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Determination of growth, physio-biochemical activity and alkaloid yield in *Catharanthusroseus*, as influenced by gamma irradiated carrageenan

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ABSTRACT

Use of natural bioactive agents obtained from radiation processed polysaccharides as growth promoting substances is an emerging technology to exploit full genetic potential of crops in terms of growth, yield, and quality of plants. A pot experiment was carried out to investigate the effects of foliar application of depolymerised form of gamma irradiated (520 kGy) irradiated carrageenan (IC) on growth (shoot and root lengths, leaf area, fresh and dry weights of plants), physio-biochemical attributes (content of chlorophyll and carotenoid, nitrate reductase and carbonic anhydrase activities and leaf -N, -P and –K content) and the alkaloid production in *Catharanthus roseus* (L) G. Don, a medicinal plant. Aqueous solutions of different concentrations (0, 10, 50 and 100 ppm) of IC were sprayed seven times on foliage, using unirradiated carrageenan (UC) and deionized water as control. Application of 50 ppm IC significantly increased the growth and the physio-biochemical characteristics. The IC (50 ppm) treatment also increased the total alkaloid content in leaves and roots by 36.8% and 38.8%, respectively. This technique of foliar spray of IC may be employed to improve the performance of plant including the alkaloid production which is of great medicinal values.

Key words: *Catharanthus roseus*, anticancer alkaloids, irradiated carrageenan, growth, yield.

INTRODUCTION

Madagascar periwinkle(*Catharanthus roseus* (L) G. Don) belonging to the family Apocynaceae is a well-known perennial medicinal herb. More than 130 alkaloids have been isolated from different parts of this plant [1].Vinblastine and vincristine are the important alkaloids extracted from this plant which inhibit the growth of certain cancer-forming cells [2]. The antineoplastic alkaloids (vincristine and vinblastine) are mainly present in leaves and antihypertensive alkaloids (ajmalicine, serpentine and reserpine) are found in roots.

It is now well documented that oligomers obtained from radiolytically degraded polysaccharides have valid applications in the field of agriculture, as plant growth promoter [3] and they have ability to trigger the plant defense responses [4]. The needs of increasing the production of plant derived alkaloids are cost effective and this method becomes more important for the increase in the alkaloid production. Keeping the medicinal value of *C. roseus* in mind, the increase total alkaloid content and its productivity is most desirable. The present study was therefore carried out to find out whether the foliar application of gamma-rays degraded carrageenan (carrageenan oligomers) could increase the growth and physio-biochemical characteristics and also the changes in its alkaloid yield in different parts of the plant. To our knowledge, this is the first ever report where, a detailed effect of gamma IC has been carried out at morphological, physiological, biochemical and alkaloid level in *C. roseus*.

MATERIALS AND METHODS

Experimental material

The authentic and healthy seeds of *Catharanthus roseus*were procured from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. The experiment was conducted in simple randomized block design in earthen pots (25 × 25 cm) in the natural environmental conditions inside the net house of the Department of Botany, Aligarh Muslim University, Aligarh (27° 53′N latitude, 78° 51′E longitude, and 187.45 m altitude).

Carrageenan Treatment

Different concentrations of gamma IC solution [0 ppm (control), 10 ppm, 50 ppm, 100 ppm together with the UC] were employed. Each treatment was replicated four times, and each replicate pot carried one plant. Growth and biochemical attributes were determined at 90 days after planting. Solid material of k-carrageenan (Sigma Aldrich, USA) was sealed in a glass tube with atmospheric air.

Gamma Rays Exposure

The samples of carrageenan were irradiated in Co-60 Gamma Chamber, GC-5000 supplied by BRIT, Mumbai, India, at a dose rate of 2.4 kGy/h. The samples were irradiated at 250 kGy. Different aqueous concentrations of IC were finally prepared as spray treatments using double distilled water.

Effect of Gamma rays on Morphological behaviour

The effect of gamma rays was evaluated at morphological level in terms of length of shoot and root of the plant in centimetre. The clean and blot-dried plants were used to record shoot length and then they were oven-dried at 80^0C for 48 h to determine the plant dry weight.

Chlorophyll Estimation

Total chlorophyll and carotenoids contents in fresh leaves were estimated using the method of [5]. The fresh tissue from interveinal leaf area was grinded using mortar-pestle containing 80% acetone. The optical density (OD) of the pigment-extract solution was recorded at 662 and 645 nm (for the contents of chlorophyll a and b, respectively) and at 470 nm (for carotenoids content) using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan). The photosynthetic pigments were expressed as mg g^{-1} FW.

Nitrate reductase activity

The nitrate reductase (NR) activity was estimated by the intact tissue method given by [6]. The method is based on the reduction of nitrate to nitrite as per the following biochemical reaction

$$NO_3 + NADH + H^+ \xrightarrow{NR} NO_2 + NAD^+ + H_2O$$

The nitrite formed was determined spectrophotometerically. 200 mg of fresh chopped leaves were transferred to each of the plastic vials containing the reaction mixture [2.5 mL of phosphate buffer (pH 7.5), 0.5 mL of 0.2 M potassium nitrate solution and 2.5 mL of 5% isopropanol], and then incubated for 2 h in dark at 30^oC. To 0.4 mL of the incubated mixture, 0.3 mL each of 1% sulphanilamide and 0.02% N-(1-naphthyl) ethylenediaminedihydrochloride (NED-HCl) was added. The test tubes were kept for 20 min at room temperature for maximum colour development. The OD of colored solution was recorded at 540 nm using the spectrophotometer. The NR activity was expressed as nM NO_2^- g^{-1} FW h^{-1}.

Carbonic anhydrase activity

The activity of carbonic anhydrase (CA) was determined in the fresh leaves using the method of [7]. 200 mg of fresh leaf tissue was transferred to petri plates, followed by incubation of the leaf tissue in 10 mL of 0.2 M cystein hydrochloride solution for 20 min at 4˚C. Thereafter, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.02% bromothymol blue was added to the homogenate. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. CA activity was expressed as µM CO_2 kg^{-1} leaf FWs^{-1}.

Estimation of leaf nutrient contents

Leaf samples, taken from each treatment at 90 days after sowing (90 DAS), were digested for the estimation of leaf-N, -P and -K contents. The leaves were dried in a hot air oven at 80ºC for 24 h. The dried leaves were grinded using mortar-pestle, followed by passing the content through a 72 mesh to get a fine leaf-powder. 100 mg of the leaf-powder was carefully transferred to a digestion tube. To it, 2 mL of AR (analytical reagent) grade concentrated sulphuric acid was added. The mixture was heated on a temperature-controlled Kjeldahl assembly at 80ºC for about 2 h and then cooled for about 15 min at room temperature. To it, 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added drop by drop, followed by heating the content gently. This step was repeated until the content of the digestion

tube turned colorless. The peroxide-digested leaf-material (aliquot), thus prepared, was used to estimate per cent content of N, P and K in the leaves on dry weight basis.

Estimation of Leaf Nitrogen content
Leaf-N content was estimated in the aliquot according to the method of [8] with a slight modification made by [9]. A 10 mL of the aliquot was poured into a 50 mL volumetric flask, followed by additions of 2 mL of 2.5 N sodium hydroxide and 1 mL of 10% sodium silicate solutions in order to neutralize the excessive acid and prevent turbidity, respectively. A 5 mL aliquot of this solution was poured into a 10 mL graduated test tube and then a 0.5 mL of Nessler's reagent was added. Regarding estimation of leaf-N content, the optical density of the solution was recorded at 525 nm, using the spectrophotometer.

Estimation of Leaf phosphorus content
The method of [10], with a slight modification, introduced by [11], was used to estimate the leaf-P content in the aliquot. A 5 mL of the aliquot was poured into a 10 mL graduated test tube, followed by additions of 1 mL of molybdic acid (2.5%) and 0.4 mL of 1-amino-2-naphthol-4-sulphonic acid. The content was kept at room temperature for color development, followed by making the volume up to 10 mL with double distilled water. The OD of the solution was recorded at 620 nm for leaf-P estimation, using the spectrophotometer.

Estimation of Leaf Potassium content
Potassium content in the aliquot was determined according to [12] with the help of a flame-photometer (C150, AIMIL, India), using a specific filter for K emission spectrum. The test solution (aliquot) was discharged through an atomizer in the form of a fine mist into a chamber, where it was drawn into the flame. Combustion of the element (potassium) produced light of a particular wavelength [λ max for K = 767 nm (violet)]. The light produced was passed through the appropriate filter to impinge upon a photoelectric cell that activated a galvanometer leading to a digital display of the potassium content in the leaf as per the emission spectrum constructed.

Total alkaloid contents
Total alkaloid contents were estimated as described by [13] in the dry leaves/ roots powder. 500 mg of the fine powder of leaves/roots was taken in a 100 ml round bottom flask , to this known volume of ethyl alcohol was added and the mixture was refluxed for 6 hours .The mixture was then filtered and a 50 ml of diluted HCL was added to the filtrate. The mixture was transferred to a separating funnel to which 50 ml of diethyl ether was added. The mixture was again transferred into a separating funnel with 50 ml of diethyl ether layer was decanted. To the decant, anhydrous solution carbonate was added. The mixture was again decanted in a reweighed dry porcelain dish and evaporated till dryness. The weight of this porcelain dish was then taken again.

Data Analysis
The data were analyzed statistically according to randomized block design using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values of the results were statistically compared using Duncan's Multiple Range Test (DMRT) at $P<0.05$.

RESULTS

Growth characteristics
Foliar application of various concentration of IC proved better than control in terms of growth attributes (Table 1). Application of 50 ppm of IC proved better than other IC concentrations. It significantly increased the shoot length (19.71%), root length (26.5%), leaf area (79.23%), fresh (28.33 %) and dry (23.80 %) weights of the plant respectively when compared to the water sprayed control plants.

Photosynthetic pigments
Of the tested concentrations (0, 10, 50 or 100 ppm) of IC, 50 ppm showed the maximum increase in the photosynthetic pigments. As compared to the control and Un 10, foliar spray of IC at 50 ppm significantly ($P \leq 0.05$) enhanced the total chlorophyll (13.3 %, 12.0 %) and carotenoid (10.70 %, 8.69 %) contents, thereafter it declined respectively. Similarly, the application ofUC, proved better over control but was less effective than IC at 50 ppm (Table 2).

NR activity
Foliar spray of IC applied at different concentrations (0, 10, 50 or 100 ppm) showed a significant ($P \leq 0.05$) difference in NR activity. A linear increase in NR activity was noticed from control to 50 ppm (IC). However, foliar application of 50 ppm of IC enhanced the NR (24.23 %, 16.38 %) enzyme activity as compared to control and UC

(Table 2).In addition to this, UC proved best over the water sprayed control, but less effective when compared with IC 50 ppm respectively.

CA activity
Like the NR activity, The activity of CA was also positively affected by the IC application. Of the tested concentrations of carrageenan maximum CA activity (22.02%) was reported from IC 50 ppm treated plant, thereafter, it showed decline at IC 100 ppm respectively when compared to water sprayed control plants. A significant ($P \leq 0.05$) variation in CA activity against different treatments has been shown in table 2.

Leaf NPK content
Table 2 showed the variation in NPK content quantified from treated and untreated plants at 90 DAP respectively. A linear progressive increase in NPK values with the increase of IC concentration was noticed up to 50 ppm as compared to control and Un 10, thereafter, it started to decline significantly ($P \leq 0.05$).Therefore, amongst the different concentration of IC, 50 ppm proved to be highly effective for the enhancement of N, P and K content by 13.17 %, 10.87 % and 14.79 % respectively when compared to water sprayed control plants (Table 2).

Alkaloid content
Foliar application of IC also altered the total alkaloid content and its yield when compared with control and untreated carrageenan in leaf and roots of *Catharanthus* plants at 90 DAP respectively. A linear increase in alkaloid content in both leaf and roots was noticed with increasing IC concentration, but this enhancement was only reported up to 50ppm IC, thereafter, it declined. Fig. 1 showing a comparative account of total alkaloid yield in leaf and roots grown under different concentrations. Maximum total alkaloid content was isolated form root respectively (Fig. 1). In terms of percent, the 50 ppm IC increased the alkaloid content by 36.8 % in leaves and 38.8 % in roots when compared to the water sprayed control plants.

DISCUSSION

From the present study it is observed that the foliar spray of IC exhibited significant effect to improve the growth attributes as compared to the water sprayed plants. Application of 50 ppm of IC significantly ameliorated shoot length, root length, fresh and dry weight of the plant. The plant growth enhancement might be due to IC induced changes in the processes like cell division, cell differentiation and morphogenesis [14,15]. These results are in agreement with those who has reported the foliar application of IC increased all growth attributes in fennel [16]. The growth-promotingeffects of degraded natural polysaccharides on growth and yield characteristics of various crops have been reported by several other workers [17, 18, 19, 20, 21]. Moreover, the plant growth promotion effect of polysaccharides including alginates, carrageenan and chitosan in their depolymerised form has also been proved recently by various authors [22, 23, 16].

Application of deploymerised form of carrageenan (IC) at 50 ppmincreased total chlorophyll and carotenoids contents at 90 DAP as compared to the control.Increment in the chlorophyll content due to application of IC might be ascribed to a favorable effect of IC application on photosynthesis as well as on the overall growth of the plant**.** In fact, various workers have reported positive effect of irradiated sodium alginate (ISA) regarding photosynthetic pigments and rate of net photosynthesis [23,24,21,16].

The CA enzyme is one of the most abundant zinc containing protein in plants. It has an active role in photosynthesis, which is evident by its presence in all photosynthesizing tissues. It catalyzes the reversible hydration of CO_2 to carbonic acid, thereby increasing the availability of CO_2 to Rubisco in photosynthesis [25]. The application of IC at 50 ppm proved optimum for the CA activity. Such a plant response to IC application is expected because the deploymerised natural polysaccharides have been reported to increase the stomatal conductance significantly [16], which might facilitate the diffusion of additional amounts of CO_2 through the stomata to be acted upon by CA, resulting in the enhanced CA activity. Further, a probable reason for the enhancement of CA activity could be the IC-mediated *de novo* synthesis of CA, which might involve transcription/translation of the genes associated as has been reported for other degraded natural polysaccharides [26]. Expectedly, the enhancement of CA activity in the IC-treated plants might be responsible for the enhanced rate of CO_2 fixation (not measured in this study) that could have resulted in significant increase in fresh and dry weights of the plants.

Leaf –N, -P and –K contents were also significantly enhanced by the application of IC 50 ppm. In conformity with these results, a significant increase in the uptake of these elements (N and P) at an IC concentration of 50ppm in *Foeniculum Vulgare* Mill has been investigated [16] earlier. Such an impact, if IC application could be ascribed to the IC-mediated increase in overall growth of plants, which accordingly demanded for higher uptake of these nutrients from the soil, leading to their significant accumulation in the leaves. Moreover, IC, applied at 50 ppm,

increased the NR activity maximally. N and P mediated increase in the uptake of various nutrients and the resultant increase in the NR activity has earlier been established under normal conditions [27]. Thus, one of the reasons of IC-enhanced NR activity in this study might be the IC-enhanced leaf-N,-P and –K contents. Thepositiveeffectof IC applicationonNRactivityhasalsobeenreportedpreviouslyin case of *Foeniculm Vulgare* Mill [16]. Additionally, the increase in NR activity of plant with increasing carrageenan concentrations in the present investigation is in agreement with the findings reported by in *Artemisia annua* L [23].

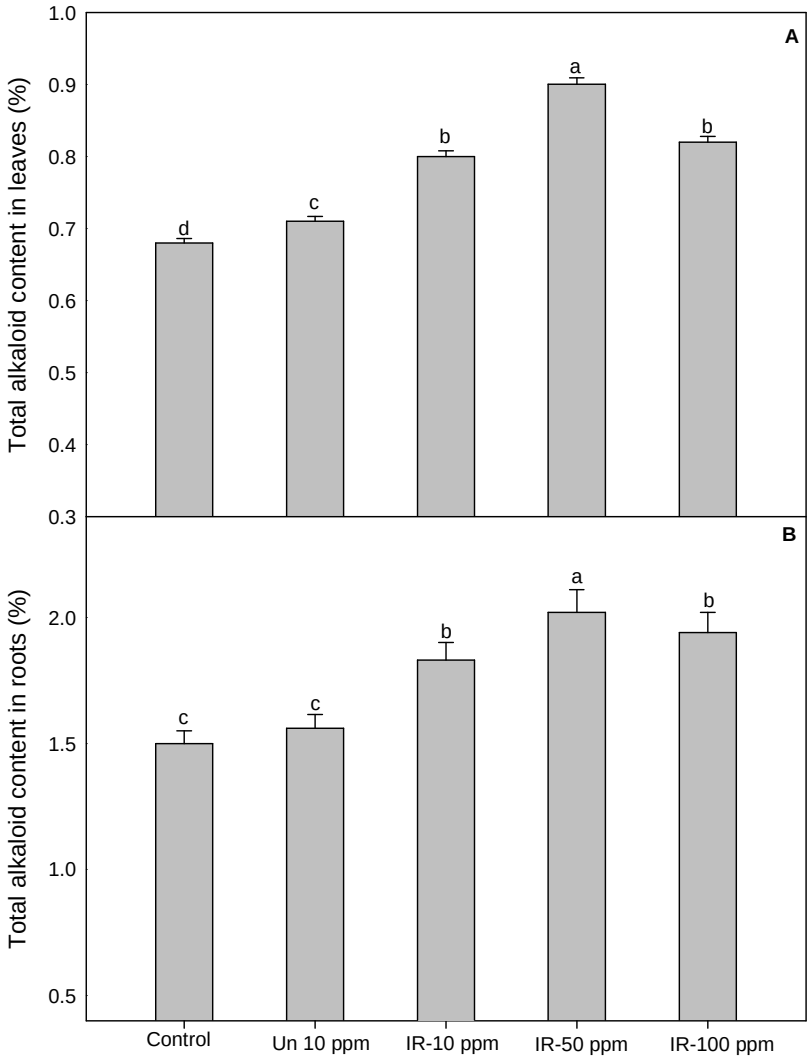

Figure 1: Effect of irradiated carrageenan on total alkaloid content in leaves (A) and roots (B) of *Catharanthusroseus* (Each bar represents means of four replicates ± SE). Means followed by the same letter(s) in the histogram not significantly different (p ≤ 0.05).

Table 1: Effect of irradiated carrageenan on growth parameters of *Catharanthusroseus* (Means of four replicates ± SE). Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05).

Growth Parameters	Irradiated carrageenan concentration (ppm)				
	Water (Control)	Un 10	IR-10	IR- 50	IR-100
Shoot length plant^{-1}(cm)	28.4±0.92^{d}	33.0±0.99^{c}	34.0±0.87^{b}	36.0±0.86^{a}	31.7±78^{d}
Root length plant^{-1}(cm)	20.0±0.40^{c}	18.0±0.48^{b}	19.0±0.29^{b}	25.3±0.33^{a}	15.3±0.44^{c}
Leaf area plant^{-1} (cm^{2})	8.86±0.13^{c}	12.94±0.27^{bc}	13.5±0.38^{b}	15.88±0.36^{a}	11.93±0.19^{c}
Fresh weight plant^{-1} (g)	73.4±1.09^{d}	79.7±1.17^{c}	84.0±1.19^{b}	94.2±1.16^{a}	84.3±1.14^{b}
Dry weight plant^{-1} (g)	19.28±0.43^{c}	19.02±0.46^{c}	18.67±0.53^{b}	23.87±0.34^{a}	20.78±0.47^{bc}

Table 2: Effect of irradiated carrageenan on biochemical parameters of *Catharanthusroseus* (Means of four replicates ± SE). Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05).

Biochemical parameters	Irradiated carrageenan concentration (ppm)				
	Water (Control)	Un 10	IR-10	IR-50	IR-100
Total chlorophyll content	0.99±0.93^{d}	1.00±0.95^{c}	1.03±0.87^{b}	1.12±0.85^{a}	1.04±88^{bc}

(mg g^{-1} FW)					
Total carotenoid content (mg g^{-1} FW)	0.271±0.03^{c}	0.276±0.51^{c}	0.281±0.32^{b}	0.300±0.32^{a}	0.285±0.44^{b}
NR Activity (nM NO_2^- g^{-1} FWh^{-1})	221.6±11.07^{d}	235.2±11.15^{c}	252.4±18.17^{b}	275.3±13.15^{a}	256.2±20.12^{b}
CA Activity [µmol $CO_2Kg^{-1}(FW)s^{-1}$]	4.54±0.43^{c}	4.76±0.45^{c}	4.93±0.52^{bc}	5.54±0.43^{a}	5.18±0.46^{b}
Leaf-nitrogen content (%)	2.96±0.10^{c}	2.97±0.13^{c}	3.15±0.66^{b}	3.35±0.46^{a}	3.19±0.16^{b}
Leaf-phosphorus content (%)	0.322±0.014^{c}	0.327±0.017^{c}	0.336±0.019^{bc}	0.357±0.022^{a}	0.343±0.017^{b}
Leaf-potassium content (%)	3.11±0.32^{c}	3.16±0.44^{c}	3.32±0.36^{b}	3.57±0.35^{a}	3.35±0.36^{b}

According to the previous report the irradiated alginate promoted the alkaloid content in *Papaver somniferum*[24]. Therefore, an increase in growth and physiological parameters of treated plants was expected to increase in alkaloid content. These studies shows that the irradiated oligosaccharides act as the plant elicitors like plant growth regulators (PGRs) and by these PGR_S influence the secondary metabolites production [23]. Also, the alkaloid content in plant tissues was affected by the rate of biosynthesis and catabolism and varies with respect to plant development, diurnal variations, functionally different parts of plants and environmental factors [28]. In present investigation, exposure of IC 50 ppm enhanced the level of total alkaloids in both leaf and root when compared to the other tested IC treatments. Therefore, an increase in the contents of these alkaloids is in accordance with the known fact that exogenous application of plant growth regulators evokes the intrinsic genetic potential of the plant causing increase in enzyme activities, uptake of nutrients, enhanced photosynthesis and improved translocation of photosynthates and other metabolites to the reproductive parts [29].

The application of IC resulted in significant improvement in growth, physiological, biochemical attributes and alkaloid contents. Based on the results it can be concluded that 50 ppm proving the best IC concentration in order to promote physiological and biochemical attributes of plants and the alkaloids production in *C. roseus*. However, further investigations are further needed to comprehend the mechanism and mode of action of carrageenan-derived oligomers in plants.

CONCLUSION

The application of IC resulted in significant improvement in growth, physiological, biochemical attributes and alkaloid contents. Based on the results it can be concluded that 50 ppm proving the best IC concentration in order to promote physiological and biochemical attributes of plants and on the alkaloids production in *Catharanthus roseus* L. However, further investigations are required to comprehend the mechanism and mode of action of alginate-derived oligomers in plants.

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