and anatomical structure were obtained from (5,10 mg/l sodium azide for 28, 7 days) and (60, 30mg colchicine for 28 days).

Keywords: Sodium azide, *Eustoma grandiflorum*, tissue culture, colchicine and anatomy.

1. INTRODUCTION

Lisianthus (*Eustoma grandiflorum*) flowers is one of most important cut flowers, very attractive, it is ideal for bed, border, pots and for rock garden and considered one of the very promising plants that have an economic important for exportation to obtain a suitable income. It is an annual or perennial plant, belongs to the Gentianceae [1]. Recently, a variety of cultivars had been developed with many traits such as flowering throughout the year, heat tolerance, flower color, flower size and form [2].

Generally, propagation of Eustoma grandiflorum is by seed or cutting, but the quality is not uniform due to the variations in flowering time, plant height and the number of flowers. Propagation of Eustoma grandiflorum by tissue culture technique are relatively low. Several factors like genotype, media, plant growth regulators and type of explants should effect the success of the micropropagation method, most of plant growth regulators that have been used were BA, KIN, NAA and IBA [3],[4]

The genetic variation created is useful because it helps population to survive and change over time. Mutagenesis is the process occur changes in the genetic information of an organism not caused by genetic segregation but induced by chemical and physical agents [5]. Colchicine is an alkaloid found in the seeds and corms of autumn crocus (Colchicum automnale); it is classified as an antimitotic agent, inhibiting microtubule formation during mitosis and has been used for chromosome doubling for induction of polyploidy.

Sodium azide (Na N_3) is a chemical mutagen and considers as one of the most powerful mutagens in plants. Its application on plant is easy, inexpensive and creates mutation improve their traits. The efficiency of mutant production depends on many conditions such as concentration of sodium azide and treatment duration [6].

The aim of this work to determine the favored type of cytokine (BA, 2ip and Kin.) in the culture medium to obtain *in vitro* culture sufficient to study the effect of various concentrations of colchicine and sodium azide as chemical mutagens on *Eustoma grandiflorum* propagated *in vitro* as well as flowering of acclimatized plants, biochemical and cytological behaviors.

2. MATERIALS AND METHODS

The experiment was conducted during years 2014 and 2016 at the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza and Tissue Culture Laboratories of Ornamental Plants and Woody Trees and Biotechnology Departments, National Research Centre (NRC), Egypt to investigate the effect chemical mutagens (colchicine and sodium azide) on in vitro and in vivo behaviors as well as flowering of acclimatized plants, biochemical and cytological behaviors of Eustoma grandiflorum plant.

Plant materials and surface sterilization.

Plants of Lisianthus (Eustoma grandiflorum) were obtained from greenhouse of National Research Centre on 2014 and prepared by washing the lateral buds as explants under running tap water and a few drops of hand washing liquid for 20 min. After three times rinses with distilled water, explants were surface sterilized in 70% (v/v) ethanol for 1min, then in 20% commercial sodium hypochlorite solution and one drop of tween 20 (polyoxy ethylene sorbitonmonolaurate) for 10 min after that the explant were rinsed three times with autoclaved distilled water followed with 7-min in 0.1g/l HgCl2, and rinsed three times with autoclaved distilled water.

Culture media and culture conditions

The explants were cultured on MS medium (free hormones) [7] supplemented with 25g/l sucrose and 8g/l agar then adjusted to PH 5.6± 0.2, the medium was atuoclaved at 121°C and 1.5 Kg/cm2 then, the cultures were incubated under 30 µmol m-2s-1 of light and 16 hr. photoperiod. After one month from culture explants, the shootlet nodal stems were used for in vitro propagation.

Proliferation of shootlet explants

Shootlet nodal stems were cultured on medium supplemented with different cytokinins [6-benzyl amino purine (BA), 6-ã,ã- dimethyl allyl amino purine riboside (2ip) and Kinetin (N-6 Fouryla denin) (Kin)] at concentration of 0.4mg/l. The obtained shoots were repeat subcultured and the mean of two subcultures data was calculated. Characters including shoot number, shoot length and number of leaves formed per shootlet after 45days from each subculture under control and cytokinins treatments.

Chemical mutagenesis

Different concentrations of colchicine (30, 60, 120 and 240 mg/l) and sodium azide (5, 10,15 and 20 mg/l) were Filter sterilized using 1% dimethyl - sulfoxide (DMSO), then supplemented to MS media under complete sterile condition to culturing the shootlets for 7, 14 and 28 days. After each treatment period, the shootlets were transferred to MS medium free (without growth regulator). The mutagenesis treated shoots were incubated under the above mentioned conditions and subcultured three times, four weeks after third subculture; the In vitro

morphological characters were recorded: Number of shootlets/explant, shootlets length (cm), number of leaves/shootlet, rooting (%), number of roots/shootlet and root length (cm).

Acclimatization stage under mutagenesis effect

The in vitro rooted plantlets were successfully transplanted (after the above mentioned mutagenesis treatments) to the greenhouse of National Research Centre using growth media contained of perlite and peatmoss (1:1). Morphological characters (Survival %, number of branches, height of branches/plant (cm), number of leaves/branch and leaf area (cm2) were recorded after two months.

After three to four months from acclimatization process, flowering characters (Days to flower bud initiation, days to bloom, flowering percentage (%), number of flower buds/plant, number of flowers/plant, flower diameter (cm), bloom stem length (cm), peduncle length (cm), days to flower senescence (from blooming), number of petals/flower, petals area (cm2), number of stamens, fresh and dry weights of flower (gm.) were recorded.

Chemical analysis Extraction and determination of Photosynthetic and Anthocyanin pigments

Half gram of fresh samples were sectioned into minute pieces in three replicates and freshly macerated and extracted with 80% cold methanol for 24 hours at 0ºC, then extracted twice 80% methanol for 24 hours. The combined extract was filtered and adjusted to a known volume for quantifying the endogenous chlorophyll- a &-b and carotenoids. According to [8]. As for anthocyanin pigment, the extraction was done with ethanolic hydrochloric acid solution (85 ml ethanol 95% + 15 ml 1.5 N HCl) according to method of [9]. The anthocyanin was quantified by using spectrophotometric measurement of tissues extracts at 535 nm. The amounts of pigments were calculated as mg/ 100 g F.W.

Anatomical structure Leaf anatomy

Samples were taken from thirty leaves on the plant. In the laboratory; samples were cleaned with tap water, cut into suitable parts, killed and fixed in FAA. Solution (100 ml formalin, 50 ml acetic acid, and 500 ml ethyl alcohol 95% and 350 ml distilled water), according to [10].Sectioning at thickness of 20µ was performed by using a rotary microtome. Sections were mounted in Canada balsam then examined microscopically and microphotography according to [11].

Statistical analysis

The data were analyzed through analysis of variance ANOVA and the treatments means were compared for significance by Duncan's New Multiple Range Test at 0.05% level of probability [12] using COSTATV-63.***6.1***

3. RESULTS AND DISCUSSION

Culture establishment stage Effect of cytokine type on in vitro shootlet proliferation of Eustoma grandiflorum

The tabulated data in Table (1) and Fig. (1) showed that, both number of shootlets/explant and number of leaves/shoot were in highest values by (10.77 and 66.67 respectively) obtained with MS culture medium supplemented with BA at the rate of 0.4 mg/l as compared to control which gave (0.663 and 19.11) and other cytokines (Zip and Kin) at the same concentrations. Our results are similar to those found by [13],[1],and [14]. Also the longest shoots (2.49 cm) were obtained with MS culture medium supplemented with Kin (0.4 mg/l) and followed by 2ip treatment which recorded (2.16 cm), while the shortest shoots (0.99 cm) was noticed with BA as

compared to control. Results go in line with those obtained by [15],[16] and [17].These could be explained by cytokinine which play an important role to stimulate cell division and cell elongation to activate RNA synthesis and stimulate protein synthesis as well as enzyme activity [13].

Table 1. Effect of cytokinin type on *in vitro* shootlet proliferation and elongation of *Eustoma grandiflorum*

Number of leaves/shoot	Shoot length (cm)	Number of shoot/explant	Characters Cytokinins (0.4 mg/l)
19.11 ^{b}	1.99 ^{ab}	0.663^{b}	**Control**
66.67 ^{a}	0.99 ^{b}	10.77 ^{a}	**BA**
15.44 ^{b}	2.16 ^{ab}	1.77 ^{b}	**2ip**
13.99 ^{b}	2.49 ^{a}	2.55 ^{b}	**Kin**

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

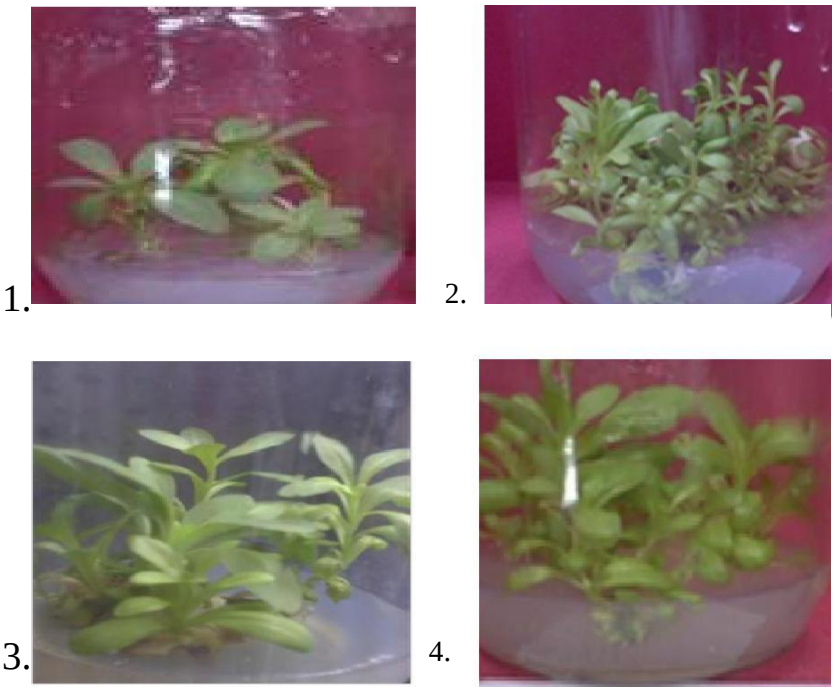


Fig.1: *In vitro* shootlet proliferation of *Eustoma grandiflorum* plant affecting by MS culture medium supplemented with various cytokinins types: 1. Control (MS free hormones), 2. MS+BA (0.4mg/l), 3.MS+2ip (0.4mg/l) and 4.MS+Kin (0.4mg/l).

Chemical mutagenic Effect of colchicine and sodium azid in multiplication stage on shooting and rooting behaviors of Eustoma grandiflorum plant

The effect of various chemical mutagenesis on the *in vitro* multiplication of *Eustoma grandiflorum* plant Table (2) was reported that the number of shootlets formed per explant was significantly reduced by most treatments except for 10 mg/l of sodium azid for long time (28 days) followed by 20 mg/l of sodium azid at the same period (28 days)

achieved 4.886 and 2.976, respectively comparing with control (1.163). Contrary, the effect of chemical mutagenic at various concentrations generally recorded increase in most cases of shootlets length, number of leaves/ shootlet, rooting%, roots number/shootlets and root length (cm).

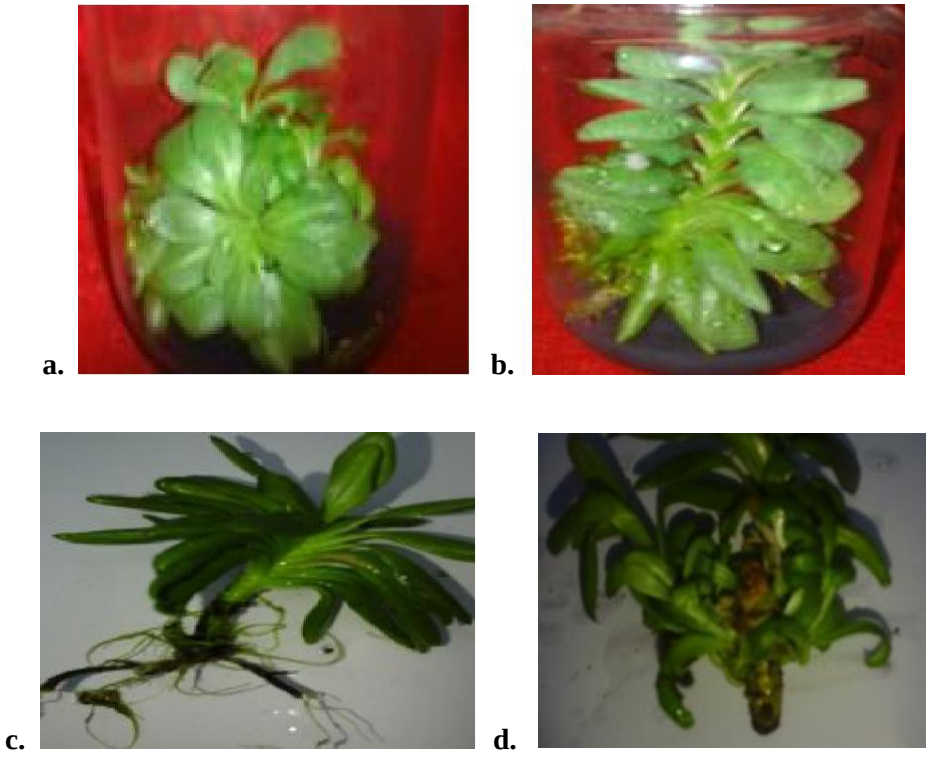


Fig.2. a. control , b. 240mg/l colochicine for 7 days, c. 5mg/l sodium azide for 14 days and d. 15mg/l sodium azide for 14 days.

Sodium azid treatment (10 mg at 28 days) recorded the highest values in shoots and rooting parameters (shootlets length, leaves number/shootlet, rooting %, roots number and root length compared with untreated explants giving (4.833, 40.33, 88, 3.99. and 8.24, respectively.) With regard to the colchicine treatments, the highest values of shooting and rooting (previous parameters) were obtained from supplemented media with 240 mg/g colchicine. It recorded (2.443, 5.583, 28,100, 3.88 and 3.66, respectively) comparing with control and other treatments. These results are agreement with those obtained by [18] and [19] . Mutagens have able to enter the cell of living organisms to interact with DNA and produce the toxic effects associated with their mutagenic properties. Moreover, chemical mutagens induce physiological damages mutations and chromosomal aberration in M1 individuals which can be detected and measured from emergence of seedlings, survival reduction, plant reduction [20].

Table 2. Effect of colchicine and sodium azid on *in vitro* shooting and rooting behavior *Eustoma grandiflorum* plant in invitro multiplication stage.

Length of root(cm)	NO .of Roots/ shootlets	Rooting (%)	Number of leaves/ shootlet	Length shootlets (cm)	Number of shootlets/ explant	Time. exposure (Days)	Characters Concentration(mg/l)
0.61^{fgh}	0.776^{bcd}	22.7^{def}	13.11^{gh}	1.67^{e}	1.163^{cdef}	0	**Control**
0^{h}	0^{d}	0^{f}	17.11^{defgh}	3.833^{abcd}	0.776^{def}	7	**30mg/l colchicine**
2.78^{bc}	1.443^{bcd}	44.3^{bcdef}	22^{bcdefg}	3.406^{bcde}	1.446^{bcdef}	14	
0.72^{efgh}	1.883^{bc}	66.7^{abcd}	19.11^{bcdefg}	3.25^{bcde}	1.22^{cdef}	28	
2.77^{bc}	1.55^{bcd}	76^{abc}	22.11^{bcdefg}	2.916^{bcde}	1.33^{bcdef}	7	**60mg/l colchicine**
2.49^{bcde}	1.216^{bcd}	44.3^{bcdef}	20.663^{bcdefg}	3.22^{bcde}	1.663^{bcde}	14	
2.27^{bcdef}	1.443^{bcd}	66.7^{abcd}	21.886^{bcdefg}	3.26^{bcde}	2.163^{bcde}	28	
1.77^{cdefgh}	1.106^{bcd}	44.3^{bcdef}	16.89^{efgh}	3.083^{bcd}	0.883^{cdef}	7	**120mg/l colchicine**
0.5^{fgh}	0.443^{bcd}	22^{def}	21.556^{bcdefg}	4.833^{ab}	1.886^{bcdef}	14	
0^{h}	0^{d}	0^{f}	17.553^{cdefgh}	3.083^{bcde}	2^{bcdef}	28	
3.66^{b}	3.88^{a}	100^{a}	28.003^{b}	5.583^{a}	2.443^{bcd}	7	**240mg/l colchicine**
2.55^{bcd}	1.113^{bcd}	44.3^{bcdef}	26.663^{bc}	3.053^{bcde}	2.33^{bcd}	14	
2.44^{bcde}	1.33^{bcd}	55.6^{abcde}	19.223^{bcdefg}	2.25^{de}	0.5^{ef}	28	
0.267^{gh}	0.609^{bcd}	44.4b^{cdef}	15.556^{fgh}	2.75^{cde}	1.33^{bcdef}	7	**5mg/l sodium azide**
1.778^{cdefgh}	2.223^{b}	66^{abcd}	18.443^{cdefg}	2.75^{cde}	1.443^{bcdef}	14	
2.61^{bc}	3.77^{a}	77.6^{abc}	22.89^{bcdef}	2.176^{de}	2.11^{bcdef}	28	
1.942^{bcdefg}	1.55^{bcd}	33^{cdef}	25.336^{bcde}	4.25^{abc}	2^{bcdef}	7	**10mg/l sodium azide**
0.279^{gh}	0.67^{bcd}	22^{def}	16.33^{efgh}	3.416^{bcde}	1.667^{bcdef}	14	
8.243^{a}	3.99^{a}	88^{ab}	40.33^{a}	4.833^{ab}	4.886^{a}	28	
0^{h}	0^{d}	0^{f}	9.33^{h}	1.667^{e}	1.22^{cdef}	7	**15mg/l sodium azide**
0^{h}	0^{d}	0^{f}	18.89^{bcdefg}	3.167^{bcde}	1.83^{bcdef}	14	
0^{h}	0^{d}	0^{f}	13.22^{gh}	1.833^{e}	1.33^{bcdef}	28	
0^{h}	0^{d}	0^{f}	14.556^{fgh}	2.916^{bcde}	0.443^{f}	7	**20mg/l sodium azide**
0.78^{defgh}	0.83^{bcd}	33^{cdef}	23.663^{bcdef}	2.096^{de}	2.5^{bc}	14	
0.556^{fgh}	0.223^{cd}	11^{ef}	26.223^{bcd}	2.75^{cde}	2.996^{b}	28	

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Effect of colchicine and sodium azied in vivo acclimatization stage on shooting and rooting behavior of Eustoma grandiflorum plant

It could observe from Table (3) the decreasing in almost of morphological parameters of adapted plants by most cases of chemical mutagenesis (colchicine and sodium aide treatments).The lowest values of survival%, branches number, branches length (cm), leaves number and leave area (cm^2) (16 % and 0.88 were recorded from 240/mg

colchicine for 7 days and (0.67, 8.33 and 0.42) with 30 mg colchicine for 7 days in comparison with control plants, respectively. On the contrary, the highest survival %, number of branches, branches length (cm) and leaf area/plant (cm^2) (70,% 2.50,4.93 and 4.52) were achieved from plants treated by 5 mg/1 of sodium azid for 28 days followed by 10 mg/l sodium azid for 7 days. Which recorded (66%,2.33, and 4.40) While 60 mg colchicine for 7 days gave the significant maximum value of leaves number by (35.33) followed by 30 mg colchicine for 28 days treatments, giving (28) compared with control which gave (21), respectively. Our results were agreement with those obtained by [21],[22] and [23].The simulative effect of chemical mutagenic like (EMS or Sodium azide) could be attributed to cell division rates and led to activation of growth hormones like auxin [24]. The low concentrations of colchicine have stimulatory effect on some of the morphological character [25].

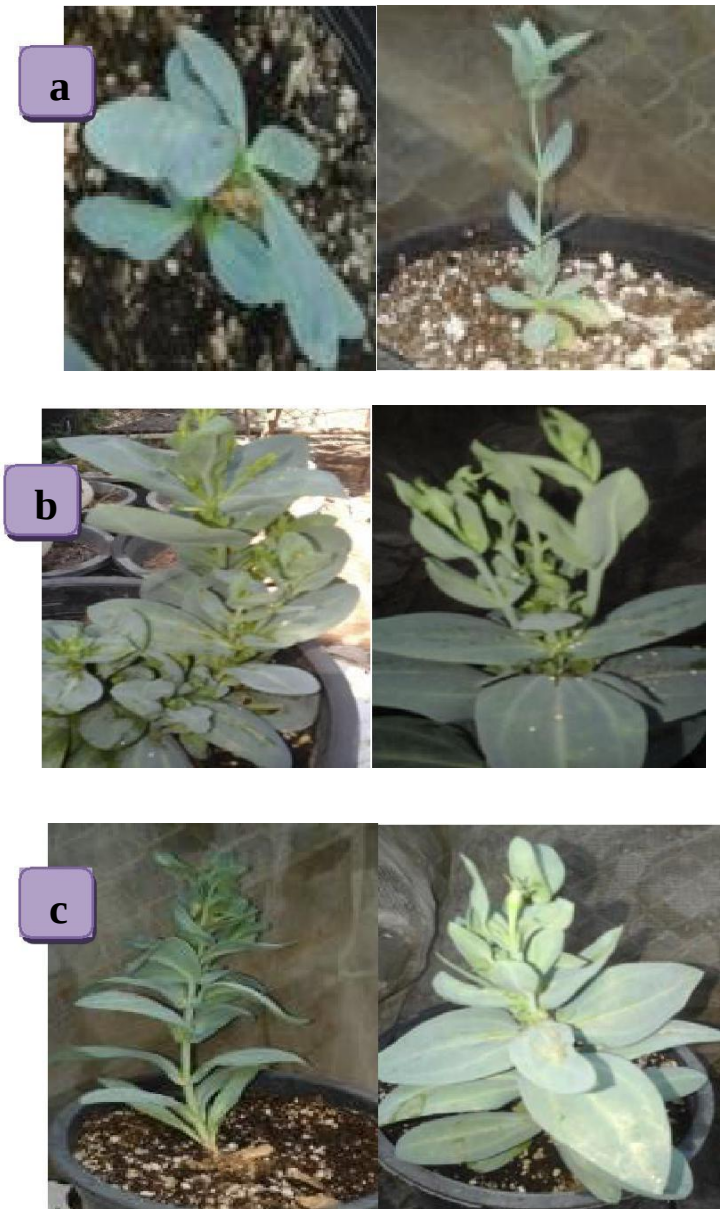


Fig.3. a. control, b. 5mg/l sodium azide for 28 days and c. 10mg/l sodium azide for 14 days

Table 3. Effect of colchicine and sodium azide on morphological characters of *Eustoma grandiflorum* adapted plant

Leaf area (cm^2)	Number of leaves per plant	Height of Branches (cm)	Number of branches	Survival (%)	Time. Exposure (Days)	Characters treatments
1.71^{bc}	21_{bcdefg}	1.83^{bcd}	1.67^{abc}	50^{f}	0	**Control**
0.42^{c}	8.33^{i}	0.67^{d}	1_{bc}	25^{i}	7	**30mg/colchicine**
1.4^{bc}	14.33_{defghi}	1.67^{bcd}	2_{abc}	33^{h}	14	
1.08^{bc}	28^{ab}	2.76^{abcd}	2.33^{ab}	62^{c}	28	
0.98^{bc}	35.33^{a}	3.83^{abc}	2.33^{ab}	56^{e}	7	**60mg/colchicine**
1.63^{bc}	$17.67_{bcdefghi}$	1.8^{bcd}	1.33^{abc}	41^{g}	14	
1.56^{bc}	21.33_{bcdefg}	2.25^{abcd}	1.33^{abc}	25^{i}	28	
1.78^{bc}	25.67^{abcd}	2.83^{abcd}	1.67^{abc}	50^{f}	7	**120mg/colchicine**
0.91^{bc}	$16.33_{bcdefghi}$	2.67^{abcd}	1_{bc}	33^{h}	14	
1.98^{b}	11.33^{fghi}	1.83^{bcd}	1.33^{abc}	25^{i}	28	
1.17^{bc}	13.67^{efghi}	1.93^{bcd}	0.88^{c}	16^{j}	7	**240mg/g colchicine**
0.90^{bc}	13_{fghi}	1.5^{bcd}	1.33^{abc}	25^{i}	14	
0.42^{c}	21.67^{bcdef}	1.83^{bcd}	1_{bc}	50^{f}	28	
1.29^{bc}	27.33^{abc}	3.83^{abc}	2_{abc}	50^{f}	7	**5mg/l sodium azide**
1.59^{bc}	25_{abcde}	3_{abcd}	1.33^{abc}	58^{d}	14	
4.52^{a}	$19.33_{bcdefghi}$	4.93^{a}	2.5^{a}	70^{a}	28	
0.97^{bc}	23_{bcdef}	4.4^{ab}	2.33^{ab}	66^{b}	7	**10mg/sodium azide**
1.71^{bc}	$20.33_{bcdefgh}$	1.5^{bcd}	1.33^{abc}	41^{g}	14	
0.73^{bc}	$15.67_{cdefghi}$	1.83^{bcd}	1.33^{abc}	25^{i}	28	
0.41^{c}	8.67^{hi}	0.67^{d}	1_{bc}	25^{i}	7	**15mg/sodium azide**
0.62^{bc}	15_{defghi}	3.16^{abcd}	1.67^{abc}	33^{h}	14	
1.04^{bc}	22.5_{bcdef}	1.4^{cd}	1_{bc}	25^{i}	28	
1.09^{bc}	13.33^{efghi}	1.5^{bcd}	1_{bc}	25^{i}	7	**20mg/sodium azide**
1.50^{bc}	12.5^{fghi}	1.5^{bcd}	1_{bc}	25^{i}	14	
0.46^{bc}	9.67^{ghi}	1.67^{cd}	1_{bc}	25^{i}	28	

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Floral characters

Data presented in Table (4) indicated that all colchicine and sodium azide treatments inhibited flowering process except the concentration of 5mg/l sodium azide for two durations 14 and 28 days. While most of colchicine treatments successfully acclimated until the morphological characters were recorded but some concentration reached to budding stage such as 30 mg/l of colchicine for 28days, 60mg/l for 7 days and 240mg/l of colchicine for 14days (formed bud initiation flower and then dead before full flowering). Treated *Eustoma grandiflorum* plantlets with 5mg/l of sodium azide for 28 days caused significant earliness in flowering about (37 days than control and also recorded increase in most floral characters (number of flowers buds/plant, number of flower/plant, flower diameter (cm),bloom stem length (cm) peduncle length (cm), number of petals/flower, petals

area (cm^2) and fresh and dry weight of flower) except (Days to bloom, days to flower senescence and number of stamens) recorded decrease compared with control. While 5mg/l of sodium azide for 14 days recorded decreased on most cases of floral characters, contrary (days to bloom, bloom stem length (cm),peduncle length (cm),number of petals/flower and petals area (cm^2) recorded unsignificant increased compared with untreated Eustoma plantlets. Our results were agreement with those obtained by [19] and [26]. Sodium azide had affected the genes that are responsible for transfer from vegetative to reproductive phase in the plant through stimulating the flower and fruit associated with hormone that consequently rapidly early flowering [25] and [27].

Table 4. Effect of chemical mutagenesis (sodium azide) on floral characters of *Eustoma grandiflorum* adapted plants

5mg/lsodium azide(28day)	5mg/l sodium azid (14 day	Control (0)	Concentration (mg/l) Character
96.67[b]	134[a]	134[a]	**Days to flower bud initiation**
124.33[b]	165[a]	148[a]	**Days to bloom**
18.51[a]	10.11[a]	18.51[a]	**Flowering percentage (%)**
11[a]	3[bc]	3.67[b]	**No .of flower buds/plant**
8[a]	5[a]	6.67[a]	**No. of flowers/plant**
4.16[a]	3.13[b]	3.23[ab]	**Flower diameter (cm)**
9.5[a]	5.5[b]	3.5[b]	**Bloom stem length (cm)**
7.5[a]	5.83[ab]	5.25[b]	**Peduncle length (cm)**
7.33[b]	8[b]	12.5[a]	**Days to flower senescence (from blooming)**
17.67[a]	11.67[b]	10.33[b]	**No. of Petals/flower**
4.98[a]	4.37[a]	4.30[a]	**Petals area per flower (cm^2)**
4.33[a]	5[a]	5[a]	**No. of Stamens per flower**
0.57[a]	0.27[a]	0.54[a]	**F.W. of flower (gm.)**
0.13[a]	0.043[c]	0.089[b]	**D.W. of flower (gm.)**

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% l

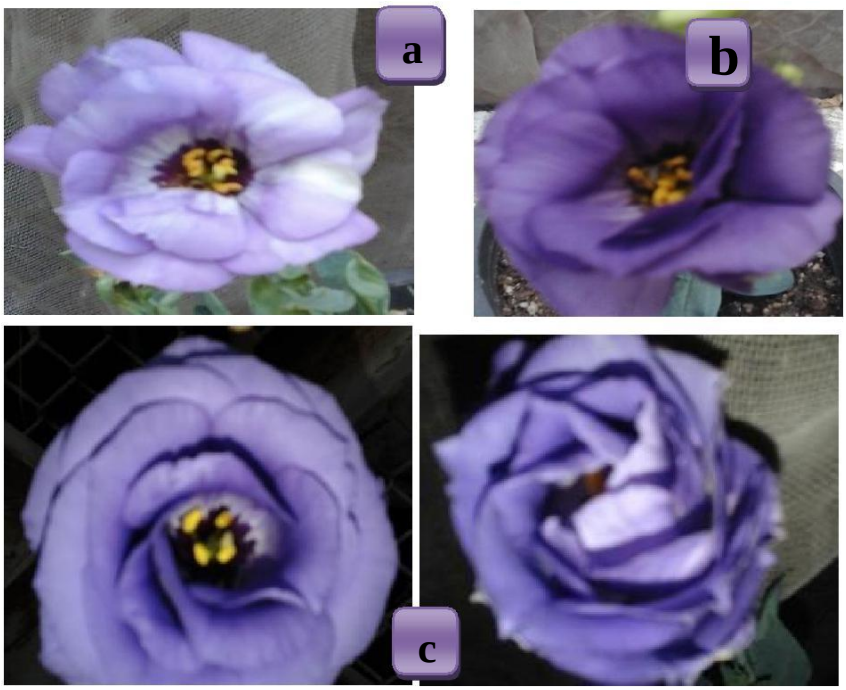


Fig.4. a. Control plant, b. 5mgl/ sodium azide for (14 day) and c. 5mg/l sodium azide for (28 days).

Photosynthetic pigments: From results outlined in Table (5) and Fig (5), the maximum values of chlorophyll (a) content were observed with 10mg/l sodium azide for 14 and 28 days giving (134.22 and 120.10 (mg/ 100g F.W), repectively. while chlorophyll (b) content were obtained with 120 mg/l of colchicie for 28days, 5mg/l of sodium azide for 7and 28 days and 10 mg/l of sodium azide for 14 and 28day by (55.96,58.13, 62.32,51.81 and 59.83(mg/100g F.W),respetively as compared to control (8.20 mg/g F.W) . The concentrations 5mg/l of sodium azide for 7 days and 10 mg/l for 14 days recorded high concentration of carotenoids content by (146.75 amd 146.49 (mg/100 g F.W), respectively.Total chlorophyll (a + b) show the same trend of chlorophyll a content. Our results were agreement with those obtained [25] who found that seeds treated with the high concentrations of colchicine increased total chlorophyll content in plantlets of *Seame indicum*, while low concentration of sodium azide increased total chlorophyll.

Table 5. Effect of colchicine and sodium azide on Photosynthesis pigments (chlorophyll a, b and carotenoids) of *Eustoma grandiflorum* plant (mg/100g.F.W.)

Total Chlorophyll (a +b) (mg/100g.F.W.)	Carotenoids	Chlorophyll (mg/100gF.W.) b	Chlorophyll (mg/100gF.W.) a	Time. Exposure (Days)	Characters Concentration(mg/l)
26.14^{fgh}	72.50^{cd}	8.20^{bcd}	17.94^{efg}	0	**Control**
33.77^{defgh}	76.11^{cd}	0.46^{d}	33.31^{cdefg}	7	**30ml/colchicine**
17.01^{gh}	66.83^{cd}	5.70^{bcd}	11.31^{fg}	14	
49.97^{cdefg}	70.33^{cd}	15.73^{bcd}	34.24^{cdefg}	28	
43.94^{cdefgh}	77.45^{cd}	11.81^{bcd}	32.13^{cdefg}	7	**60mg/l colchicine**
16.2^{gh}	72.13^{cd}	3.36^{cd}	12.84^{efg}	14	
57.68^{cdef}	79.72^{c}	17.12^{bcd}	40.56^{cde}	28	
27.53^{fgh}	86.77^{c}	6.99^{bcd}	20.54^{defg}	7	**120mg/l colchicine**
32.11^{efgh}	71.50^{cd}	3.49^{cd}	28.62^{cdefg}	14	
72.96^{cd}	116.84^{b}	55.96^{a}	17$_{efg}$	28	
52.19^{cdefg}	79.52^{c}	14.41^{bcd}	37.78^{cdef}	7	**240mg/l colchicine**
31.28^{efgh}	83.03^{c}	6 $_{bcd}$	25.28d^{efg}	14	
70.14^{cde}	83.36^{c}	22.69^{bc}	47.45^{bcd}	28	
129.29^{b}	146.73^{a}	58.13^{a}	71.16^{b}	7	**5mg/l sodium azide**
27.56^{fgh}	58.76^{cd}	9.32^{bcd}	18.23^{efg}	14	
131.88^{b}	115.46^{b}	62.32^{a}	69.56^{b}	28	
46.69$_{cdefgh}$	47.97^{de}	11.21^{bcd}	35.47^{cdefg}	7	**10mg/sodium azide**
186.03^{a}	146.49^{a}	51.81^{a}	134.22^{a}	14	
179.95^{a}	138.79^{ab}	59.85^{a}	120.10^{a}	28	
7.94^{h}	26.06^{e}	0.61^{d}	8.56^{g}	7	**15mg/sodium azide**
42.31$_{cdefgh}$	77.44^{cd}	10.59^{bcd}	31.72^{cdefg}	14	
39.35^{defgh}	65.57^{cd}	6.41^{bcd}	32.93^{cdefg}	28	
80.15^{c}	88.42^{c}	24.51^{b}	55.64^{bc}	7	**20mg/l sodium azide**
34.32^{defgh}	57.72^{cd}	5.74^{bcd}	28.58^{cdefg}	14	
46.74$_{cdefgh}$	81.16^{c}	14.23^{bcd}	32.51$_{cdefg}$	28	

Anthocyanin pigment

Data in Table (6) show the decease and non singificant variations in anthocyanin content of *Eustoma grandiflorum* petals due to sodium azide for neither 14 nor 28 days when compared to control (untreatrd plants). This result was agreement with obtained by [28] they show the highest total anthocyanine pigments content with 0.1% sodium bezoote. In this investigation results also showed significant variations (increase or decline) in photosynthetic and anthocyanin pigments content in shootlets of *Eustoma grandiflorum* plantlets treated with different concentrations of colchicine and sodium azid. These results similar to [25],[28] and [19].

The reduction of photosynthetic and anthocyanin pigments may be due to sensitivity of chloroplasts to chemical mutagenic concentrations or may be attributed to degradation of these pigments mutagenic or increase chlorophylase activities.

Table 6. Effect of colchicine and sodium azide on anthyocynin pigment of *Eustoma grandiflorum* plant(mg/100 g.F.W.).

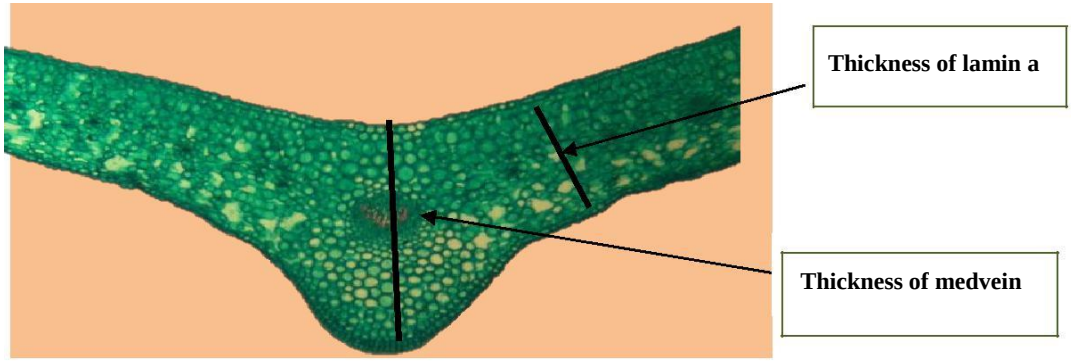
charachter	**Treatments**
201.76[a]	**control**
143.41[a]	**5mg/l sodium azide(14 day)**
143.23[a]	**5 mg/l sodium azid(28 day)**

Anatomical structure Leaf anatomy

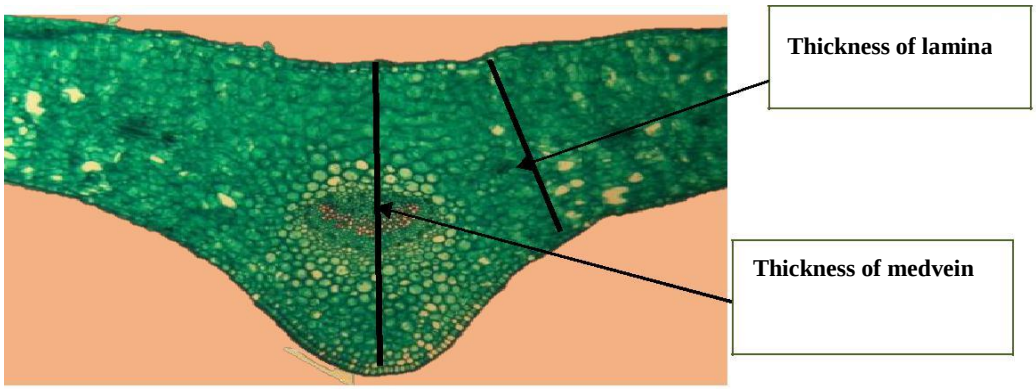
The results as shown in Table (7) and illustrated Fig.(5, 6) indicated that shootlets of *Eustoma grandiflorum* treated with low doses 5 mg/l sodium azid (28 days) achieved increase in all leaf anatomical structure parameters compared with untreated plantlets.[29] mentioned that under the influence of chemical mutagens change in physiological process activities apparently affected nucleic acid metabolism and the concentration of mutagen caused damage of genetic material and also stoped cell division which reflecting on floral, growth, anatomical parameters and chemical constituents.

Table 7. Effect of sodium azide on leaf anatomy of *Eustoma grandiflorum* adapted plants

Wide of vascular bundle (ì)	**Length of vascular bundle (ì)**	**No. of vascular**	**No. of xylem rows**	**Thickness of lamina (ì)**	**Thickness of midvein (ì)**	**Characters Concentration (mg/l)**
28	**32**	**44**	**13**	**70**	**105**	**Control**
33	**60**	**82**	**21**	**115**	**154**	**5mg/l sodium azide (28day)**



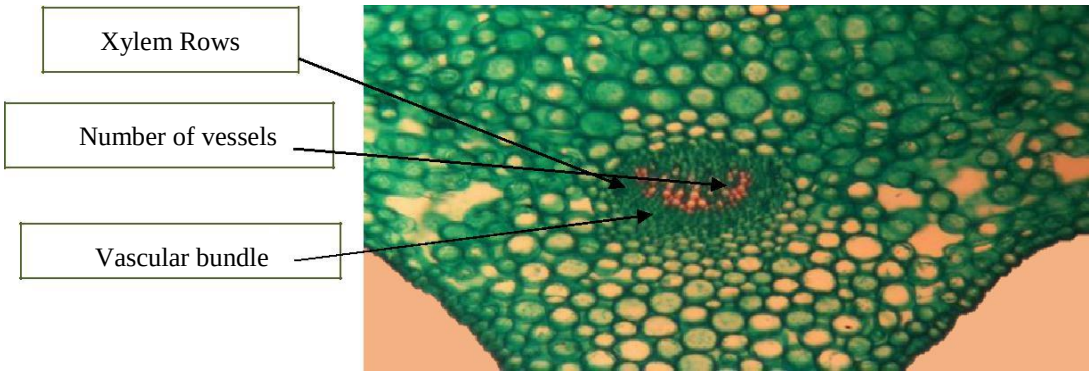
a. Untreated plant (Control)



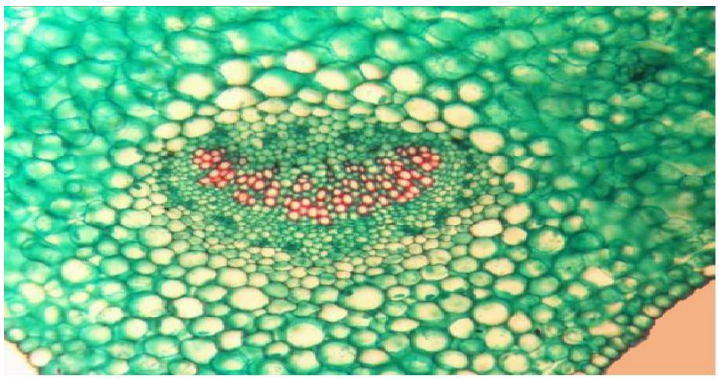
b. Plant treated with 5mg/l sodium azide for 28 days

Fig. 5, 6: Shows leaf anatomy of *Eustoma grandiflorum* treated with 5mg/l of sodium azide for 28 days.a. Control plant and b. 5mg/l sodium azide

Fig.5. Light microphotograph showing transverse sections through the blade of the third in vitro plant leaf developed on main stem of *Eustoma grandiflorum* plantlets.(x10)(Bar=0.05ml).TM=Thickness of medvein, and TL=Thickness of lamina



a. Untreated plant (Control)



b. Plant treated with 5mg/l sodium azide for 28 days

Fig.6. Light microphotograph showing transverse section through the blade of the third *in vitro* plant leaf developed on the main stem of *Eustoma grandiflorum* plantlets. The section shows vascular bundle, (number of vessels and number of xylem rows. (x40)(Bar=0.05ml). VR=vascular bundle, XR=Xylem Rows and NV=Number of vessels.

REFERENCES

[1] Kunitake, H.; Nakashima, T.; Mori, K.; Tanaka, M. and Mii, M. (1995). Plant regeneration from mesophyll protoplasts of Lisinanthus (*Eustoma grandiflorum*) by adding activated charcoal into protoplast culture medium. Plant Cell tissues and Organ Culture., 43:59-65.

[2] Harbaugh, B.K. (2006). Lisianthus *Eustoma grandiflorum*. In flower breeding and gentics, anderson N.O. (ed). Springer, Netherlands, 645-663.

[3] Pati,P.K.;Rath,S.P.;Sharma,M.;Sood,A. and Ahuja,P. (2005). *In vitro* propagation of rose-a review.Biotech.Adv.,24,94-114.

[4] Nhut,D.T.; Hai, N.T. and Phan,M.X.(2010).A highly efficient protocol for micropropagation of *Begonia tuberous*.In: protocols for in vitro propagation of ornamental plants, Jain, S.M.,Ochatt,S.J.(ed).Springer protocols,Humana Press,15-20.

[5] Roychowdhury, R. and Tah, J.(2011). Assessment of chemical mutagenic effect in mutation breeding programme for M1 generation of carnation (*Dianthus caryophyllus*). Research in Plant Biology, 1(4):23-32.

[6] Al-Qurainy, F. and Khan, S. (2009). Mutagenic effect of sodium azide and its application in crop improvement. World Applied Sci., 6:1589-1601.

[7] Murashing,T. and Skoog (1962).A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. plant., 15:473-497.

[8] Saric, M.R. Kastrori; Cupina,T.C. and Gerir, I. (1967).Vasiverzit et Novon Sadu. Praktikum Izfiziologize Biljaka- Beogard, Haucua Anjiga, 215p.

[9] Fuleki, T. and Francis, F. J. (1968). Quantitative method for anthocyanins. J. Food Sci., 33: 72.

[10] Johansen, D.A. (1940). Plant microtechnique. MC. Graw. Hill Book Company New York.

[11] Corgen, J.N. and Widmamayer, F.B. (1971).The effect of gibberellic acid on flower differentiation date of bloom, and flower hardiness of poach. J. Amer. Sci., 96:54-57.

[12] Duncan, D.B. (1955). Multiple range and multiple F-tests. Biometrices,11:1-42.

[13] Kulaeva, O.N. (1980). In: Skoog, F. (ed) 1980. Proc.10thInt. Conf. Plant growth substances, Madison, Wisconsin, 1979. Springer-Verlag. Berlin, Heidelberg, New York, pp:119-128.

[14] Khorrami, M.R.; Bohluli, S.Z.; Shoor, M.; Hamidoghli, Y.; Ramezani, A.S.; Kharabian, A.M. and Kaviani, B. (2012). Callus induction and organogenesis capacity from lamina and petiole explants of *Anthurium andreanum* Linden (Casino and Antadra). AJCS., 6(5):928-937.

[15] Abou Dahab, A.M. (2006). *In vitro* propagation of *Hydrangea macrophylla* Thunb. Arab. J. Biotech., 10 (1):161-178.

[16] Ahmed, A. H.; Kaviani, B.; Tarang, A. and Sahar, B. Z. (2011). Effect of different concentrations of kinetin on regeneration of ten weeks *Matthiola incana*. POJ., 4(5):236-238.

[17] Mustafa, N.S. and Rania, A. T. (2012). Influence of plant growth regulators and subculturing on *in vitro* multiplication of some fig (*Ficus carica*) cultivars. Journal of Applied science Research.,8(8):4038-4044.

[18] Sajjad, S.; Jaskani, M.J.; Mehmood, A.; Ahmed, I and Abbas, H. (2013). Effect of colchicine on *in vitro* polyploidy induction in *African marigold* (*Tagetes erecta*). Pak. J. Bot., 45(3)1255-1258.

[19] Ali, A.; Yubey, K.;Deka, U.K. and Tomar, S.M.S. (2014). Effect of sodium azide an seed germination and related agro-metrical traits in M1 lentil (*Lens culinaris* Medik) generation. Journal of Agricultural Sciences.10 (3):95-102.

[20] Kumar, V.A.; Kumari, R.U.; Amutha, R.; Kumar, T.S.; Hepziba, S.J and Kumar, C.R.A.(2009). Effect of chemical mutagen on expression of characters in arid legume pulse – cowpea (*Vigna unguiculata* L. Walp.). Research Journal of Agriculture and Biological Sciences, 5 (6): 1115 - 1120.

[21] Tharawoot, T.; Samyam, W and Vessabutr, S. (2012). Colchicine induced polyploid of *in vitro Impatiens patulacraib*.Thal. J. Botany.,4 (2555):75-80.

[22] Naheed, A. (2014). Effect of physical and chemical mutagens on morphological behavior of tomato (*Lycopersicon esculentum* L.) cv. 'Rio Grande' under heat stress conditions. Scholarly Journal of Agricultural Science., 4(6):350-355.

[23] Sungkaew, K.; Taychasinpitak, T.; Wongchaochants, S.; Sukprasert, P.and Kikuchi, S. (2015). Effect of gout drug treatments on survival rate and morphological change of *Lindernia sp*. *in vitro*. International transaction journal of engineering, Mangement & applied science &Technologies.,6 (4):191-201.

[24] Joshi, N .; Ravindran, A. and Mahajan, V. (2011). Investigations on chemical mutagen sensitivity in onion (*Allium cepa* L.). Int J Bot 7: 243-248.

[25] Mensah, J.K.;Obadoni, B.O.;Akomeah, P.A.;Ikhajiagbe, B. and Ajibolu, J. (2007). The effect of sodium azide and colchicine treatments on morphological and yield traits of sesame seed (*sesame indicum* L.). African Journal of Biotechnology., 6(5)534-538.

[26] Singh, P.K.; Sadhukhan, R.;Dudhan, A.S.;Kumar, V. and Sarkar, H.K. (2015). Preliminary study on mutagenic effect of EMS on tuberose (*Polianthes tuberosea* L.)., Environment &Ecology.,33(3A):1386-1390.

[27] Sadashiv, B.N.;Vikram, B.D. and Konddiram, D.N. (2012). Effect of mutagens on seed germination, plant survival and quantitative characters of horsegram (*Macrotyloma uniflorum* (LAM) VERDC).International Journal of life Science &Pharma Research.,4(2):129-136.

[28] Shiema, A.; Kudachikarv, V.B.; Vanajakshi and Ravi, R. (2013). Effect of combined preservation techniques on the stability and microbial quality and retention of anthocyanins in grape pomace stored at low temperature. Journal of Science and Technology., 50(2):332-338.

[29] Borzouei, M. and Kafi, H.(2010). Effects of gamma radiation on germination and physiological aspects of wheat (*Triticum aestivum* L.) seedlings. Pakistan Journal of Botany., 42: 2281-2290.