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In vitro laser radiation induces mutation and growth in *Eustoma grandiflorum* plant

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Abstract

Background: *Eustoma grandiflorum* is a new floral crop for the international flowers market, moderately cold-tolerant annual or biennial plant. A large number of seedlings can be produced by seed propagation but the quality is not uniform due to variations in the flowering time, plant height, and the number of flowers. Propagation of *Eustoma grandiflorum* plant by tissue culture technique is relatively low. Inducing mutations is one of the powerful tools for breeding biotechnology. Laser mutagenesis is an easy and new tool. The goal of the present work was to investigate the influence of laser irradiation on in vitro growth, anatomy, flowering, chemicals composition, and gene mutagenesis.

Results: The most of morphological, floral parameters, total chlorophyll, carotenoids, and anthocyanin pigment contents in the flower recorded increment by most treatments of laser types. The highest survival percentage of acclimatized plants (95%) and highest values of number of branches and branches length (cm) were obtained from treated plantlets by 20 min of green laser, while most of highest floral parameters, anthocyanin pigment contents in flower, and anatomical structural parameters recorded increasing using 20 min of blue laser and 20, 25 min of green and red laser, respectively. Contrary, the lowest values of photosynthetic pigments and carotenoids were obtained from 20 min of green laser.

Conclusions: The current research concluded that laser irradiation has remarkable effect on plant morphology, flowering, chemical constituents, and gene mutagenesis.

Keywords: Laser rays, *Eustoma grandiflorum*, Tissue culture and anatomy

Introduction

Eustoma grandiflorum (family, Gentianaceae) is considered cut flowers in the international flowers market. The plant is a moderately cold-tolerant annual or biennial plant native to the southern part of the USA and Mexico (Roh and Lawson 1988). This plant attains to 50–75 cm in height with 20–40 flowers. By nature, *Eustoma grandiflorum* initially forms a rosette and grows very slowly during the winter, stems elongate in the spring, and it flowers in summer (Roh et al. 1989). *Eustoma grandiflorum* is commonly propagated by seed or cutting. A large number of seedlings can be produced by seed propagation but the quality is not uniform due to variations in flowering time, plant height, and the number of flowers. Propagation of *Eustoma grandiflorum* by tissue culture technique is relatively low. Several factors like genotype, media, plant growth regulators, and type of explants should effect the success of the micropropagation method, and most of plant growth regulators that have been used were 6-benzyle amino purine (BA), n-6-foural adenine (KIN), naphthalene acetic acid (NAA), and indole butyric acid (IBA) (Pati et al. 2005; Nhut et al. 2010).

Laser has been discovered in the past century and has been applied in the society from its conception until today. Among its application is its use in agriculture as a biostimulator device. The laser light at low intensity produces biostimulation when used on seeds and seedling plants (Chen et al. 2005). The basis of laser stimulation mechanism in any plant physiological stage is the synergism between the polarized monochromatic laser beams and the photoreceptors (Bielozierskich and Zolotariewa

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1981; Koper et al. 1996). There are many facts that indicate the biostimulating action of laser radiation on various organs and tissues in plants (Anisimov et al. 1997). Plants absorb light via their photoreceptors and control all stages of plants development (Spalding and Folta 2005).

Protein molecular weight determination via SDS-PAGE is universally used method and it can be economically used for assessing genetic variation (Ranjan et al. 2013; Awatef 2017).

Mutagenesis experiment permits to increase possibilities of variability creation with high ornamentation (Cantor et al. 2002). Mutation is a natural process which creates changes in DNA sequences. The genetic variation created is useful because it helps population to survival and change over time. Mutagenesis is the process where changes occur in the genetic information of an organism not caused by genetic segregation but induced by chemical and physical agents (Roychowdhury and Tah 2011).

Materials and methods

The experiment was conducted from 2014 to 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 of laser as physical mutagens on in vitro and in vivo propagation behaviors as well as flowering of acclimatized plants, biochemical and cytological behaviors of *Eustoma grandiflorum* plant.

Plant materials and surface sterilization

Eustoma grandiflorum plants were obtained from greenhouse of National Research Centre on 2014 and were 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 rinsing with distilled water, explants were surface sterilized in 70% (*v*/*v*) ethanol for 1 min, then in 20% commercial sodium hypochlorite solution and one drop of tween 20 (polyoxy ethylene sorbitonmonol aurate) for 10 min, and after that the explants were rinsed three times with autoclaved distilled water followed with 7 min in 0.1 g/l $HgCl_2$, and finally rinsed three times with autoclaved distilled water.

Culture media and culture conditions

The explants were cultured on MS (Murashing and Skoog 1962) medium (free growth regulators) supplemented with 25 g/l sucrose and 8 g/l agar then adjusted to pH 5.6 ± 0.2; the medium was autoclaved at 121 °C and 1.5 kg/cm^2, then the cultures were incubated under 30 μmol m^{-2} s^{-1} of light and 16 h photoperiod. After 1 month from culture explants, the shootlet nodal stems were used for in vitro propagation.

Proliferation of shootlet explants under effect of various cytokinins types

Shootlet nodal stems were cultured on MS medium supplemented with different cytokinins [6-benzyl amino purine (BA), 6-γ,γ-dimethyl ally amino purine riboside (2ip) and Kinetin (N-6-fouryl adenine) (Kin)] at the concentration of 0.4 mg/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 were calculated after 45 days from each subculture under control and cytokinins treatments.

In vitro multiplication stage under physical mutagenesis effect laser radiation

Three types of laser (green, blue, and red) were used for five exposure times (0, 5, 10, 20, and 25 min) as described in Table 1.

Acclimatization stage under mutagenesis effect

The in vitro rooted plants were successfully transplanted (after the above-mentioned mutagenesis treatments) to the greenhouse of National Research Centre (17/2/2015) using growth media which contained perlite and peatmoss (1,1). Morphological characters (survival %, number of branches, height of branches/plant (cm), number of leaves/branch, and leaf area (cm^2)) were recorded after 2 months.

After 3 to 4 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 (cm^2), number of stamens, fresh and dry weights of flower (g)) were recorded.

Determination of protein molecular weight via SDS-PAGE is universally used method and it can be economically used for assessing genetic variation (Ranjan et al. 2013).

Electrophoretic analysis of protein provides information concerning the structural genes and their regulatory systems that control the biosynthetic pathways of that protein. Each polypeptide band represents the final products of transcriptional events occurring due to active structural genes (Sadia et al. 2009).

The objective of the present study was 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 doses of laser as physical

Table 1 Source of laser types used for mutagens

System properties	Blue light (helium cadmium laser)	Red light (helium neon laser)	Green light (argon laser)
Laser wavelength	460 nm	650 nm	530 nm
Output power	16.2 mW	50 mW	8 mW

Power (in units of Watts (W) and milliwatts (mW)

mutagens on *Eustoma grandiflorum* propagated in vitro as well as flowering of acclimatized plants, biochemical and cytological behaviors.

Chemical analysis

Extraction and determination of photosynthetic pigments

According to Saric et al. (1967), the color density was measured using (spectronic) at 660, 640, and 440 nm wave length against the blank methanol.

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 the method of Fuleki and Francis (1968).

Analysis of protein profile of leaf by SDS-PAGE

Protein concentration in the supernatant samples was estimated according to the method of Bradford (1976) and gels were made according to Laemmli (1970).

Anatomical structure

Leaf anatomy

The preparation of leaf section was carried out according to the methods described by Johansen (1940) and Corgen and Widmamayer (1971). Leaf section was mounted in Canda balsam then examined microscopy and microphotography. The following parameters were recorded: number of bundles, dimension of bundles (length−wide) (μ), thickness of midvien (μ), thickness of lamina (μ), number of xylem rows, and number of vessels.

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 (Duncan (1955) using COSTATV-63.

Results

Laser radiation

In vitro vegetative growth behaviors

The results tabulated in Table 2 show the maximum number of shootlets per explants (3.67) which was observed with 20 min of blue laser treatment while the minimum value (1.11) was recorded when the shootlets were subjected to blue laser radiation for 5 min; we can also notice that most of laser types (green, blue, and red laser radiation) for any exposure times (5, 10, and 25 min) led to increase in shoot multiplication of *Eustoma grandiflorum* except for blue laser radiation for 5 min when compared with control (1.22).

Longest shootlets 4.22, 3.99, and 4.27 cm were produced from exposing *Eustoma grandiflorum* shootlets to green and blue laser for a short time exposure of 5 min, which was produced as well at long time exposure of red laser (20 min) as compared to control which recorded 3.22 cm.

The highest number of leaves per shootlet (34 leaf/shootlet) appeared with red laser for 5 min as compared to control which recorded 24.55 leaf/shootlet.

All laser radiation treatments had not significant effect on rooting percentage of *Eustoma grandiflorum* plant as compared to control.

Table 2 Effect of laser radiation on in vitro shooting and rooting behaviors of *Eustoma grandiflorum* plant

Characters Doses (min)	Number of shootlets per plant	Length of Shootlets (cm)	Number of leaves/ shootlets	Rooting (percentage)	Number of roots/ shootlet	Length of roots (cm)
Control	1.22bc	3.22ab	24.55bc	67a	2.77bcd	3.12d
5 min green	3.11abc	4.22a	28.55bc	89a	4.106abcd	4.44bcd
10 min green	2.106abc	1.88b	27.44abc	78a	4.77abc	15.91a
20 min green	1.33abc	3.49ab	29.33abc	89a	2.99abcd	6.38bc
25 min green	2.22abc	2.61ab	26.22abc	89a	3.99abcd	3.71cd
5 min blue	1.11c	3.99a	20.89c	100a	5.77a	3.086d
10 min blue	3.23abc	3.22ab	22.77c	89a	5.55ab	3.21cd
20 min blue	3.67a	2.77ab	23.44bc	89a	3.89abcd	1.84d
25 min blue	3.55ab	2.49ab	25.44abc	78a	2.33cd	4.33bcd
5 min red	3.23abc	3.74ab	34a	67a	3.22abcd	7.38b
10 min red	1.33abc	2.77ab	21.88c	89a	2.99abcd	3.44cd
20 min red	3.106abc	4.27a	32.77ab	89a	3.21abcd	2.25d
25 min red	1.806abc	3.64ab	24.55bc	89a	1.66d	4.88bcd

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

The highest number of roots (5.77 root/shootlet) was obtained with blue laser for 5 min; however, the minimum value (1.66) of root/shootlet was observed with red laser for 25 min as compared to control which gave 2.77 root/shootlet and other treatments.

Regarding the effect of laser radiation on length of roots as affected by different types of laser radiation and various times exposure, data showed that the longest roots (15.91 cm) resulted from irradiation of shoots with green laser for 10 min as compared to control which resulted to 3.12 cm.

Acclimatization stage

Morphological characters

Data in Table 3 showed the effect of laser radiation on survival percentage of adapted plantlets. The maximum percentage (95%) was observed with green and blue laser radiation for 25 min as compared to control (51.6%). The best results for the number of branches per plant were obtained from the green laser for the long time exposure (25 min) and red laser for short time exposure (5 min) which recorded 4 branch per plant as compared to control (1.67 branch per plant).

The tallest branches (11, 11.16, and 12.16 cm) resulted from different types of laser radiation such as green, blue, and red for various times (10, 5, and 20 min., respectively) as compared to control (1.86 cm).

The highest number of leaves per branch (39.67 leaf/branch) was resulted with green laser for 25 min as compared to control (17.67 leaf/branch).

Data presented in Table 3 pointed out that the highest leaf area (3.70 cm^2) was resulted from using green light of laser for 20 min compared to control (0.962 cm^2) and other treatments.

Floral characters

It is evident from the data presented in Table 4 that red laser radiation for 5 min delayed bud flower initiation. The longest period 198 days was resulted from red light laser treatments for 5 min followed by 164 days from red light for 10 min as compared to control which took 177 days to form bud flower initiation. Contrary, the shortest period (94.67 days) was obtained with blue light of laser for 20 min.

Shootlets with various types and different laser radiation led to delay bloom bud formation. The longest duration (192.33, 192, 203.67, and 192 day) was observed with 25 min blue light, 5 min green light, 5 min red light, and 10 min red light laser irradiation, respectively with no significant difference between them and control (183.33 day).

Considering the flowering percentage of *Eustoma grandiflorum* **plant,** Table 4 showed that among different treatments, shootlets exposure to blue and red light laser radiation for 20 min gave the best results (59.25%, 4.83 cm and 72.07%, 4.5 cm) of flowering percentage and flower diameter compared to control (18.51% and 2.33 cm) and other treatments respectively.

The highest number of flower buds per plant and the highest number of flowers per plant (12.33 buds/plant and 8.33 flower/plant) were resulted with blue laser radiation for 20 min, while control treatment formed (2.33 bud/plant and 1.67 flowers/plant) respectively.

Table 3 Effect of laser radiation on morphological characters of *Eustoma grandiflorum* adapted plants

Characters Dose (min)	Survival (percentage)	Number of branches/plant	Height of branches (cm)	Number of leaves/branch	Leaf. area (cm2)
Control	51.6d	1.67b	1.86bc	17.67def	0.962d
5 min green	56.67d	1.67b	2bc	27bcde	1.32cd
10 min green	63.67cd	2b	11a	38ab	2.42abcd
20 min green	84.67ab	1.33b	1.5c	17.33ef	3.70a
25 min green	95a	4a	3.33bc	39.67a	2.86ab
5 min blue	63.67cd	1b	11.16a	23.33cdef	2.22abcd
10 min blue	64cd	1.67b	1.67bc	19.67def	2.12abcd
20 min blue	84ab	2b	11a	29.67abcd	2.32abcd
25 min blue	95a	2.33b	2.6bc	23cdef	3.17ab
5 min red	65cd	4a	3.16bc	32.33abc	2.37abcd
10 min red	74.67cd	1.33b	4.33b	26.67bcde	2.77abc
20 min red	75.33bc	1b	12.16a	25.33cde	2.95ab
25 min red	64cd	1b	1.16c	12.33f	2.15bcd

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 4 Effect of laser radiation on floral characters of *Eustoma grandiflorum* adapted plants

Character	Control	Blue (min.)				Green (min.)				Red (min.)			
Dose (min.)	(0)	5	10	20	25	5	10	20	25	5	10	20	25
Days to flower bud initiation	177ab	120.33de	134cde	94.67e	103de	133cde	145bcd	122cde	143bcd	198a	164abc	131.33cde	105de
Days to bloom	183.33a	152.67b	144.33b	102d	192.33a	192a	148b	133.5cd	150.33b	203.67a	192a	135bc	113cd
Flowering percentage (%)	18.51c	21.55bc	10.11c	59.25a	9.37c	9.37c	37.03b	37.03b	10.85c	9.37c	9.37c	72.07a	25.92bc
No .of flower buds/plant	2.33cd	7.33b	6bc	12.33a	4.33bcd	2.33cd	5.67bc	5bcd	3.33bcd	1d	3bcd	1.67cd	4bcd
No. of flowers/plant	1.67c	6ab	4bc	8.33a	3.33bc	1c	5.67ab	2.67bc	1.33c	1c	1c	1.67bc	3.67bc
Flower Diameter (cm)	2.33c	2.83bc	2.16c	4.83a	2.16c	2.13c	4.46a	3.8ab	2.86bc	2.13c	2.33c	4.5a	3.83ab
Bloom stem length (cm)	6.5def	8cd	3.5ef	29a	2.5f	2.16f	8.16cd	10.8bc	2.16f	2.3f	2.4f	6.83cde	14b
Peduncle length (cm)	3.67d	6.16c	2.67d	8.5ab	3d	2.83d	6.16c	7.5bc	3.16d	2.9d	2.9d	6.6c	9.7a
Days to flower senescence (from blooming)	3d	10.33ab	4cd	12.67a	3.67cd	3d	9abc	6.67bcd	10ab	3d	3d	7.67abcd	7bcd
No. of petals/flower	11def	14cde	8.33f	20ab	9f	8.33f	17.33bc	24a	9.67f	9f	10ef	15cd	21.67a
Petals area (cm^2)	3.76c	1.95cd	1.79d	7.58a	1.98cd	2.04cd	5.72b	5.71b	3.09cd	1.98cd	1.9cd	5.54b	6.44ab
No. of Stamens per flower	4c	5bc	3c	6.67ab	3c	3c	7a	4.67c	4.33c	3c	3.67ab	6.67ab	4.67c
F.W. of flower (g)	0.41bcd	0.24cd	0.16d	1.42a	0.13d	0.15d	0.62bc	0.72b	0.18d	0.031d	0.038d	0.76b	1.33a
D.W. of flower (g)	0.095bcde	0.095bcde	0.058cd	0.14ab	0.44cde	0.025e	0.081bcde	0.12abc	0.051cde	0.015e	0.028de	0.11bcd	0.19a

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

The longest bloom stem length (29 cm) and fresh weight of flower (1.42 g) were obtained with 20 min of blue light of laser radiation followed by 25 min of red light of laser radiation which reached to 14 cm in height and 1.33 g, while the shortest bloom stem length (2.3 cm) and fresh weight of flower (0.03 g) was obtained with 5 min of red light of laser radiation as compared to control (6.5 cm and 0.41 g) respectively.

The longest peduncle length (9.7 cm) was obtained with 25 min red light of laser radiation as compared to control (3.67 cm) while the shortest peduncle (2.9 cm) was observed with 5 min and 10 min of red light of laser radiation. The peduncle length was increased gradually with increasing time exposure times.

The treated shootlets with 20 min of blue light laser radiation delayed the flower senescence to 12.67 day and recorded the highest petal area (7.58 cm^2) as compared to control (3 days and 3.76 cm^2) respectively. While the maximum number of petals (24 and 21.67 petal/flower) were observed with 20 min of green light and 25 min of red light laser radiation as compared to control (11 petal/flower).

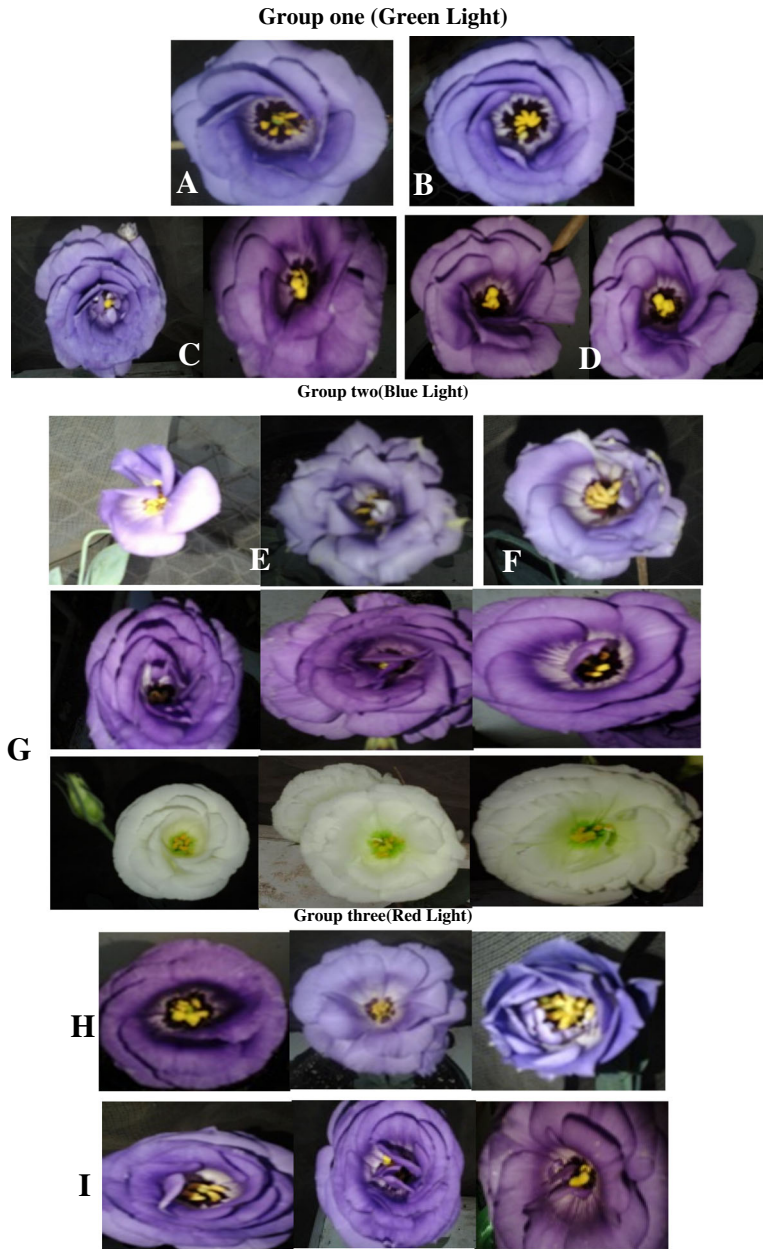

Fig. 1 Changes in *Eustoma grandiflorum* flowers color and form after radiation with different doses of laser radiation. **a** Control (untreated plants), **b** 10 min green light, **c** 20 min green, **d** 25 min green, **e** 5 min blue, **f** 10 min blue, **g** 20 min blue, **h** 20 min red light, and **i** 25 min red

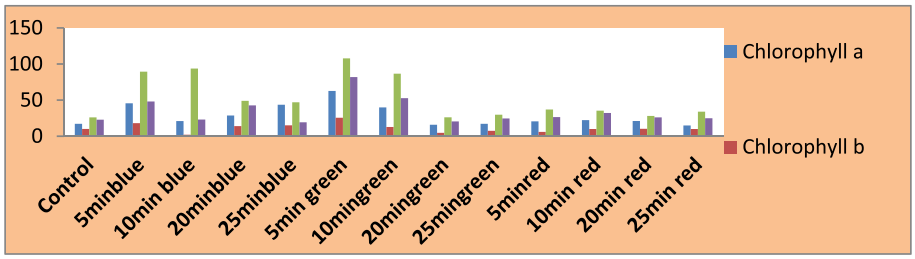

Fig. 2 Effect of laser irradiation on photosynthetic pigments content of in vitro *Eustoma grandiflorum* plants

The maximum number of stamens (seven stamens/flower) was recorded with 10 min of green laser. While the best results of dry weight also was observed with 25 min of each of blue and red light of laser rays comparing with control and other treatments.

Changes in *Eustoma grandiflorum* flowers color and form after irradiated with different types and exposure times of laser radiation

The changes range of flower color and form were varied as shown in (Fig. 1) as follows.

The flowers of unirradiated plants were characterized by violet (light purple) color in the middle of the petals, dark purple in the flower center, and white or brown color on the edges of the petals; the ovary is closed with green color and the stamens is yellow (Fig. 1a). The irradiation with green laser for 10 min resulted in very light purple flowers, wide and big stamens, and small light yellow ovary (Fig. 1b). While exposing plants to the same type of laser (green laser) for 20 min (Fig. 1c) caused variation in colors in the flowers such as the first flower was very light purple, the second one was very dark purple, but increasing this time exposure to 25 min (Fig. 1d) changed the color to light purple flowers with arranged and compact petals. Blue irradiation laser treatment for 5 min (Fig. 1e) resulted in light purple flowers planned with light white color; the petals were varied in the shape from triangle to round and sometimes serrate. The ovary was small and greenish yellow. Further, 10-min (Fig. 1f) exposer of blue laser led to very light purple flowers brushed with white color in the edges of external petals, while increasing this time exposure to 20 min (Fig. 1g) was a very important factor to produce two different colors which were creamy white and dark purple with big petals. The third type of laser (red) resulted in dark purple flowers for the exposure time of 20 min (Fig. 1h), but increasing this time to 25 min (Fig. 1i) led to flowers varied in the darkness of purple color from light to dark purple reddish and planed in white color.

Photosynthesis pigments

Data presented in (Fig. 2) indicated that shootlets irradiated with short exposure time of green laser radiation had the best results for pigments content as compared to control and other treatments. The maximum values of chlorophyll (a, b), carotenoids, and total chlorophyll (a + b) (62.78, 25.52, 107.81, and 88.3 mg/100 mg, respectively) were resulted from green light of laser for 5 min.

Anthocyanin pigment

Data in (Fig. 3) indicated that anthocyanin content of *Eustoma grandiflorum* petals were significantly increased

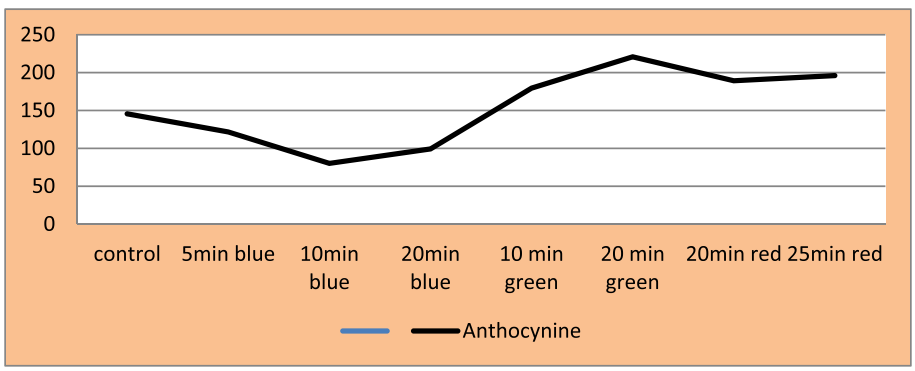

Fig. 3 Effect of laser radiation on anthocynine pigments content of *Eustoma grandiflorum* adapted plant (mg/100 g F.W)

Table 5 SDS-PAGE profile of total proteins extracted from *Eustoma grandiflorum* plants as respond to different leaser light irradiation

Band No.	MW (kDa)	Green light (min)				Red light (min)				Blue light (min)			
		1	2	3	4	5	6	7	8	9	10	11	12
1	75					+	+	+	+	+	+	+	+
2	66	+	+	+	+	+	+	+	+	+	+	+	+
3	57		+	+	+					+	+	+	+
4	47	+	+	+	+	+	+	+	+	+	+	+	+
5	39	+	+	+	+	+	+	+	+	+	+	+	+
6	31	+	+	+	+	+	+	+		+	+	+	+
7	27	+	+	+	+								
8	25	+	+										
9	23					+	+	+	+	+	+	+	+
10	20	+	+		+								
11	19			+									
12	14	+	+	+	+	+	+	+	+	+	+	+	+
Total No.		8	9	8	8	7	7	7	6	8	8	8	8

Total protein extracted from leaves samples ($n = 3$); + means protein band is appearance

by using dose of laser radiation and reached up to the maximum values (220.84 and 196.07 mg/100 g F.W.) with both doses (green for 20 min and red light laser for 25 min) as compared to control giving (145.67 mg/100 g F.W.).

Protein fraction

Total proteins were extracted from leaves of control and different laser lights (red, green, and blue) for four different irradiation times of *Eustoma grandiflorum* plants after flowering and analyzed by SDS-PAGE. These protein alterations based on changes in polypeptides molecular weights (MWs), appearance of new bands, and disappearance of some bands. SDS-PAGE analysis revealed the total of 12 polypeptides bands with different bands MWs that ranged from 75 to 14 kDa as shown in Table 5 and Figs. 4 and 5; out of those 12 polypeptides bands, 4 common bands with all laser light irradiations and with different time at MWs 66, 47, 39, and 14 kDa appeared. SDS-PAGE generated two common bands (75 and 23 kDa), which disappeared with the green light and appeared with red and blue laser irradiated plants. On the other hand, irradiated plants with green laser light showed five protein profiles that appeared at 57, 27, 25, 20, and 19 kDa, which did not appear with red or blue laser irradiated plants. The maximum number of bands (nine bands) was found with green light laser at 10-min irradiation. While, the minimum numbers of bands (six bands) were found with red light laser at 25 min irradiation.

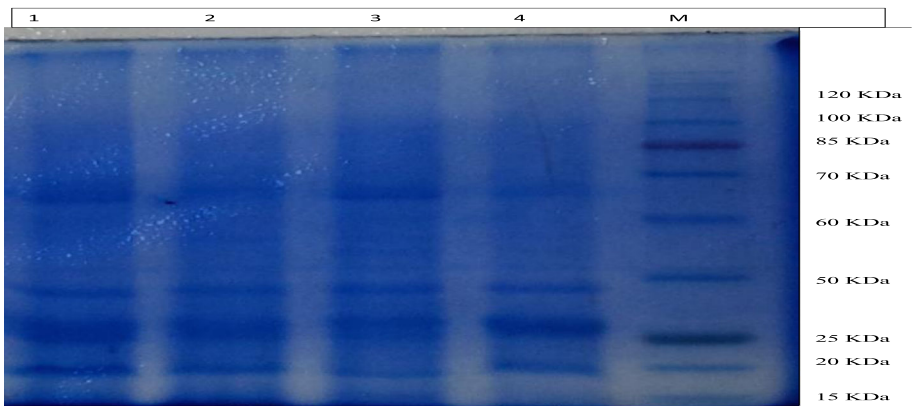

Fig. 4 Patterns of SDS-PAGE electrophoretic protein of *E. grandiflorum* plants as respond to leaser green light irradiation for different time. 1. 5 min, 2. 10 min, 3. 20 min, 4. 25 min, M. protein marker

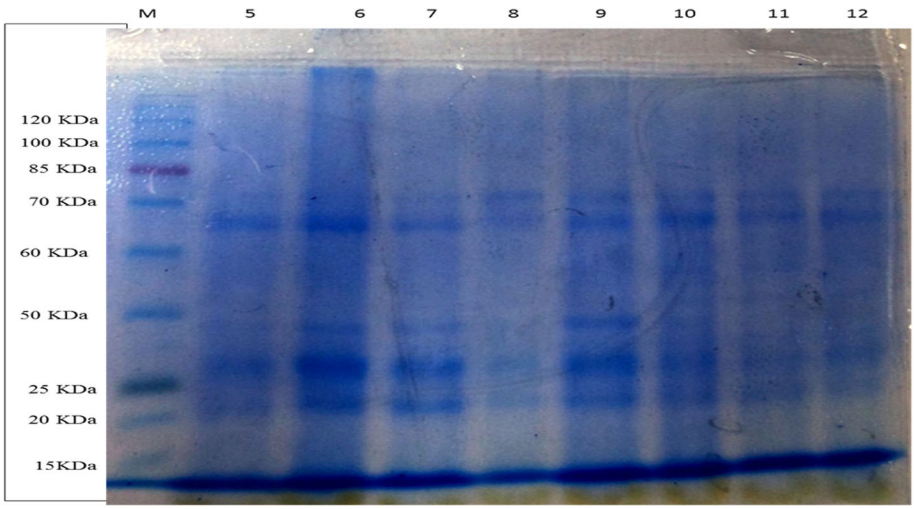

Fig. 5 Patterns of SDS-PAGE electrophoretic protein of *E. grandiflorum* plants as respond to leaser red and blue light irradiations for different time. For red light: 5. 5 min, 6. 10 min, 7. 20 min, 8. 25 min. For blue light: 9. 5 min, 10. 10 min, 11. 20 min, 12. 25 min and M. protein marker

Leaf anatomical structure

The results presented in (Table 6) and (Figs. 6 and 7) indicated that the highest thickness of midvein, **t**hickness of lamina**,** number of xylem rows, number of vessels and diminution of vascular bundle (length – wide) obtained with 20 min cadmium and 25 min helium neon laser which recorded (125, 80, 55, 340, 50, and 47 and 114.5, 82, 35, 336, 50, and 40) respectively as compared with control and other treatments, We can notice that shootlets of *Eustoma grandiflorum* plant exposed to short time (exposure time of 5 min cadmium laser and exposure time 10 min argon laser) led to decrease in thickness of midvein (79 and 75 μ) as compared to control (105 μ).

Discussion

The goal of the present work was to investigate the influence of laser irradiation *Invitor* on growth, anatomy, flowering, chemicals composition, and gene mutagenesis.

According to data presented in vitro growth ability, we can notice that most of laser types (green, blue, and red laser radiation) for any exposure times (5, 10, and 25 min) led to increase in shoot multiplication of *Eustoma grandiflorum* except for blue laser radiation for 5 min when compared with control (1.22). The data go in line with those obtained by Danaila et al. (2011) on *Petunia hybrid* and *Dianthus caryophyllus* plants and Hwida et al. (2012) on *Balanites aegyptiaca* and *Cotoneaster horizontalis*. Lobna et al. (2014), Rania et al. (2015), and Hwida et al. (2012) mentioned that the maximum shootlets number per explant of *Balanites aegyptiaca* were observed with red laser treatments. Therefore, the mechanism influence of laser irradiation is most likely attributed to light and electromagnetism effects. Longest shootlets were produced from exposing *Eustoma grandiflorum* shootlets to green and blue laser for short time exposure 5 min produced as well as long time exposure of red laser (20 min) as compared to

Table 6 Effect of laser radiation on leaf anatomy of *Eustoma grandiflorum* adapted plants

Character Dose (min)	Thickness of midvein (μ)	Thickness of lamina (μ)	No. of xylem rows	No. of vessels	Length of vascular bundle (μ)	Wide of vascular bundle (μ)
Control	105	70	13	44	32	28
10 min green	75	55	11	18	35	25
20 min green	90	59	20	80	48	26
25 min green	80	60	9	21	27	19
5 min blue	79	63	10	26	22	15
20 min blue	125	80	55	340	50	47
20 min red	92	55	28	170	30	27
25 min red	114.5	82	35	336	50	40

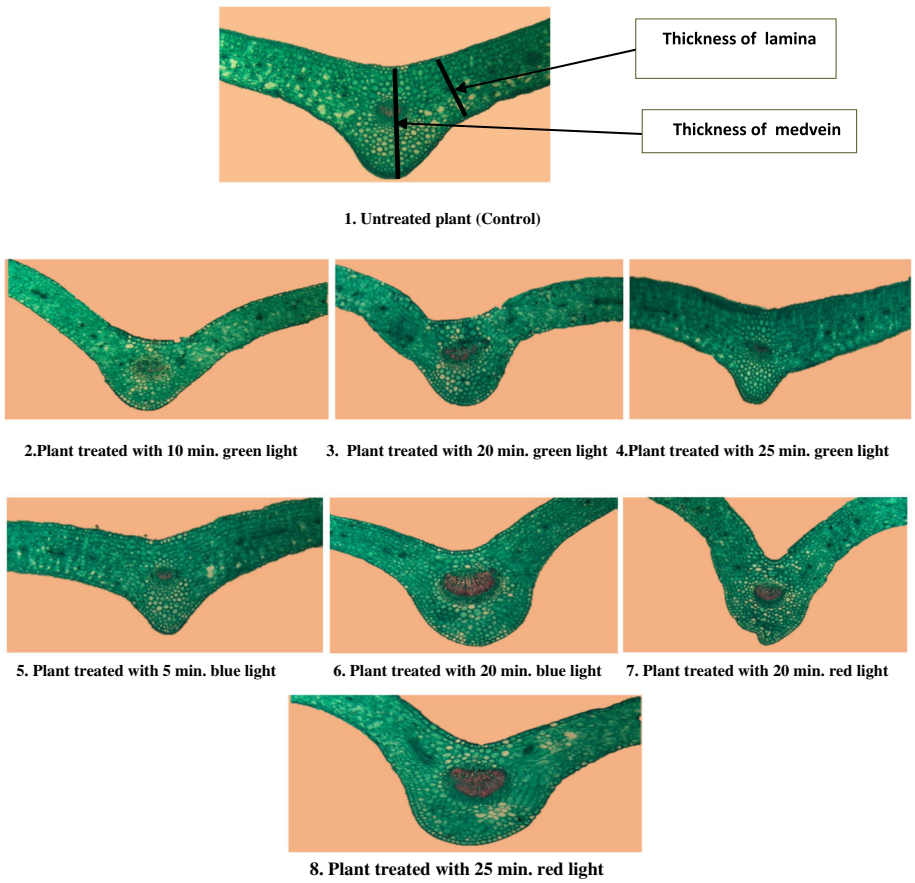

Fig. 6 Shows leaf anatomy of *Eustoma grandiflorum* under effect different types of laser radiation and time exposure. (1) Control, (2) 10 min green light, (3) 20 min green light, (4) 25 min green light, (5) 5 min blue light, (6) 20 min blue light, (7) 20 min red light, and (8) 25 min red light. Light microphotograph showing transverse sections through the blade of the third *in vitro* plant leaf developed on main stem of *Eustoma grandiflorum* plantlets (x10) (Bar = 0.05 ml). *TM* thickness of medvein, *TL* thickness of lamina

control. These results were in agreement with Lobna et al. (2014), Sahar et al. (2014), and Ali et al. (2014). The cell elongation resulted by laser treatments increased gibberellic acid which increased the cell vacuoles (Mahmoud and brahem 2000).

The highest number of leaves per shootlet appeared with red laser for 5 min as compared to control. These results were confirmed by researchers such as Danaila et al. (2011), Hwida et al. (2012) Lobna et al. (2014), and Rania et al. (2015). Using He-Ne laser beam stimulation related to higher activity of some enzymes in treated biological material Dobrowolski et al. (1987).

All laser radiation treatments had not significant effect on rooting percentage of *Eustoma grandiflorum* plant as compared to control. Some studies similar with our study like Hanna and Babelewski (2014)mentioned that the laser radiation did not affect percentage of rooted cutting. This was due to the first reason type and concentration of auxin.

The highest number of roots was obtained with blue laser for 5 min. However, the minimum of root/shootlet was observed with red laser for 25 min as compared to control. These results were confirmed by Hwida et al. (2012), Metwally et al. (2013) and Rimal et al. (2014).

Regarding the effect of laser radiation on length of roots as affected by different types of laser radiation and various times exposure, data showed that the longest roots were resulted from irradiation of shoots with green laser for 10 min as compared to control. Our study was confirmed by Lobna et al. (2014), Hwida et al. (2012), and Metwally et al. (2013).

In this investigation, results in acclimatization stage also showed that the best results for the number of branches per plant were obtained from the green laser for the long time exposure (25 min) and red laser for short time exposure (5 min). These results similar to Osman et al. (2009) and Aguilar et al. (2015) reported that laser radiation could cause enhancement of enzyme activity. Also may be the endogenous content of GA and role in cell elongation, where GA may cause cell elongation by induction of enzymes that weaken the cell wall (Macleod and Millar 1962).

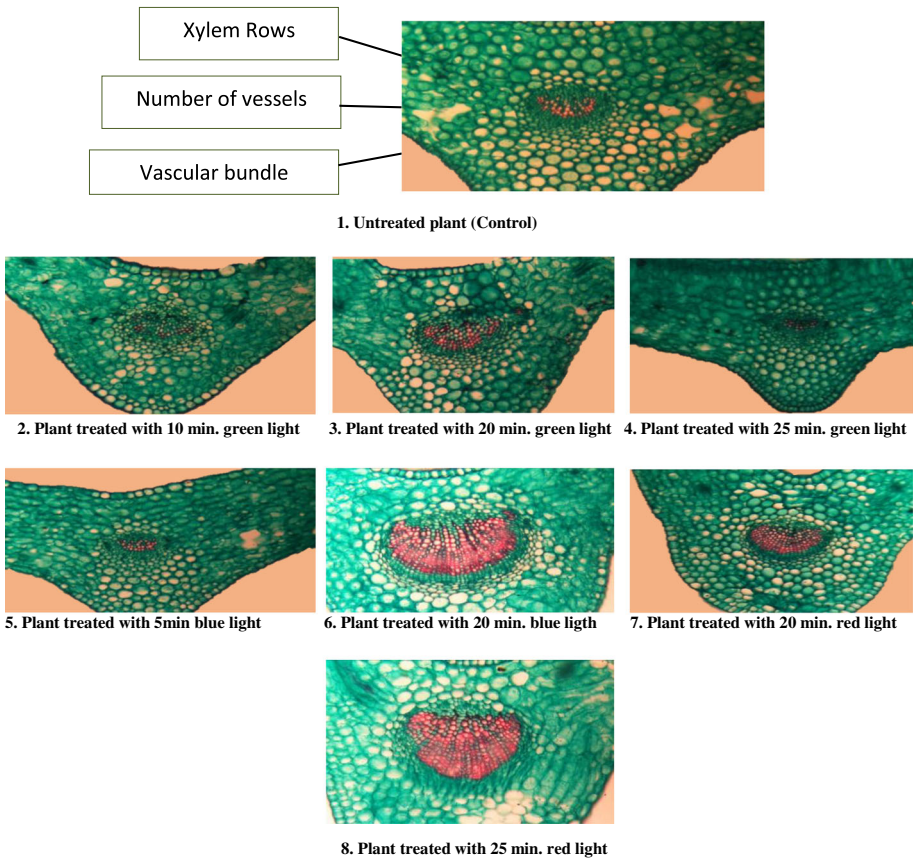

Fig. 7 Light microphotograph showing transverse section through the blade of the third in vitro plant leaf developed on the main stem of *Eustoma grandiflorum* plantlets. The section shows vascular bundle, (number of vessels and number of xylem rows. (× 40)(Bar = 0.05 ml). *VR* vascular bundle, *XR* xylem rows, *NV* number of vessels

The tallest branches were resulted from different types of laser radiation such as green, blue, and red for various times (10, 5, and 20 min, respectively) as compared to control. Our study agree with (Aladjadjiyan 2002) who mentioned that the He-Ne laser irradiation could raise the activities of superoxide dismutase (SOD) and Ascorbate peroxidase (Apx) enzymes in plants which resulted in accelerating the plant physiological metabolism and increased plant growth.

The highest number of leaves per branch was resulted with green laser for 25 min as compared to control. Osman et al. (2009) found that the best number of leaves and number of branches produced from application of laser treatments for 20 min for both seasons. Noha and El Ghandoor (2011) expressed the stimulated seedling length, average number of leaves for longer treatment time with laser application for 30–120 min. The highest leaf area was resulted from using green light of laser for 20 min compared to control and other treatments. These results were confirmed by some researchers such as Rybiński and Garczyński (2004), Al-sherbini et al. (2015), and El-Kereti et al. (2013) who revealed that the increase in leaf area may be reflected by the effect of rays on cell division which continues to all parts of plant at vegetative stage or may be the main biological active gibberellic acid formation is promoted by laser radiation.

It is evident from our result that red laser radiation for 5 min delayed bud flower initiation. The longest period was resulted from red light laser treatments for 5 min. Contrary, the shortest was obtained with blue light of laser for 20 min. These results were agreed with Podleoeny (2002) and Metwally et al. (2013).

Considering the flowering percentage of *Eustoma grandiflorum* plant, results showed that among different treatments, shootlets exposure to blue and red light laser radiation for 20 min gave the best results of flowering percentage and flower diameter compared to control and other treatments.

The highest number of flower buds per plant and the highest number of flowers per plant were resulted with blue laser radiation for 20 min. These finding were in agreement with El-Tobgy et al. (2009) and Osman et al. (2009).

The longest bloom stem length and fresh weight of flower were obtained with 20 min of blue light of laser radiation as while the shortest bloom stem length and fresh weight of flower was obtained with 5 min of red light of laser radiation as compared to control.

The longest peduncle length was obtained with 25 min red light laser radiation as compared to control while the shortest peduncle was observed with 5 min and 10 min of red light laser radiation. The peduncle length was increased gradually with increasing time exposure times.

The treated shootlets with 20 min of blue light laser radiation delayed the flower senescence to 12.67 day and recorded the highest petal area as compared to control these results online with Metwally et al. (2013) and Ritambhara and Girjesh (2013). While the maximum number of petals were observed with 20 min of green light and 25 min of red light laser radiation as compared to control.

The maximum number of stamens was recorded with 10 min of green laser. Mohammed (2005) found that irradiated *Salvia officinalis* plant with different doses and time exposure of He-Ne laser produced higher yield of herb compared to other types of laser. While the best results of dry weight also was observed with 25 min of each of blue and red light of laser rays comparing with control and other treatments. These results were agreed with Sahar et al. (2014).

Data presented indicated that shootlets were irradiated with short exposure time of green laser radiation resulted the best results for pigments content as compared to control and other treatments. The maximum values of chlorophyll (a, b), carotenoids, and total chlorophyll (a + b) were resulted from green light of laser for 5 min as compared with control. Our results were agreed with some researchers such as Lobna et al. (2014) Al-sherbini et al. (2015), Rania et al. (2015), and Dziwulska et al. (2016).

Our data indicated that anthocyanin content of *Eustoma grandiflorum* petals were significantly increased by using dose of laser radiation and reached up to maximum values with both doses (green for 20 min and red light laser for 25 min) as compared to control. Kurata et al. (2000) found that blue and red laser radiation was able to stimulate anthocyanin production.

The results presented indicated that the highest thickness of midvein, thickness of lamina, number of xylem rows, number of vessels, and diminution of vascular bundle (length – wide) were obtained with 20 min cadmium and 25 min helium neon laser as compared with control and other treatments. The results were mentioned by Hwida et al. (2012) and Bedour et al. (2012).

In general, the plant growth is associated with some factors such as enzymes and hormones like cytokinin and gibberellic acid (GA_3). The primary study observed that the red light of laser radiation have important role on GA_3 formation and the endogenous content of GA_1 according to Kamiya and Martinez (1999). The GA_3 response for cell elongation and other effects such as weaken the cell wall, production of proteolytic enzymes, increase of auxin content, increase of concentration of sugar, raising the osmotic pressure in cell soap. This elongation of cell which is treated with laser radiation led to increase of plant height, number of branches, and number of flower according to Lobna et al. (2014) and Rania et al. (2015), Using of laser radiation increased the nitrogen content that caused increase in protein content which is necessary for increasing plant organs such as branches and number of umbels (Osman et al. 2009). According to Mahmoud and Brahem (2000), they indicated that the cell number increased by laser radiation that increased nucleic acids and phospholipids membranes as well as increased phosphorus and potassium contents that led to cell elongation irradiated with laser radiation.

Conclusions

The current research concluded that the laser irradiations can affect plant morphology, flowering, chemical constituents, and gene mutagenesis. The highest survival % of acclimatized plants (95%) and highest values of number of branches, branches length (cm), were obtained from treated plantlets by 20 min green laser, while most of the highest floral parameters, anthocyanin pigment contents in flower, and anatomical structural parameters recorded increased by using 20 min blue laser and 20, 25 min of green and red laser respectively. Contrary, the lowest values of photosynthetic pigments and carotenoids were obtained from 20 min green exposure time.

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Authors' contributions

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Ethics approval and consent to participate

The manuscript does not contain studies involving human participants, human or animal data, and animal or human tissue.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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