Modulation of physiological activities, active constituents and essential oil production of *Mentha arvensis* L. by concomitant application of depolymerised carrageenan, triacontanol and 28-homobrassinolide

M. Naeem, Tariq Aftab, Mohd. Idrees, Minu Singh, Akbar Ali, M. Masroor A. Khan, Moin Uddin and Lalit Varshney

**Abstract**

Cornmint (*Mentha arvensis* L.) constitutes most important source of therapeutic agents used in the alternative systems of medicine. The essential oil of cornmint has wide applications in pharmaceutical, agrochemical and flavoring industries worldwide. This study was conducted to explore the individual as well as combined effect of the best foliar concentrations of gamma-irradiated carrageenan (IC), triacontanol (TRIA) and 28-homobrassinolide (HBR) on growth, yield and quality of cornmint. Foliar application of IC, TRIA and HBR, applied individually on plants, significantly improved the plant attributes studied. However, combined application of these plant growth regulators (PGRs) was more effective compared to their individual application. In comparison to other applied treatments and the control, the combination of three PGRs (80 ppm IC + 10⁻⁶ M TRIA + 10⁻⁷ M HBR) proved to be the best for most of the growth, physiological, biochemical and agronomic parameters studied. Combined application of the tested PGRs excelled the control in per plant yield of menthol, menthone and menthy acetate by 135.9 and 134.1%, 180.0 and 161.1% and by 225.0 and 187.5% at 100 and 120 days after planting, respectively.

1. Introduction

Gamma-rays irradiation degrades the natural polysaccharides, such as chitosan, carrageenan and sodium alginate, into smaller oligomers with comparatively low molecular weight. Oligomers, obtained from radiolytically degraded polysaccharides, including those of irradiated carrageenan (IC), have valid applications as plant growth promoter in the field of agriculture (1, 2). Application of the degraded polysaccharides (in the form of oligomers) on foliage of the plants promotes various biological and physiological activities, including plant growth in general (1–3), seed germination, shoot elongation, root growth, flower production, antimicrobial activity, amelioration of heavy metal stress, synthesis of phytoalexins, etc. (1–5).

Carrageenans are composed of D-galactose units linked alternately with α-1,4 and β-1,3 linkages. They are mixtures of water-soluble, linear and sulfated galactans. The use of carrageenan (C₁₂H₂₁₀O₂₁S), to promote plant growth and, thereby, augment the amount of essential oil (EO) along with the desired active constituents in medicinal and aromatic plants, is inexpensive as well as safe. Triacontanol (TRIA), a long-chain primary alcohol (C₃₀H₆₁OH), has been realized as a potent plant growth-promoting substance regarding a number of agricultural and horticultural crops. TRIA, being a plant growth promoter, has been reported to improve the plant growth as well as yield and quality characteristics of various crops (6, 7), in addition to increase the rate of several biochemical and physiological processes (6, 7).

Recently, brassinosteroids (BRs) have emerged as a new group of growth promoting phytohormones. 28-homobrassinolide (HBR) is one of the several brassinosteroids, the role of which in enhancing growth, productivity and quality of plants, via improving various physiological processes, has been established both under stress and normal conditions (8–11).

Out of a large number of EO bearing plants, cornmint (*Mentha arvensis* L.) constitutes most important source of therapeutic agents used in the alternative systems of medicine. It is a stimulant, tonic and vermifuge; in addition, it has anti-spasmodic, diaphoretic, stomachic, carminative, antiviral, antifungal, antibacterial and choleretic
properties. In fact, the cornmint oil has wide applications in pharmaceutical, agrochemical and flavoring industries worldwide (2, 3, 11).

Keeping the importance and increasing demand of cornmint essential oil (EO) in mind, this study was conducted to find out the combined effect of IC, TRIA and HBR in order to get the best response of cornmint crop in terms of productivity, physiological activities, active constituents and production of EO. There is no information till date regarding the effect of cumulative application of IC, TRIA and HBR on cornmint crop.

2. Materials and methods

2.1. Plant materials and growth conditions

The pot experiment was conducted on cornmint in the natural conditions of the net house at Botany Department, Aligarh Muslim University, Aligarh, India. Prior to transplantation, each pot was filled with 5 kg of homogenous mixture of soil and cowdung manure (4:1). Physico-chemical characteristics of the soil were: texture-sandy loam, pH (1:2) 7.5, E.C. (1:2) 0.48 dS m⁻¹, available N, P and K 102.4, 7.8 and 145.9 mg kg⁻¹ of soil, respectively. A uniform recommended basal dose of N, P and K (25:11:21 mg kg⁻¹) and HBR (10⁻⁷ M) was carried out at 10 days interval using earthen pots (25 cm diameter × 25 cm height). Each treatment was replicated five times and each replicate had three plants. Thus, each treatment consisted of fifteen pots, each pot contained a single healthy plant. The pots were watered as required.

The best foliar dose of each of the PGRs (IC, TRIA and HBR) employed was determined on the basis of earlier findings (2, 11, 26). The individual as well as combined application of optimized concentrations of IC (80 ppm), TRIA (10⁻⁶ M) and HBR (10⁻⁷ M) was carried out at 10 days interval when the plants were at 2–3 true leaves stage to find out the agricultural response of cornmint crop. Totally, five foliar sprays of IC, TRIA and HBR were applied to the crop using a hand sprayer. Un-irradiated carrageenan was not tested in this study as it gave significantly equal effect with that of water spray control (2). Seven spray treatments, viz. (i) Control (distilled water), (ii) 80 ppm IC, (iii) 10⁻⁶ M TRIA, (iv) 10⁻⁷ M HBR (v) 80 ppm IC+10⁻⁶ M TRIA, (vi) 80 ppm IC+10⁻⁷ M HBR (vii) 80 ppm IC+10⁻⁷ M TRIA + 10⁻⁷ M HBR] were applied. The plants were sampled at 100 and 120 days after planting (DAP).

2.2. Irradiation and GPC (Gel permeation Chromatography) analysis

Solid material of k-carrageenan (Sigma Aldrich, USA) was purchased from Sigma-Aldrich, USA. The samples of carrageenan were irradiated (Cobalt-60, GC-5000) in a Gamma Radiation Chamber (BRIT, Bhabha Atomic Research Centre, Mumbai, India) to 250 kGy gamma radiation dose at a dose rate of 2.4 kGy/h. GPC of carrageenan samples were done on DIONEX ULTIMATE 3000 machine and the experimental conditions were as follows: mobile phase-water, flow rate: 1.5mL/min, column PL-Aquagel, mixed bed column, 300 mm × 10 mm, 20 micro liter loop injection (11). The molecular weight of the un-irradiated commercial k-carrageenan sample was estimated to be about 100,000. Polyvinyl alcohol polymers of known molecular weight were used as standards. Different aqueous concentrations of IC were finally prepared using double distilled water for the spray treatments.

2.3. Scanning electron microscopy (SEM) analysis

The morphology structure of the carrageenan samples were examined using the Scanning Electron Microscope (Philips XL 30 ESEM, Jeol, Japan). The samples were coated with gold. Scanning electron microscopy and elemental analysis was performed for un-irradiated as well as irradiated carrageenan samples (Figures 3a, b) at Ultra Sophisticated Instrumentation Facility (USIF), Aligarh Muslim University, Aligarh, India.

2.4. Determination of growth attributes

The growth attributes viz. plant height, leaf-area, leaf-yield per plant and fresh and dry weights of plants were determined at 100 and 120 DAP. Plant height was measured using a meter scale. Leaf-yield was recorded by weighing all plant leaves using an electronic balance. Potted plants from each treatment were uprooted carefully followed by measuring the height and fresh weight per plant. The plants were dried in a hot-air oven at 80°C for 24 hours prior to recording the plant dry weight. Only 10% of the randomly selected total leaves of each sample (consisting of five plants) were used to determine the leaf area using graph paper sheet (12). The mean area per leaf, thus determined, was multiplied with the total number of leaves to measure the total leaf area per plant.

2.5. Determination of physiological attributes

2.5.1. Estimation of total chlorophyll and carotenoids contents

Total content of chlorophyll and carotenoids in the leaves was estimated using the method of Lichtenthaler and Buschmann (13). The fresh tissue from the interveinal area of leaf was grinded with 100% acetone using mortar-pes- tle. The optical density (OD) of the pigment solution was recorded at 662, 645 and 470 nm to determine chlorophyll a, chlorophyll b and total carotenoids content, respectively,
2.5.2. Determination of net photosynthetic rate and stomatal conductance

Net photosynthetic rate and stomatal conductance were determined employing the youngest fully expanded randomly selected leaves from the five replicates; measurements were made on sunny days at 1100 hours using an Infra Red Gas Analyzer (IRGA, Li-Cor 6400 Portable Photosynthesis System Lincoln, Nebraska, USA) at 100 and 120 DAP.

2.5.3. Determination of carbonic anhydrase (CA) activity

The activity of carbonic anhydrase (E.C. 4.2.1.1) was measured in the fresh leaves selected randomly, using the method described by Dwivedi and Randhawa (14). Two hundred mg of the leaves (chopped leaf-pieces) were transferred to Petri plates. The leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride solution for 20 minutes at 4°C. The solution adhering at the cut surfaces of the leaf pieces was removed with the help of a blotting paper, followed by their transfer immediately to a test tube containing 4 mL of phosphate buffer (pH 6.8). To it, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue were added. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. The enzyme activity was expressed as μM CO₂ kg⁻¹ leaf FW s⁻¹.

2.5.4. Total phenol content

Total phenol content was estimated by the method described by Sadasivam and Manickam (15). Five hundred mg of oven-dried leaves were grinded with 10 times volume of 80% ethanol, using mortar-pestle. The homogenate was centrifuged at 10,000 rpm (10,062×g) for 10 minutes at 4°C. Thereafter, the supernatant was evaporated to dryness, adding 5 mL of double distilled water (DDW). Later, 0.5 mL of Folin-Ciocalteau Reagent and 2 mL of 20% Na₂CO₃ solution were added to each test tube. The OD of the solution, thus obtained, was measured at 650 nm against a reagent blank. Using the standard curve, the content of total phenols in the test samples was determined as mg phenol per 100 g of dry leaves.

2.6. Yield parameter

Herbage yield of the crop was measured by weighing the total biomass per plant excluding the roots.

2.7. Isolation of essential oil

The EO of cornmint leaves was extracted and determined gravimetrically according to (16). Fresh leaves were collected from each treatment pot. Later, they were chopped together. Sufficient quantity (100 g) of chopped leaf-pieces was taken for EO estimation. The EO content in the leaves was extracted by distillation method for 3 hours, using a Clevenger’s apparatus. The extracted oil was dried over anhydrous sodium sulphate and preserved in sealed glass vials at 4°C for the GLC analysis of the oil.

2.7.1. Compositional analysis of essential oil using Gas Chromatography

The active constituents of the EO, namely, menthol, menthone and menthyl acetate, were analyzed using Gas Liquid Chromatography (GLC, Nucon 5700, New Delhi, India). The oil samples were subjected to GLC analysis equipped with an AT-1000 stainless steel column (3 m Length × 3 mm ID, packed with 10% AT-1000 on Chromosorb WHP, 100–120 mesh), a flame ionization detector and an integrator. Nitrogen was used as the carrier gas. The flow rates of nitrogen, hydrogen and oxygen were maintained at 0.5, 0.5 and 5 mL s⁻¹, respectively. The temperature schedule of GLC was as follows: detector temperature 240°C; injector temperature 230°C. The oven temperature was programmed from 90°C to 240°C at 4°C/min. The sample size was 2 μL for all the measurements. The identification of the active constituents was based on retention time of the particular constituent in the GLC column. The active constituents were quantified in per cent content, comparing their peaks with the peaks obtained from the reference standards reported in the book of Adams (17).

2.7.2. Determination of specific gravity of essential oil

The specific gravity of the cornmint EO was determined at 25°C with a ‘specific gravity bottle’. The weight of distilled water and the essential oil was determined using a specific gravity bottle at room temperature (25°C). The same bottle was emptied and dried. It was filled with the oil up to the mark and weighed, maintaining the room temperature at 25°C. The exact weight of the oil was determined by subtracting the weight of the empty bottle from the total weight of the bottle filled with the oil. The specific gravity was determined according to Afaq et al. (18).

2.7.3. Determination of refractive index of EO

The refractive index of the EO was determined according to Jenkins et al. (19) employing an Abbe’s Refractometer (Sipcon, New Delhi, India). Two to three drops of oil were placed on the double prism, clamping the prisms together firmly. The instrument was adjusted until the border line between light and dark halves of the view-field...
All the physiological and biochemical attributes were significantly affected by the single application of IC, TRIA and HBR at both the growth stages. However, the combination of IC, TRIA and HBR at 80 ppm, 10−6 M and 10−7 M significantly increased plant height, leaf area, leaf yield per plant, and plant fresh and dry weights by 62.8% and 67.1%, 51.9 and 53.4%, and by 59.3 and 65.3%, respectively, when compared to the control (Table 1).

### 3.2. Growth attributes

The influence of the IC, TRIA and HBR sprays was significant on plant height, leaf area, leaf yield per plant, and plant fresh and dry weights at 100 and 120 DAP. The selected spray concentrations at 100 and 200 M of HBR proved more effective than the other applied treatments in enhancing the growth attributes. This treatment combination (80 ppm of IC, 10−6 M of TRIA and 10−7 M of HBR) proved more effective than the other applied treatments (Table 1). The mean of three readings was designated as the refractive index of EO. The refractive index of the EO was expressed as N = \frac{n}{\sin \theta}; where, ND = \frac{n}{\sin \theta} denotes the index of the light refraction for the ‘D’ line (sodium light) measured at 24°.

### 3.3. Growth attributes

This study indicated that the optimized spray concentrations of IC, TRIA and HBR applied individually enhanced all the attributes significantly; however, their combined application proved the best, increasing all the studied attributes, including active constituents, at both the stages maximally. However, specific gravity and refractive index of the EO were not significantly increased by either alone or combined application of the treatments at 100 and 120 DAP. The control showed the lowest effect in this study.

### 3.1. Growth attributes

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### 2.8. Statistical analysis

The data were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA) according to simple randomized design. Means were compared using Duncan's Multiple Range Test (DMRT) at P < 0.05. Standard error was also employed in this regard.

### Table 1. Effect of foliar sprays of IC, TRIA and HBR on growth attributes of cornmint (Mentha arvensis L.) at 100 and 120 DAP.

| Growth attributes | DAP | Control | IC | TRIA | HBR | IC+TRIA | HBR | IC+TRIA | IC+TRIA +HBR |
|-------------------|-----|---------|----|------|-----|---------|-----|---------|--------------|
| Plant height (cm) | 100 | 68.26 ± 1.15g | 87.20 ± 1.14f | 112.25 ± 1.17c | 94.55 ± 1.19e | 113.60 ± 1.20b | 98.60 ± 1.24d | 115.12 ± 1.50a |
| Leaf-area per plant (cm²) | 120 | 80.60 ± 1.21d | 106.75 ± 1.73c | 134.15 ± 1.05b | 116.10 ± 1.15e | 135.26 ± 1.32b | 118.26 ± 1.34c | 136.26 ± 1.42c |
| Leaf-yield per plant (g) | 100 | 14.56 ± 0.164g | 19.62 ± 0.160f | 23.20 ± 0.132d | 22.92 ± 0.130e | 23.50 ± 0.128b | 23.36 ± 0.134c | 23.70 ± 0.210a |
| Dry weight per plant (g) | 120 | 65.98 ± 1.52g | 93.48 ± 0.160f | 96.50 ± 1.09e | 92.30 ± 2.19d | 96.84 ± 1.53b | 94.50 ± 1.62c | 101.2 ± 1.45a |
| Fresh weight per plant (g) | 120 | 65.98 ± 1.52g | 93.48 ± 0.160f | 96.50 ± 1.09e | 92.30 ± 2.19d | 96.84 ± 1.53b | 94.50 ± 1.62c | 101.2 ± 1.45a |

Notes: *Treatment concentrations: IC 80 ppm, TRIA 10−6 M, HBR 10−7 M. Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05). Means of five replicates ± SE.
Foliar application of optimized concentration of IC, TRIA and HBR applied alone increased the carbonic anhydrase (CA) activity. In this study, the combined application of IC, TRIA and HBR improved the CA activity by 25.8 and 29.4% compared to the control at 100 and 120 DAP, respectively (Figure 2A). Similarly, it improved the level of leaf-phenolic content by 9.0 and 12.5% compared to the control at 100 and 120 DAP, respectively (Figure 2B).

### 3.3. Yield and quality attributes

Combined application of 80 ppm of IC, 10^{-6} M of TRIA and 10^{-7} M of HBR was significantly better than their single application for the yield and quality attributes too. The concomitant application (80 ppm of IC + 10^{-6} M of TRIA + 10^{-7} M of HBR) enhanced the herbage yield maximally, surpassing the control by 59.3 and 64.4% at 100 and 120
in promoting seed germination, shoot elongation and growth and development (1–4, 21–23). In this study, the application of IC enhanced the leaf-area, which might obviously provide increased opportunity for light harvesting leading to the accumulation of enhanced plant dry matter, compared to the control (Table 1). This study showed significant improvement in plant growth attributes by the application of radiation-derived oligomers of IC. Depolymerized carrageenan has been reported to promote valuable biological functions (21). It is believed that the plants have capacity to recognize the oligomers or oligosaccharides, derived from the depolymerized natural polysaccharides, which regulate growth, physiological activities, development and defense responses of plants (1–3). It seems that there might be specific structural and size requirement of the oligosaccharides for inducing a range of effects in plants (24). However, the phenomenon by which the radiolytically degraded oligomers of carrageenan and other natural polysaccharides stimulate the physiological processes related to the promotion of plant growth still needs further investigations.

According to El-Rehim (25), application of irradiated sodium alginate (ISA) resulted in improvement in the plant root growth and augmented shoot elongation too; thereby, it might have increased the plant productivity in response to improved growth and physiological parameters. The IC might presumably be expected to enable the plants to respond in somewhat similar mode regarding plant growth, photosynthetic pigments and photosynthesis (1).

There are various plant growth promoters, which have direct or indirect influence on growth and development of the plant. Degraded oligosaccharides might be comparable with endogenous growth elicitors that probably could function as a signal to trigger the synthesis of different enzymes and activate various plant responses exploiting the gene expression (20). It is well established that polysaccharides such as sodium alginate, carrageenan and chitosan, in their depolymerized form, are effective in promoting seed germination, shoot elongation and growth and development (1–4, 21–23). In this study, the application of IC enhanced the leaf-area, which might obviously provide increased opportunity for light harvesting leading to the accumulation of enhanced plant dry matter, compared to the control (Table 1). This study showed significant improvement in plant growth attributes by the application of radiation-derived oligomers of IC. Depolymerized carrageenan has been reported to promote valuable biological functions (21). It is believed that the plants have capacity to recognize the oligomers or oligosaccharides, derived from the depolymerized natural polysaccharides, which regulate growth, physiological activities, development and defense responses of plants (1–3). It seems that there might be specific structural and size requirement of the oligosaccharides for inducing a range of effects in plants (24). However, the phenomenon by which the radiolytically degraded oligomers of carrageenan and other natural polysaccharides stimulate the physiological processes related to the promotion of plant growth still needs further investigations.

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There is enough evidence regarding the growth-promoting effect of TRIA (6, 7, 26–28) and HBR (10, 29, 30). Improvement in shoot height and leaf area index due to combined spray application might be expected to

![Figure 2](image-url). Effect of foliar sprays of IC, TRIA and HBR on carbonic anhydrase activity (A) and total phenolic content (B) of cornmint (Mentha arvensis L.) studied at 100 and 120 DAP. Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05). Error bars (σ) show SE.
Table 2. Effect of foliar sprays of IC, TRIA and HBR on yield and quality attributes of cornmint (*Mentha arvensis* L.) at 100 and 120 DAP.

| Yield and quality attributes | DAP  | Control   | IC      | TRIA    | HBR     | IC+TRIA  | IC+HBR   | IC+TRIA+HBR |
|------------------------------|------|-----------|---------|---------|---------|----------|----------|-------------|
| Herbage yield per plant (g)  | 100  | 36.62 ± 0.12f | 48.73 ± 0.15e | 56.48 ± 0.23d | 56.60 ± 0.23d | 57.40 ± 0.23b | 57.18 ± 0.23c | 58.35 ± 0.21a |
| 120                          | 52.60 ± 0.22a | 71.65 ± 0.26f | 85.59 ± 0.20e | 85.72 ± 0.21a | 85.87 ± 0.24b | 85.60 ± 0.25b | 86.46 ± 0.22a |
| Essential oil-content (%)    | 100  | 0.644 ± 0.02g  | 0.840 ± 0.02f  | 0.910 ± 0.01e  | 0.885 ± 0.002a | 0.912 ± 0.002b | 0.896 ± 0.001d | 0.918 ± 0.002a |
| 120                          | 0.950 ± 0.01d  | 1.298 ± 0.02f  | 1.180 ± 0.002e  | 1.304 ± 0.002d | 1.296 ± 0.002d | 1.310 ± 0.002d | 1.310 ± 0.002d |
| Essential oil-yield per plant (mL) | 100  | 0.256 ± 0.001f | 0.440 ± 0.001e | 0.542 ± 0.003d | 0.528 ± 0.003d | 0.550 ± 0.002b | 0.540 ± 0.003c | 0.555 ± 0.002b |
| 120                          | 0.476 ± 0.001d | 0.886 ± 0.002a | 0.998 ± 0.004e | 1.012 ± 0.002b | 0.992 ± 0.003c | 1.028 ± 0.002a | 1.038 ± 0.002a |
| Specific gravity of essential oil (g/cm³) | 100  | 0.893 ± 0.001a | 0.890 ± 0.001a | 0.894 ± 0.001a | 0.892 ± 0.001a | 0.890 ± 0.001a | 0.890 ± 0.001a | 0.890 ± 0.001a |
| 120                          | 0.892 ± 0.001a | 0.890 ± 0.001a | 0.894 ± 0.001a | 0.892 ± 0.001a | 0.890 ± 0.001a | 0.890 ± 0.001a | 0.890 ± 0.001a |
| Refractive index of essential oil | 100  | 1.462 ± 0.001a | 1.463 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a |
| 120                          | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a | 1.462 ± 0.001a |

Notes: *Treatment concentrations: IC 80 ppm, TRIA 10⁻⁶ M, HBR 10⁻⁷ M.*
Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05). Means of five replicates ± SE.

Table 3. Effect of foliar sprays of IC, TRIA and HBR on content and yield of active constituents of cornmint (*Mentha arvensis* L.) at 100 and 120 DAP.

| Content and yield of active constituents | DAP  | Control   | IC      | TRIA    | HBR     | IC+TRIA  | IC+HBR   | IC+TRIA+HBR |
|-----------------------------------------|------|-----------|---------|---------|---------|----------|----------|-------------|
| Menthol content (%)                     | 100  | 80.52 ± 0.02f | 84.72 ± 0.02e | 82.18 ± 0.023e | 85.74 ± 0.024c | 84.74 ± 0.021d | 85.80 ± 0.019b | 86.00 ± 0.020c |
| 120                                     | 80.65 ± 0.02e | 85.26 ± 0.02d | 82.12 ± 0.029e | 86.30 ± 0.026c | 85.28 ± 0.024d | 86.50 ± 0.016b | 86.64 ± 0.021c |
| Menthol yield per plant (mL)            | 100  | 0.206 ± 0.004d | 0.373 ± 0.003d | 0.445 ± 0.003d | 0.453 ± 0.004d | 0.463 ± 0.002b | 0.463 ± 0.002b | 0.486 ± 0.002c |
| 120                                     | 0.384 ± 0.003a | 0.755 ± 0.004a | 0.820 ± 0.005a | 0.828 ± 0.005d | 0.863 ± 0.003c | 0.858 ± 0.002b | 0.899 ± 0.003a |
| Menthone content (%)                    | 100  | 3.96 ± 0.020a | 3.96 ± 0.012d | 4.80 ± 0.03b | 4.37 ± 0.024e | 4.82 ± 0.017b | 4.39 ± 0.029a | 4.93 ± 0.020a |
| 120                                     | 3.92 ± 0.010a | 3.95 ± 0.020b | 4.21 ± 0.026b | 4.49 ± 0.028a | 4.25 ± 0.020c | 4.50 ± 0.024b | 4.55 ± 0.018a |
| Menthone yield per plant (mL)           | 100  | 0.010 ± 0.001d | 0.017 ± 0.002e | 0.026 ± 0.004d | 0.023 ± 0.004b | 0.026 ± 0.002b | 0.024 ± 0.002c | 0.028 ± 0.002a |
| 120                                     | 0.018 ± 0.001f | 0.035 ± 0.002e | 0.042 ± 0.004f | 0.043 ± 0.004b | 0.043 ± 0.001b | 0.045 ± 0.002c | 0.047 ± 0.001a |
| Menthyl acetate content (%)             | 100  | 1.60 ± 0.012c | 1.61 ± 0.022d | 2.23 ± 0.020c | 1.80 ± 0.023c | 2.24 ± 0.020b | 1.82 ± 0.018a | 2.29 ± 0.019a |
| 120                                     | 1.64 ± 0.010a | 1.64 ± 0.021c | 2.20 ± 0.029b | 1.91 ± 0.026b | 2.20 ± 0.023b | 1.90 ± 0.021c | 2.26 ± 0.016a |
| Menthyl acetate yield per plant (mL)    | 100  | 0.004 ± 0.001f | 0.007 ± 0.002e | 0.012 ± 0.002a | 0.009 ± 0.002b | 0.012 ± 0.001b | 0.009 ± 0.001b | 0.013 ± 0.001a |
| 120                                     | 0.008 ± 0.001f | 0.014 ± 0.002c | 0.022 ± 0.002a | 0.018 ± 0.002b | 0.021 ± 0.001b | 0.019 ± 0.001b | 0.023 ± 0.002a |

Notes: *Treatment concentrations: IC 80 ppm, TRIA 10⁻⁶ M, HBR 10⁻⁷ M.*
Means within a column followed by the same letter(s) are not significantly different (p ≤ 0.05). Means of five replicates ± SE.
contribute the enhanced values of dry weight of plants applied with single spray of TRIA or HBR in this study (Tables 1 and 2). In fact, when TRIA was applied in combination with IC and HBR, it proved much effective in comparison to their single application in this regard (Table 1).

When foliage of the plants was sprayed with IC, TRIA and HBR, it elevated the content of both chlorophyll and carotenoids in comparison to the control (Figure 1). The significant enhancement in the chlorophyll content might have resulted in increased photosynthetic rate in this study (Figure 2). The increase in photosynthetic rate due to application of degraded polysaccharides like ISA has, in fact, been indicated by several workers (2, 4, 5). The ISA has also been reported to induce cell signaling, leading to stimulation of various physiological processes in various plants, including ISA-mediated improved content of photosynthetic pigments and enhanced net photosynthetic rate (1, 3). We found the same effect of IC, as that of ISA, in this study.

Earlier studies have revealed an increase in the rate of both CO₂ fixation and photosynthesis in different plants as a result of TRIA application (7, 31–33). Further, in this study, increased photosynthesis was parallel with the elevated levels of leaf chlorophyll and carotenoids contents (Figures 1 C and D), indicating the important role of TRIA in this regard (7). The application of 28-homobrassinolide also resulted in elevated carbon dioxide fixation as compared to the untreated control plants (Figure 1). Similarly, the exogenous application of HBR (100 μM) and 24-epibrassinolide (EBL) (3 μM) resulted in a significant increase in the CO₂ fixation in geranium (29). In line with our studies, there are several reports regarding the positive effects of brassinosteroids on chlorophyll levels in plants (11). Carbonic anhydrase activity was positively affected by the IC alone as well as by a combination of IC, TRIA and HBR. The activity of the enzyme increased to the maximum extent at 120 DAP (Figure 2). In this regard, our findings are similar to those that claim the synthesis of certain enzymes in tissue culture studies following addition of alginate derived oligomers (21–24). This study revealed that the leaves treated with TRIA improved the CA activity considerably at both of the sampling stages (Figure 2A). The enhancement of CA activity due to TRIA application might also be ascribed to the de novo synthesis of CA, which might involve the genes associated with its transcription and translation in the cell. The enhancement of CA activity in the HBR-treated plants might presumably be due to the enhanced rate of CO₂ fixation that, accordingly, could have been responsible for significant increase in the fresh and dry weights of HBR-treated plants (Table 1).

The irradiated carrageenan alone increased the level of leaf phenolic content at both the sampling stages (Figure 2B). However, combined effect of IC with TRIA and HBR was much pronounced in increasing the level of phenolic content in the leaves at 100 as well as 120 DAP (Figure 2B). The positive results obtained in this regard in response to IC application might be ascribed to the specific role of carrageenan oligomers obtained by irradiation with Co-60 gamma rays (1). In addition, TRIA application alone improved the leaf-phenolic content at both the sampling stages. However, TRIA, applied with IC and HBR, increased the total phenolic content maximally in this study (Figure 2B). The leaf phenolic content reflects the free radical scavenging capability of the plant that may help the plant to maintain the normal growth at later growth stages, at which frequent production of free radicals takes place, inducing the bad effects of aging (34). The significant effect of TRIA on phenol content has also been reported by Kumaravelu et al. (35) and Naeem et al. (6) regarding green gram and Japanese mint, respectively.

The significant increase in the above mentioned yield parameters of the IC treated plants might possibly culminate in maximization of the leaf-yield and herb-age-yield of the cornmint plant employed (Tables 1 and 2). Presumably, the improved content and yield of EO in
TRIA treated plants could be due to the enhanced rates of photosynthesis and improved translocation of photosynthates and other metabolites to the reproductive organs as indicated by the photosynthetic model for oil production in *Mentha piperita* L. (6). The positive role of TRIA in increasing growth, yield and quality together with improving the physiological processes in various medicinal plants including *Artemisia annua* L. (36), *Coriandrum sativum* (37), *Cymbopogon flexuosus* (31), *Mentha arvensis* (6), and *Pelargonium* species (38) has been confirmed by various researchers. The effect of TRIA on EO yield might also be mediated through the TRIA-improved growth and metabolism as revealed by our study (Table 2). Expectedly, TRIA-improved secondary metabolism might also contribute to elevate the levels of EO in cornmint crop. Thus, this study might provide more insight to the role of the plant growth regulators and other growth promoting substances with regard to the secondary metabolism of cornmint. Ries (7) suggested that TRIA, like other plant hormones might activate enzymes or alter the permeability of a membrane, which could trigger a cascading effect resulting in increased metabolism and the enhanced accumulation of various critical intermediate compounds. The increase in the EO content in lavender, spearmint, Japanese mint, geranium and coriander as a result of application of EBL, ethrel, gibberellic acid and TRIA might support our studies in this context (7, 29, 37–39). Furthermore, the positive effect of HBR on cornmint EO yield might be attributed to the HBR-improved overall plant growth and metabolism as revealed by our study (Table 2). Thus, HBR-enhanced plant growth, photosynthesis and the overall plant metabolism might have accounted significantly for enhanced EO accumulation in this study. In this regard our results corroborate the findings of Aftab et al. (36), Idrees et al. (37) and Naeem et al. (38) regarding various medicinal and aromatic plants which were positively influenced by different plant growth regulators. A positive effect of HBR on cornmint EO yield and its components (menthol, menthone and methyl acetate) has also been reported by Naeem et al. (11). However, combined effect of IC with TRIA and HBR was much effective in increasing the level of content and yield of EO in the fresh leaves at 100 as well as 120 DAP (Table 2).

**5. Conclusion**

The cumulative effect of the optimized spray-concentration of IC, TRIA and HBR could significantly improve the growth attributes, physiological activities, herbage yield, content and yield of EO and the active constituents of cornmint plant. Further, this study revealed that the combined application of the IC, TRIA and HBR was positively significant to increase the desired production of cornmint EO compared to the single application of these growth promoting substances. We hope that the concomitant application of these substances may be applied in future to achieve the desired quality of several medicinal and aromatic plants. Further, this technique may be safely adopted for boosting up the growth, yield and quality of other medicinal and other crop plants.

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