Effect of nonionizing rays on growth, chemical constituents, and molecular aspects of *Catharanthus roseus* plant

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Background and objective
Catharanthus roseus (L.) is a perennial tropical plant and is considered one of the important ornamental and medicinal plants of the family Apocynaceae. It is used as an anticancer and antidiabetic agent. *Catharanthus* leaves are used for menorrhagia and rheumatism. The laser rays are used as a physical method to improve the germination, the plant growth, and the vigor of the seeds. The study aimed to evaluate vegetative growth, flowering, and marker gene on *Catharanthus* leaves and to investigate its chemical constituents under the effect of laser rays.
Materials and methods
The pot experimental study was carried out at greenhouse of National Research Centre, Giza, Egypt, on *C. roseus*. Two types of laser [helium cadmium (He-Cd) and argon (Ar) laser] were used at different exposure times (0, 4, 8, and 12 min), and various morphological, flowering, chemical constituents were determined. Inter-simple sequence repeat (ISSR) marker was used to illustrate the effect of He-Cd and Ar laser on *C. roseus* at different exposure time.
Results
Generally, the highest values of number of leaves, plant height, root length, fresh and dry weight of leaves, and number of days to flower, photosynthetic pigments, proline %, carbohydrate, vinblastine, and vincristine were obtained with 8 and 12 min of He-Cd laser treatments. Conversely, 4-min He-Cd and 8-min Ar laser time exposure recorded decrease in photosynthetic pigments, proline %, and carbohydrate concentration. ISSR showed that mean polymorphic percentage was 25%. ISSR investigation demonstrated that the control, He-Cd laser exposure for 8 min, and Ar laser exposure for 12 min of *Catharanthus* plant produced unique positive markers, which were found to be mutant specific.
Conclusion
It was concluded that laser rays with different time exposure had potential effect on growth, flowering, chemical constituents, and the production of *Catharanthus* plant.

Keywords:
Catharanthus roseus, ISSR, laser rays, proline, vinblastine, vincristine

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Introduction

Catharanthus roseus (L.) is a perennial tropical plant and is considered one of the important ornamental and medicinal plants; it grows throughout the world in different climate conditions. These plants belong to the family Apocynaceae, which contain a good source of antihypertension, enzymatic antioxidants, and nonenzymatic antioxidants.

The flower extract of *Catharanthus* is commonly administered as eyewash for infant's eyes, and *Catharanthus* leaves are used for menorrhagia and rheumatism [1,2]. Several studies revealed that the importance of *C. roseus* owing to its antidiabetic properties. It acts as antidiabetic through increase in glucose utilization [3].

The extracts of dried plant contain many alkaloids which can be used for medicinal purpose. Moreover, the *Catharanthus* plants are considered as a source of antitumor agents, vinblastine and vincristine, which are used in chemotherapy of leukemia disease [4].

Numerous studies have covered the improving knowledge on the antitumor alkaloids of *Catharanthus*. Vinblastine and vincristine are substances that act by inhibiting proliferation in cancer cells and are used against cancer [5,6]. There are more than 70 alkaloids that have been isolated from *Catharanthus* plant besides vinblastine and vincristine.

Laser rays belong to nonionizing radiation, which is identified by the emitted wavelength and the power.

Laser rays have been attained much attention at different parts of the world for improving growth, flowering, and plant production.

Laser rays interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology and biochemistry of plants depending on the irradiation doses. These effects include changes in the plant cellular structure and metabolism and alteration in photosynthesis [7–10].

In this concern, laser can regulate several characterisitcs of plant's function and may encourages some growth parameters [11–15].

The laser radiation is used as a physical method to improve the germination, the growth, and the vigor of seeds [16]. Biophysical methods can stimulate the seed and plants through improving the energy balance and hence activating the growth and yield processes [17,18]. It is now evident that physical methods such as laser radiation application enhance the energy account of metabolites by internal energy transformation [17].

The aim of this work was to throw more light on growth, flowering, and chemical constituents especially the antitumor alkaloids of *Catharenthus roseus* (vinblastine and vincristine) of *Catharanthus* plant by using types of laser rays [helium cadmium (He-Cd) and argon (Ar)].

Materials and methods

This study was carried out during the two growing seasons 2016 and 2017 at the green house of National Research Centre, Dokki, Egypt, and aimed to investigate the response of *Catharanthus* roses to laser treatments (He-Cd and Ar laser). This study does not involve experiments on human or animals (which needs approval of ethical committee). For cultivation, pots 25 cm in diameter and 25 cm in depth were filled with a loamy sandy soil (2:1) by volume. The experiment in both seasons consisted of seven treatments including the control. 'He-Cd' laser and 'Ar' laser were used for exposing *Catharanthus* plant seeds at the wavelengths of lasers rays 441 and 514 nm, respectively. The output powers for 'He-Cd' laser and Ar laser were adjusted nearly 16 and 8 Mw/cm^2, respectively. Seeds were treated with He-Cd laser and Ar laser, whereas the exposure time for each type was 4, 8, and 12 min. In May of both seasons, phosphorous fertilizer was added to the soil before planting; nitrogen and potassium fertilizers were added to the soil according to the recommended dose of the ministry of agriculture after 30 days from sowing. After 6 months from planting, representative plant sample was taken from three replicates randomly. The growth and flower parameters included plant height (cm), leaves number/plant, root length (cm), fresh and dry weight of leaves, and number of days to flowering.

The samples of leaves in both seasons were dried at 70°, whereas another sample of fresh leaves were sampled, and the following chemical constituents were determined:

(1) Photosynthetic pigments (mg/g FW).
(2) Chlorophyll a, b and carotenoids were determined according to the methods described by Saric *et al.* [19].
(3) Carbohydrate mg/g FW was determined according to Smith *et al.* [20].
(4) Determination of vinblastine and vincristine content as described by Nagaraja and colleagues [21–24]. The absorbance of the resulting yellow-colored azo product of vinblastine and vincristine was recorded at 430 and 440 nm, respectively. The blank sample was run simultaneously with each set of determination.
(5) Proline content was determined using fresh leaves according to Bates *et al.* [25].

The experimental layout was a randomized complete block design. Each treatment included three replicates. The recorded data (the average of two seasons) were subjected to one-way analysis of variance according to Snedecor and Cochran [26]. The values of least significant difference and the means were compared using least significant difference test at 5% levels of probability.

Genomic DNA extraction and PCR condition

Total genomic DNA of all samples (control and mutants) was extracted from green leaves using Qiagen DNeasy Plant Minikit following the protocol of the manufacturer (Qiagen Inc., Valencia, California, USA). The quality of the extracted DNA was assessed on agarose gel electrophoresis (Fig. 1). PCR was performed using four preselected Inter-simple sequence repeat (ISSR) primers based on their ability to generate reproducible and informative amplification patterns, and amplification reactions were carried out in Biometra TOne Thermal Cycler (Analytik Jena, Jena, Germany). PCR amplification was performed in 25 µl reaction mix

which contained 20–30 ng DNA template, 10 pmole of each primer, 2.5 µl of 2 mmol/l thermo dNTPs, 5 µl of 5× Promega Green GoTaq Flexi Reaction Buffer, 2.5 µl of 25 mmol/l Promega $MgCl_2$, and 0.125 µl of 5 U/µl Promega GoTaq Flexi DNA polymerase. The reaction was assembled on ice, and amplification was performed at certain conditions as follows: an initial denaturing step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, annealing at 50°C for 1 min, an extension at 72°C for 1 min, and final extension at 72°C for 7 mins. The PCR products were assessed on 1.6% agarose gel. The banding profile of ISSR was scored using Labimage program (Kapelan Bio-Imaging solution, Leipzig city, Federal State of Saxony, Germany). The polymorphism was estimated as follows:

$$\text{Percent of polymorphism} = \left(\frac{\text{Number of polymorphic bands}}{\text{Total number of bands}}\right) \times 100.$$

Results

The data presented in Table 1 indicates that laser rays had promotive effect on all of growth parameters and chemical constituents of *C. roseus*.

Growth parameters

Generally, we can observe from the data presented in Table 1 that there was significant increase in plant height with most of laser treatments. The highest values were obtained from treated plant with He-Cd laser at 12 and 8 min exposure. The increment reaches to 45.63 and 42.67%, respectively, over the control. On the contrary, the lowest values were recorded with treated plants with Ar at 12 min exposure, which recorded decrease compared with the control and other treatments. The highest values for leaves number were obtained from the treated plants with He-Cd laser rays at 8 min, followed by 4 min exposure time, which were 54.02 and 44.05% overpass the control, respectively.

Root length increased significantly owing to the treatments, and the highest effective treatments were He-Cd laser at 12 min and 8 min exposure time for Ar laser rays, increasing the values to 118.76 and 137.52% over the control, respectively. Moreover, it could be observed from the data presented in Table 1 that He-Cd (12 min exposure) and Ar (4 min exposure) significantly increased and gave the highest values for fresh and dry weight of leaves in comparison with the control and other treatments. The values for He-Cd (12 min exposure) and Ar (4 min exposure) reached to 27.40 and 28.57%, respectively, for fresh weight and 38.98 and 32.28%, respectively, for dry weight of leaves over the control. On the contrary, the plants received Ar laser rays at 8 min exposure time and He-Cd rays at 4 min exposure and resulted in slight decreases in fresh (13.50 and 14.28, respectively) and dry (5.54 and 5.61%, respectively) weight of leaves than the control.

Number of days to flower (day)

The data in Table 1 showed marked differences between the treatments and control in number of days to flowering, that is, decreasing the days to flowering means accelerating the flowering. Treating the plants with both types of lase rays mostly decreased the days to flowering (early flowering) as compared with the control. The most effective treatments were He-Cd laser at 4 and 8 min exposure time which resulted in 17 and 12 days earlier than the control. However, Ar laser at 12 min recorded 2 days earlier than control plants and it considered the lowest treatment recorded increased in number of days to flowering compared with the control and all other treatments of two laser types.

Table 1 Effect of exposure time to helium cadmium and argon laser on the growth and flowering of *Catharanthus roseus* plants (average of two seasons 2016 and 2017)

Treatments	Characteristics					
	Plant height	Leaves number	Root length (cm)	Fresh weight of leaves (g)	Dry weight of leaves (g)	Number of days to flower
Control	22.66	16.66	5.33	18.21	1.96	42.33
He-Cd 4 min	27.66	24.00	10.83	17.20	1.85	25.66
He-Cd 8 min	32.33	25.66	9.00	17.75	1.93	30.00
He-Cd 12 min	33.00	22.23	11.66	23.20	2.52	33.33
Ar 4 min	24.66	23.33	9.33	25.31	2.73	36.00
Ar 8 min	29.33	22.33	12.66	15.75	1.68	38.00
Ar 12 min	22.50	19.00	8.00	20.94	2.25	40.00
LSD at 0.5%	1.52	2.23	2.20	0.185	0.057	0.708

Ar, argon; He-Cd, helium cadmium; LSD, least significant difference.

Table 2 Effect of exposure time to helium cadmium and argon laser on photosynthetic pigments and carbohydrates percentage of *Catharanthus roseus* plants (average of two seasons 2016 and 2017)

Treatments	Characteristics				
	Chlorophyll a (mg/g.f.w.)	Chlorophyll b (mg/g.f.w.)	Total (a+b)	Carotenoids (mg/g.f.w.)	Carbohydrates (mg/g.f.w.)
Control	0.459	0.350	0.815	2.357	9
He-Cd 4 min	0.418	0.319	0.737	2.806	10.33
He-Cd 8 min	0.388	0.280	0.669	1.723	2.60
He-Cd 12 min	0.581	0.419	1.000	3.055	4.33
Ar 4 min	0.470	0.358	0.828	2.330	3.53
Ar 8 min	0.516	0.418	0.934	3.340	10.50
Ar 12 min	0.491	0.371	0.862	3.045	4.50
LSD at 0.5%	0.009	0.015	0.013	0.037	0.404

Ar, argon; He-Cd, helium cadmium; LSD, least significant difference.

Photosynthetic pigments

The data in Table 2 showed that, treating (*C. roseus*) plants with laser rays resulted in the higher chlorophyll a, b, total chlorophyll and carotenoids concentrations as compared with the control. The third dose of He-Cd laser (12 min exposure) and the doses of Ar (4, 8, and 12 min exposure) significantly increased the previously mentioned parameters, giving values of 26.57, 19.71, 22.69, and 29.61%, respectively; 2.39, 2.28, 1.59, and 12.41%, respectively; 19.42, 14.60, and 41.70%, respectively; and 6.97, 6, 5.76, and 29.18%, respectively, over the control. On the contrary, the treatment of He-Cd laser at 4 and 8 min exposure exhibited decreases in chlorophyll a, b, total chlorophyll and carotenoids contents giving a decrement of 15.46, 20, 17.91, and 26.89% below the control, respectively.

The data presented in Table 2 explained that laser ray treatments in most cases decreased carbohydrate %. He-Cd and Ar irradiation increased the percentage of soluble carbohydrates as a result of the lower dose of He-Cd with 4 min exposure and the medium dose of Ar laser at 8 min exposure as compared with control and all other treatments, respectively. The effectuated increments reached 14.77 and 16.66% over the control, respectively.

Lower level of soluble carbohydrate was recorded with He-Cd laser at 8 min and Ar laser at 4 min exposure. The decrements reached to 71.11 and 60.77% lower than untreated plant.

Proline %

The results in Table 3 show that, generally, raising laser dose increased in proline concentrations in the cells of *Catharanthus* plants. When *Catharanthus* seeds were subjected to Ar at 12 and 8 min and He-Cd at 12 min exposure, they showed the highest increase in total proline (mg/g) as compared with the untreated plant. These treatments increased the proline percentage to 232.81, 124.06, and 1.35 % over the control. On the contrary, the low dose of Ar laser at 4 min exposure decreased the values of proline content by 2.25% as compared with the control and other laser treatments.

Table 3 Effect of exposure time to helium cadmium and argon laser on proline, vinblastine and vincristine contents of *Catharanthus roseus* plants (average of two seasons 2016 and 2017)

Treatments	Characteristics		
	Proline	Vinblastine	Vincristine
Control	28.80	0.023	0.161
He-Cd 4 min	29.00	0.201	0.183
He-Cd 8 min	28.40	0.414	0-391
He-Cd 12 min	29.19	0.383	0.365
Ar 4 min	28.15	0.366	0.345
Ar 8 min	64.53	0.315	0.293
Ar 12 min	95.85	0.187	0.173
LSD at 0.5%	0.266	0.002	0.0014

Ar, argon; He-Cd, helium cadmium; LSD, least significant difference.

Alkaloids components

It can be observed from the data presented in Table 3 that, in general, treatments of two types of laser rays significantly increased the vinblastine and vincristine contents in *C. roseus* plant cell, especially with the dose of He-Cd at 8 min and 12 min exposure time. These treatments superinduced the contents, with significant increases of 1700 and 142.85, respectively, and 1565.21 and 126.70%, respectively, over the control plant. However, the higher dose of Ar laser irradiation resulted in significant slight increase in vincristine content (7.45%) as compared with the control plant and other treatments.

ISSR analysis

Four ISSR primers were used to indicate the genetic variability of the six mutants of *C. roseus* plant caused by exposure to He-Cd and Ar laser irradiation at different

exposure times as shown in Table 1. ISSR primers detected high polymorphism percentages in the amplified DNA pattern. A total number of 73 scorable amplified DNA fragments ranging from 2521 to 79bp were observed using four ISSR primers, whereas 73 fragments were polymorphic. The four primers showed mean polymorphic percentage of 25%. The highest number of polymorphic bands (25) was obtained with primer UBC-826. The lowest number of polymorphic bands (9) was recorded with the primer UBC-829 (Table 4). The data in Table 5 showed unique bands based on ISSR of *Catharanthus* plant at different exposure times to He-Cd and Ar laser irradiation. It could be observed that the control plant had unique positive marker (79 bp) with primer UBC-826 and this band was present in the control only but absent in all mutants. On the contrary, it could be observed that plant exposed to He-Cd laser for 8 min had one unique positive marker (658bp) with primer UBC-808 which is mutant specific (Table 5). Moreover, the bands of 263, 149, 263bp which amplified by primers UBC-808, UBC-826, and UBC-827, respectively, were specific only to *Catharanthus* plant exposed to Ar laser for 12 min.

Finally, with *Catharanthus* plant exposed to He-Cd and Ar laser for 4 and 12 min, no primers produced unique markers (Fig. 2).

Discussion

The presented study aimed to study the effect of laser irradiation on growth, flowering, and some chemical constituents, especially vinblastine and vincristine of *C. roseus* plant. The assessment of the response of *Catharanthus* plants to these treatments was undertaken by measuring growth parameters and some chemical constituents of the *Catharanthus* plant.

According to the data presented in our investigation, laser rays led to increase in the plant height, number of leaves, root length, and both of fresh and dry weights of treated plants in comparison with the untreated plants.

Laser radiation at different wavelengths and exposure times of He-Cd and Ar showed different effects on *Catharanthus* plants. The statistical analysis revealed that plant height and other growth parameters were increased by laser treatments by reflecting the effect of that laser on cell division, and the endogenous content of GA, the main biological active GA formation, is promoted by laser rays treatment [27,28].

The data also revealed that the increase in plant height was followed by increase in the number of leaves per plant which induced high fresh and dry weights. This may be owing to the role of GA in cell elongation by

Table 4 ISSR amplification products of DNA extracted from *Catharanthus* plant exposed to helium cadmium and argon laser after different times

Primer	5′-sequence-3′	Size range of the scorable bands (bp)	Amplicons number	Polymorphic amplicons	Polymorphism (%)
UBC-808	AGAGAGAGAGAGAGAGC	2521–210	15	15	100
UBC-826	ACACACACACACACACC	2000–79	25	25	100
UBC-827	ACACACACACACACACG	1283–208	24	24	100
UBC-829	TGTGTGTGTGTGTGTGC	1269–276	9	9	100
Total			73	73	100
Average			18.25	18.25	25

ISSR, Inter-simple sequence repeat.

Table 5 Unique bands based on ISSR from *Catharanthus* plant exposed to helium cadmium and argon laser irradiation after different times, using four primers

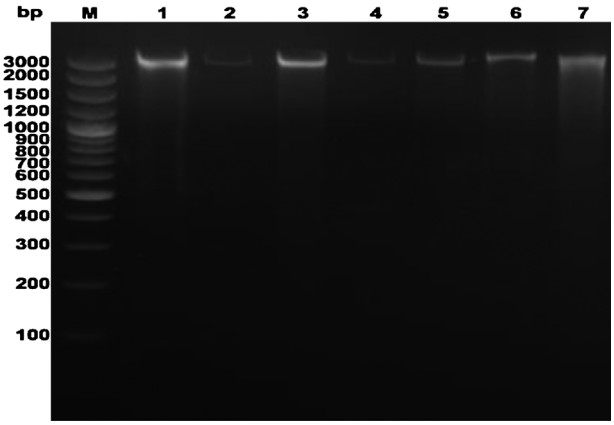
Laser beam	Primer name	UPM (bp)	UNM (bp)	Total unique marker	
				Each primer	All primers
Control	UBC-826	79	–	1	1
He-Cd 4 min		–	–	–	–
He-Cd 8 min	UBC-808	658	–	1	1
He-Cd 12 min		–	–	–	–
Ar 4 min		–	–	–	–
Ar 8 min		–	–	–	–
	UBC-808	263	–	1	
Ar 12 min	UBC-826	149	–	1	3
	UBC-827	263	–	1	

Ar, argon; He-Cd, helium cadmium; ISSR, Inter-simple sequence repeat.

induction of enzymes that weaken the cell wall [28,29].

Chlorophyll a and b content of *C. roseus* plants were increased with the application of He-Cd and Ar laser treatments. However, Ar laser was more effective than He-Cd laser in increasing carotenoids concentrations in the plant tissues, and He-Cd laser was nearly more effective on total chlorophyll (a+b). These photosynthetic pigments have a role for increasing photosynthesis processes and metabolic products in the plants such as carbohydrates and other constituents (i.e. vinblastine and vincristine). The laser rays promote GA formation which caused increase in sugar concentration, which reflects on photosynthetic pigments contents. These results support the finding of Lobna and colleagues [12,27,28,30,31].

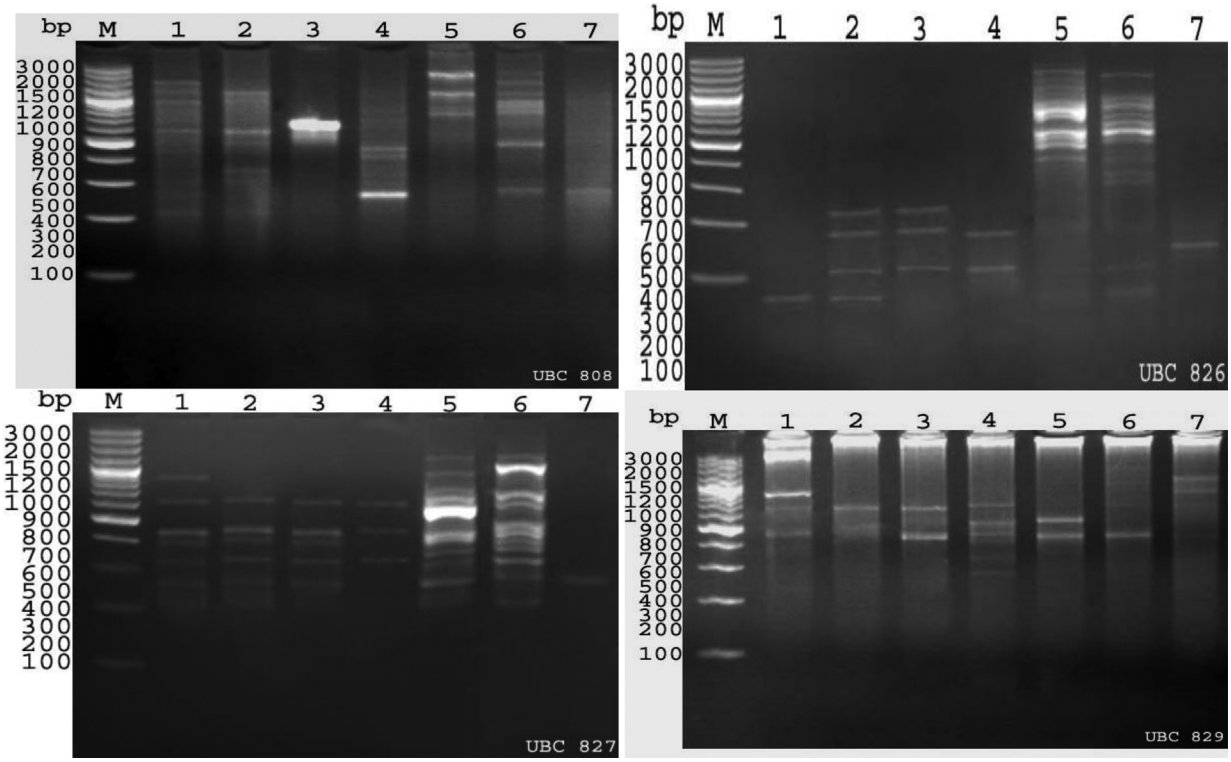
Figure 1



Agarose gel electrophoresis of total genomic DNA of Vinca plants. Original plant (1) and the induced mutants (2–7). M: 100 bp DNA ladder.

The application of the high laser ray doses treatments led to increase in the proline content in *Catharanthus* leaves. The observation hold true with Sami [28], who obtained increase in the protein content in gerbera plants covering the increase of plant organs (branches, leaves, and root length). The laser treatments increased growth regulators and protein, which may explain the increase of proline content under the higher doses of laser rays. In this respect, Sacher [32] reported that auxins induced net synthesis of RNA and protein; however, proline content had been affected by laser rays and similar results were reported by Seyed and colleagues [33–35].The application of laser treatments He-Cd and Ar enhanced the flowering, and He-Cd laser was more effective than Ar laser on fastening the flower bud initiation. These results agreed with Podleoeny [36] on *Vicia faba* minor; he obtained taller plants from the treated seeds, starting to flower

Figure 2



Agarose gel electrophoresis of ISSR profiles of *Catharanthus* plant. Original plant (1) and the induced mutants (2–7). M: 100 bp DNA ladder.

approximately 3–4 days earlier than the control. Moreover, the flowering enhancement by laser rays on *Eustoma grandiflorum* plants was reported by Awatef [31]. On the contrary, Sami [28] recorded that Ar laser delayed flowering onset of gerbera plants, whereas Metwally *et al.* [37] mentioned that treating the plants with helium neon laser (He-Ne) significantly increased the days to flowering as compared with the control plants.

High polymorphism percentages in the amplified DNA pattern using ISSR primers had been recorded. According to El-Banna *et al.* [38], the percentage of polymorphism detected by application of ISSR primers on *Nigella sativa* irradiated with gamma and laser radiation reached 68.9%, and 12 of 45 ISSR markers were found to be mutant specific.

Conclusion

Laser rays at different doses can be beneficial for inducing growth and production of *C. roseus* plant. He-Cd laser (8 and 12 min) exposure time showed the best results in vegetative growth and chemical constituent production, and it produced unique positive markers which were found to be mutant specific. Treating the plants with both types of laser enhanced early flowering as compared with the control.

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Conflicts of interest

There are no conflicts of interest.

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