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Radiolytically depolymerized sodium alginate improves physiological activities, yield attributes and composition of essential oil of *Eucalyptus citriodora* Hook

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ABSTRACT

Eucalyptus citriodora Hook. is highly valued for its citronellal-rich essential oil (EO) extracted from its leaves. Hence, escalated EO production of eucalyptus is the need of hour. Marine polysaccharides (sodium alginate) are processed through gamma radiation of particular intensity, to obtain the irradiated sodium alginate (ISA). A pot experiment was conducted to study the effect of foliar application of ISA on growth, biochemical, physiological, EO yield and composition of *E. citriodora*. The treatments were applied as: foliar spray of deionized water only (control), seed soaked with ISA (90 mg L^{-1}) and foliar spray of ISA with 30, 60, 120 and 240 mg L^{-1}. The treatment 6 (spray of ISA at 120 mg L^{-1}) showed the highest value for most of the parameters studied. It also enhanced the EO content (33.3%), EO yield (86.7%), citronellal content (63.4%) and citronellal yield (205.5%) as compared to the control.

1. Introduction

Essential oils (EOs), the complex mixtures of biologically active substances, have been used for a long time as flavoring agents and are essential constituents of a number of commercial products currently available in the market; their importance is evident due to their increasing demand in food, cosmetics and pharmaceutical industries. Recent scientific literature revealed the antimicrobial, antifungal and antioxidant potential of EOs. In view of the multiple applications of EOs, their characterization based on their chemical profiles, is of great importance. The world trade regarding EOs is expected to continue to expand tremendously in the foreseeable future, as a consequence of the growing number and preferences of consumers and a wide spectrum of the uses of these compounds (The Wealth of India: Raw materials, 2003; Weiss, 2002; Sangwan, Farooqi, Shabih & Sangwan, 2001).

Gamma radiation and electron beam are supposed to be high energy ionizing radiation. These radiations (particularly the former) can interact with various chemicals to degrade them to new chemical entities. Derivatives of some natural polysaccharides, like those of alginates, chitosan, and carrageenan, when applied to certain plants (through hydroponics, tissue culture method or foliar spray, etc.) have been shown to increase the growth of the plants and their products. The increased yields were considerably higher than those promoted through inorganic fertilizers or plant hormones applications. Polysaccharides are broken down to lower molecular weight oligomers when exposed to high energy radiation. It is much easier to control the molecular weight of such oligomers by the use of high energy radiation than by chemical methods. These oligomers, when applied to plants in the form of foliar sprays, elicit various kinds of biological and physiological activities, including promotion of plant growth, seed germination, shoot elongation, root growth, flower production, alleviation of heavy metal stress, etc. Furthermore, application of these oligomers can shorten the harvesting period of various crops and may help in reducing the use of insecticides and chemical fertilizers (Hien et al., 2000; Nagasawa, Mitomo, Yoshii & Kume, 2000; Ahni, Park, & Byun, 2001; Cabalfin, 2002; Hafeez, Muhamad, Muhammad, & Ahmad, 2003; Luan et al., 2003; Lee et al., 2003; Sabharwal, 2004). Keeping this in mind, it was hypothesized whether the production and quality of EO of eucalyptus might be enhanced by this novel, economical, ergonomical and safe technique.

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Out of several natural polysaccharides, sodium alginate (SA) is derived from brown algae, with a large available quantity in nature. Alginates have widespread application in processing of foods and drinks as well as in pharmaceutical and bioengineering industries (Gaseca, 1988; Hien et al., 2000). Alginates are composed of three types of block polymers namely polyglucuronate (poly-G), polymannuronate (poly-M) and copolymer of poly-G and poly-M in random sequences (Haug, Larsen, & Smidsrod, 1967). The radiolytically (using gamma rays) degraded natural polymers are also reported to have been used as plant growth promoters in the form of foliar sprays (Kume, Nagasawa, & Yoshii, 2002; Kume, 2006; Mollah, Khan, & Khan, 2009; Khan, Khan, Aftab, Idrees, & Naeem, 2011; Idrees et al., 2011; Aftab, Khan, Naeem, Idrees & Varshney, 2011; Sarfaraz et al., 2011; Idrees et al., 2012; Naeem et al., 2012; Aftab et al., 2013).

Eucalyptus (family Myrtaceae) represented by over 700 species distributed throughout the world, are well known for their EOs. Among them, lemon scented eucalyptus (*Eucalyptus citriodora* Hook.) is widely grown in different parts of the world for its EO that is used as perfumery and flavouring agent besides being used as pesticide (Swain, 1977; Langenheim, 1994; Hill & Johnson, 1995; Isman, 2000). It has been introduced into India for reclamation of waste lands and timber and wood production as well as for pulp, fuel and volatile oil purposes (The Wealth of India: Raw materials, 2003). The leaves are intensely aromatic releasing a number of volatile terpenes into the environment. The EO of the leaves is a powerful antiseptic agent and is used all over the world as a respiratory decongestant; it is also used for relieving colds, coughs, bronchitis, flu, pneumonia, headache and sore throats, etc. (Mhaskar, Blatter, & Caius, 2000; Vaghasiya, Nair, & Chanda, 2008). It has disinfectant action and, therefore, is applied externally to cure cuts and skin infections. It is inhaled to open blocked nasal passages. It is useful as gargles for sore throats and is taken internally for a wide range of complaints. The eucalyptus oils are the starting material for the manufacture of citronellal and derived products. *E. citriodora* oil showed analgesic, anti-inflammatory, antimicrobial (Fiori et al., 2000; Silva et al., 2003; Ramezani, 2006), acricidal (Clemente et al., 2010), larvicidal (Batish, Singh, Setia, Kaur, & Kohli, 2006; Singh, Dhiman, & Mittal, 2007; Setia, Batish, Singh, & Kohli, 2007; Habila et al., 2010), cytotoxic and natural anti-tumor product (Bhagat, Saxena & Arora, 2009), allelopathic activity (El-Rokiek & El-Nagdi, 2011) and upper respiratory tract infections (Ben-Arye et al., 2011). Perfumery industry has great interest in *E. citriodora* Hook. after the discovery of its EO in 1882. The EO of *E. citriodora* is being used in perfumery industry and the demand for its EO is stepping up since it is rich in citronellal, an aromatic chemical.

Keeping the marvelous therapeutic qualities of EO of *E. citriodora*, it seems highly desirable to increase the production and quality of this plant. Use of oligomers (as plant promoters) obtained through irradiating the sodium alginate by ionizing gamma radiations, is a novel and emerging technique in agronomy. Therefore, it was hypothesized whether performance of this plant, particularly in terms of EO production could be enhanced by using the foliar application of aqueous solution of depolymerized oligomers of sodium alginate?

2. Materials and methods

2.1. Plant material and growth conditions

A net house experiment was conducted using earthen pots under naturally illuminated environmental conditions at the Department of Botany, Aligarh Muslim University, Aligarh (27°88′ N latitude, 78°08′ E longitude, and 187.45 m altitude), India. Authentic seeds of *E. citriodora* Hook. were procured from Resource center, Central Institute of Medicinal and Aromatic Plants (CIMAP), Bangalore, India. Seeds of *Eucalyptus* were initially surface sterilized with 95% ethyl alcohol for 5 min and then soaked in 1% $HgCl_2$ solution for 15 min. Thereafter, the seeds were washed thoroughly with double distilled water before sowing. Prior to seed sowing, bags of 5.0 kg homogenous mixture of soil and farmyard manure (6:1) were prepared. Each bag was autoclaved for 30 min at 121 °C and at a pressure of 15 lbs per square inch to sterilize the soil, making it free from seeds of weeds, bacteria, fungi and viruses, etc. The autoclaved soil (5.0 kg) was filled in each pot. The soil samples were tested at the Government Soil Testing Laboratory, Quarsi Farm, Aligarh. Physico-chemical characteristics of the soil were as follow: texture–sandy loam, pH (1:2)—6.6, E.C. (1:2)—0.48 m mhos cm^{-1}, available N, P and K—98.5, 11.2 and 146.0 mg kg^{-1} soil, respectively. A uniform recommended basal dose of N (as urea), P (as single superphosphate) and K (as muriate of potash) was applied at 150.0, 125.0 and 30.0 mg kg^{-1} soil, respectively. Seeds were sown at a depth of 2 cm in earthen pots (25 cm diameter × 25 cm height).

2.2. Irradiation of sodium alginate by Co-60 gamma rays

SA samples were irradiated by gamma rays from Co-60 source at 520 Kilo Gray (kGy) in Bhabha Atomic Research Centre (BARC), Mumbai, India. Different aqueous concentrations of irradiated sodium alginate (ISA), viz. ISA 30, 60, 120 and 240 mg L^{-1}, together with deionized water (control) and un-irradiated sodium alginate (UISA; 30 mg L^{-1}), were finally prepared and used in the present study.

2.3. Gel permeation chromatography (GPC) analysis

Solid material of sodium alginate (Fig. 1a) was procured from Sigma Aldrich (USA). The mannuronic to guluronic acid residues M/G ratio is 30/70. Viscosity of sodium alginate (1% w/v) at 25 °C is 5.5 ± 2 cps (Centipoise). Sodium alginate samples were irradiated (Cobalt-60, GC-5000) in a Gamma Radiation Chamber (BRIT, Mumbai, India). The samples were irradiated by 520 kGy gamma radiation dose at a dose rate of 2.4 kGy h^{-1} (Fig. 1b). GPC of sodium alginate samples were carried out on Hitachi EMerck HPLC/GPC system using RI detector. The experimental conditions were as follows: mobile phase – water, flow rate – 1.5 mL min^{-1}, column PL—Aquagel, mixed bed column, 300 mm × 10 mm, 20 μL injection loop. The average molecular weights of the USA samples were estimated to be about 0.695 MDa (Fig. 2). Polyvinyl alcohol polymers of known molecular weight were used as standards. Different aqueous concentrations of ISA were finally prepared using double distilled water for spray treatment.

2.4. Scanning electron microscopy (SEM) analysis

The morphology structure of the sodium alginate samples were examined under a Philips XL 30 ESEM, Jeol, Japan scanning electron microscopy. The samples were coated with gold. Scanning electron microscopy and elemental analysis was performed for unirradiated as well as irradiated materials (Fig. 3a and b) at Ultra Sophisticated Instrumentation Facility (USIF), Aligarh Muslim University, Aligarh, India.

2.5. Pot culture

The experiment was conducted in pots according to complete randomized design. The effects of foliar application of ISA was determined on the basis of growth, yield and quality (content of EO and citronellal) attributes. In a Petri dish experiment, we

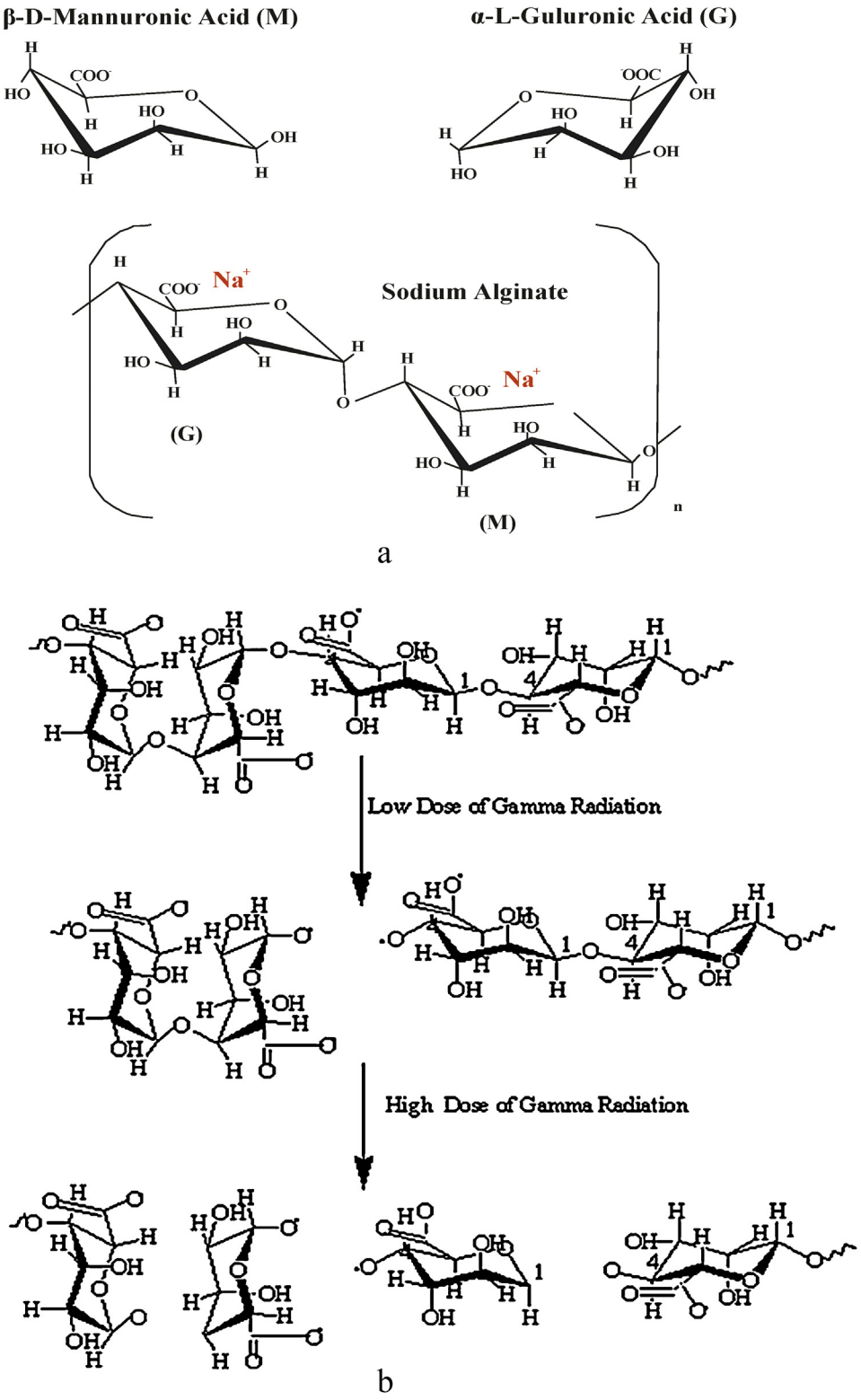

Fig. 1. (a) Schematic diagram of β-D-mannuronic acid (M units) and α-L guluronic acid (G units), and sodium alginate. (b) A typical process of alginate breaking down into the smaller particle by g-radiation (de- polymerization of alginate by g-radiation). Source: Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford,OX5 1GB,UK, License number 3293020480604, License date Dec 20, 2013

had already established that 90 mg L^{-1} ISA proved the best for most of the parameters regarding germination studies (unpublished data). Hence, in the present study, we have tested whether the *pre-soaking of seeds with 90 mg L^{-1} ISA and foliar spray of different doses of ISA could further ameliorate the performance of *E. citriodora*, particularly in terms of essential oil and citronellal production. The treatments were applied according to the following scheme of treatments:

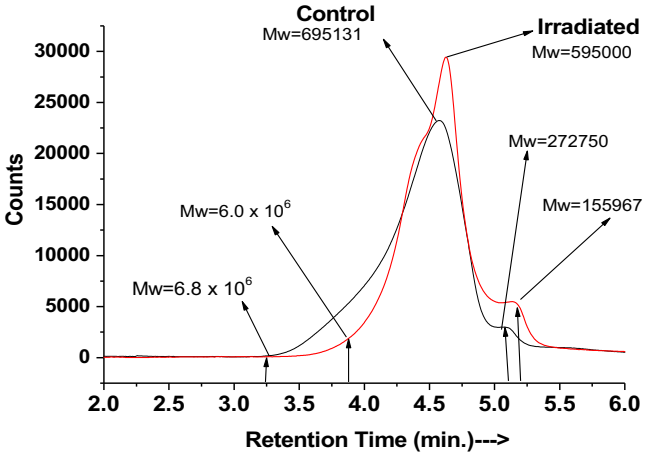

Fig. 2. GPC of unirradiated and irradiated sodium alginate (ISA). The molecular weight distribution of un-irradiated and irradiated sodium alginate. The average molecular weights of the un-irradiated sodium alginate samples were estimated to be about 0.695 MDa. The distribution curve in the GPC profile shows shifting of whole graph to higher retention time indicating radiation degradation of sodium alginate on irradiation and forming lower molecular weight oligomers. This average molecular weight of 0.695 MDa was observed in the control and 0.595 MDa for the irradiated samples.

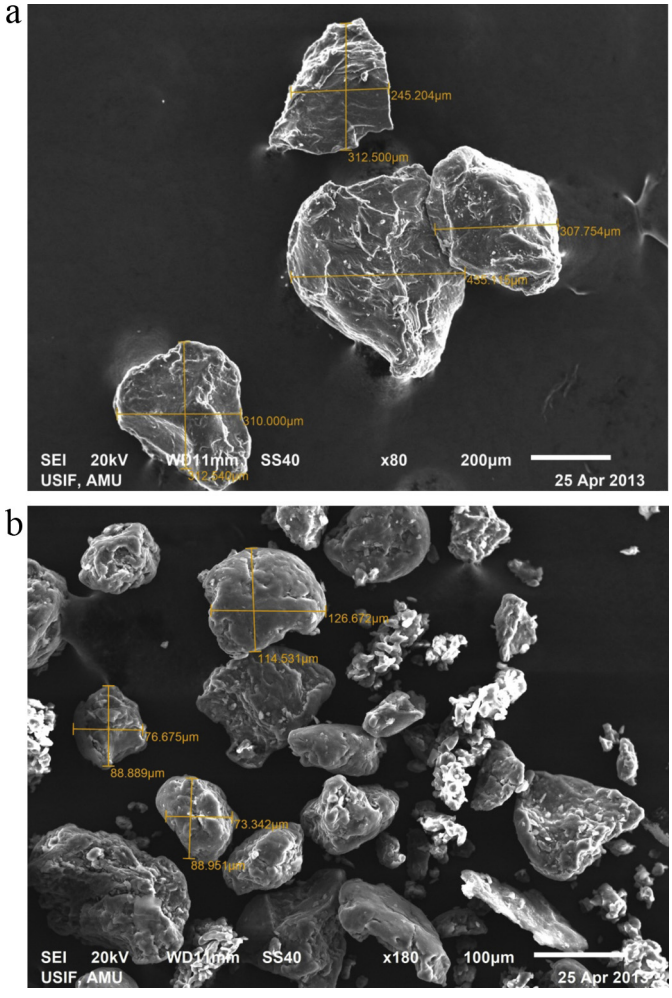

Fig. 3. (a) SEM pictures of Un-irradiated Sodium alginate. (b) SEM pictures of irradiated sodium alginate with gamma radiation.

Treatment no.	Detail of treatments
T1	No seed soaking, spray of distilled water (DW) only (Control)
T2	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray DW
T3	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray of UISA at 30 mg L^{-1}
T4	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray of ISA at 30 mg L^{-1}
T5	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray of ISA at 60 mg L^{-1}
T6	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray of ISA at 120 mg L^{-1}
T7	Pre-soaking of seeds with 90 mg L^{-1} of ISA + foliar spray of ISA at 240 mg L^{-1}

* *E. citriodora* seeds were pre-soaked in ISA solution at 90 mg L^{-1} for 12 h before planting for T2, T3, T4, T5, T6 and T7; whereas, *Eucalyptus* seeds in the T1 treatment (control) were soaked in distilled water. We sprayed UISA to check whether UISA has some significant effect on plants or not. The foliar sprays were applied to the crop at 10 days interval using a hand sprayer. All the attributes studied were determined at 270 days after sowing (DAS). Each treatment was replicated five times. Each pot contained a single healthy plant. The pots were watered as and when required.

2.6. Determination of growth attributes

The crop performance was assessed in terms of growth and other physiological parameters, yield characteristics and quality attributes. At 270 DAS, five plants of each treatment were harvested and their roots were washed carefully with tap water to remove all adhering foreign particles. Water adhering to the roots was removed with blotting paper. The height and fresh weight of plant was measured thereafter. The plants were dried in hot air oven at 80 °C for 48 h; thereafter, the dry weights were recorded.

2.7. Physiological and biochemical analyses

2.7.1. Estimation of total chlorophyll and carotenoids contents

Total chlorophyll and carotenoids contents in fresh leaves were estimated using the method of Mac Kinney (1941) and MacLachlan and Zalik (1963). The fresh tissue from interveinal leaf area was ground using a mortar and pestle containing 80% acetone. The optical density (OD) of the solution was recorded at wave length of 645 and 663 nm for chlorophyll estimation and that at 480 and 510 nm for total carotenoids content, respectively, using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan). The photosynthetic pigments, thus measured, were expressed as mg g^{-1} FW.

2.7.2. Determination of carbonic anhydrase (CA) activity

The activity of carbonic anhydrase (EC 4.2.1.1) was measured in fresh leaves, using the method as described by Dwivedi and Randhawa (1974). Chopped leaf pieces (200 mg) were weighed and transferred to Petri plates. Then, the leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride solution for 20 min at 4 °C. To each test tube, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.002% bromothymol blue were added. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. The enzyme was expressed as µM CO_2 kg^{-1} leaf FW s^{-1}.

2.7.3. Nitrate reductase (NR) activity

The activity of NR (EC 1.6.6.1) was estimated by the intact tissue assay method developed by Jaworski (1971) based on the

reduction of nitrate to nitrite according to the following biochemical reaction:

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

The amount of nitrite (NO_2^-) formed was determined spectrophotometrically. Fresh pieces of chopped leaves, weighing 200 mg, were transferred to a plastic vial. The reaction mixture, contained in each vial, carried 2.5 mL of phosphate buffer (pH 7.5), 0.5 mL of 0.2 M potassium nitrate solution were added followed by the addition of 2.5 mL of 5% isopropanol. The vials were incubated for 2 h in dark at 30 °C for the manifestation of maximum enzyme activity. After incubation, 0.4 mL of the content was transferred from the vial to a test tube. To it, 0.3 mL solution each of 1% sulfanilamide and 0.02% *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED–HCl) was added. The test tubes were kept for 20 min at room temperature for color development. The OD of the content was recorded at 540 nm using the spectrophotometer. The NR activity was expressed as nano moles of nitrite produced per gram of fresh weight of leaf tissue per hour (nM NO_2^- g^{-1} FW h^{-1}).

2.8. Estimation of N, P and K contents in leaves

Leaf samples from each treatment were digested for the estimation of leaf-N, -P and -K contents. The leaves were dried in a hot air oven at 80 °C for 24 h. The dried leaves were ground using mortar and pestle and the leaf-powder was passed through a 72 mesh. The sieved leaf-powder was used for the estimation of N, P and K contents. One hundred mg of the oven-dried leaf powder was carefully transferred into a digestion tube, to which 2 mL of AR (analytical reagent) grade concentrated sulfuric acid was added subsequently. This solution was heated on a temperature controlled assembly at 100 °C for about 2 h and then the content was cooled for about 15 min at room temperature. To the cooled content, 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added drop by drop. The addition of H_2O_2 was followed by gentle heating of the content; then, the content was cooled at room temperature. This step was repeated until the content of the tube turned colorless. The aliquot (peroxide-digested material), thus prepared, was used to estimate the per cent N, P and K contents in the leaves on dry weight basis.

2.8.1. Determination of N content

Leaf-N content was estimated according to method of Lindner (1944) with slight modification by Novozamsky, Houba, van Eck, and van Vark (1983). The dried leaf-powder samples were digested with H_2SO_4 in the digestion tubes using temperature controlled Kjeldahl assembly. A 10 mL aliquot (peroxide-digested material) was poured into a 50 mL volumetric flask. To it, 2 mL of 2.5 N sodium hydroxide and 1 mL of 10% sodium silicate solutions were added to neutralize the excess acid and prevent turbidity. A 5 mL aliquot of the peroxide-digested plant material was poured into a 10 mL graduated test tube followed by addition of 0.5 mL of Nessler's reagent. The OD (optical density) of the solution was recorded at 525 nm using the spectrophotometer.

2.8.2. Determination of P content

The method of Fiske and Subba Row (1925), with slight modification by Rorison, Spencer, and Gupta (1993) was used to estimate the leaf-P content in the peroxide-digested material. A 5 mL aliquot was poured into a 10 mL graduated test tube. To it, 1 mL of molybdic acid (2.5%) was added, followed by addition of 0.4 mL of 1-amino-2-naphthol-4-sulfonic acid. When the color of the content turned blue, the volume of the test tube was made up to 10 mL, using double distilled water. The OD of the solution was recorded at 620 nm using the spectrophotometer.

2.8.3. Determination of K content

Leaf-K content was determined in the peroxide digested plant material by a flame-photometer (Hald, 1947) (Model, C150, AIMIL, India) with the help of emission spectra using specific filter. In the flame-photometer, the solution (peroxide-digested material) was discharged through an atomizer in the form of a fine mist into a chamber, where it was drawn into a flame. Combustion of the elements produced the light of a particular wavelength [λ_{max} for *K* = 767 nm (violet)]. The light, thus produced, was passed through an appropriate filter to impinge upon a photoelectric cell that subsequently activated a galvanometer in order to get the reading.

2.9. Determination of yield and quality attributes

The yield and quality attributes were determined estimating the EO-yield per plant and citronellal yield per plant.

2.9.1. Isolation and compositional analysis of EO (Estimation of EO content)

The EO was extracted and then quantified gravimetrically according to Guenther (1972). The fresh leaves were chopped into small pieces. EO content in the leaves (100 g) was extracted by distillation for 3 h, using Clevenger's apparatus (Borosil, India). The extracted oil was dried over anhydrous sodium sulfate and preserved in sealed glass vials at 4 °C for the GC analysis of the oil.

The amount of oil obtained from plant material was calculated as:

$$\text{oil content (\% v/w)} = \frac{\text{observed volume of oil (mL)}}{\text{weight of sample (g)}} \times 100$$

2.9.2. Gas chromatography (GC) analysis

The active constituent (citronellal content) of the EO was determined using gas chromatography (Perkin Elmer Auto system XL SAIF, CDRI, Lucknow, India) equipped with the SE-30 stainless steel column (10 ft packed), flame ionization detector and integrator. Nitrogen was used as the carrier gas. GC temperature schedule was as follows: detector temperature, 250 °C; oven temperature, 160 °C; injector temperature, 250 °C. The sample size was 0.025 μL invariably. The identification of active constituent (citronellal) was based on retention time. It was quantified as the per cent content comparing their peaks with the peaks obtained from the reference standard reported in the literature (Adams, 2007).

2.10. Statistical analysis

Every pot was treated as one replicate and each treatment contained five replicates. The data were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Means were compared using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05\%$. Standard error (±S.E.) was also employed to separate the means in the tables.

3. Results

In the present study, the following results revealed that there was significant difference between T1 and T6 and hence, we have compared the effect of optimum treatment with T1 (Control) only.

3.1. Growth parameters

Application of treatment T6 (120 mg L^{-1} of ISA) proved the best among the various concentration of ISA (Table 1). Increments in shoot and root lengths by this treatment were 31.5% and 27.8% higher as compared to the control (T1) (Table 1). Treatment T6 (120 mg L^{-1} of ISA) significantly enhanced fresh and dry weights

Table 1
Effect of foliar application of different concentrations of irradiated sodium alginate on the growth parameters of *Eucalyptus citriodora* Hook. (Means of five replicates ± SE). Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Treatments/parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
Shoot length (cm)	62.00 ± 1.14^{e}	64.50 ± 1.62^{de}	67.75 ± 1.24^{cd}	70.75 ± 1.49^{bc}	74.50 ± 1.13^{b}	81.25 ± 1.19^{a}	71.30 ± 1.60^{bc}
Root length (cm)	19.25 ± 0.90^{c}	20.40 ± 0.80^{bc}	21.00 ± 0.83^{bc}	21.50 ± 0.88^{bc}	22.65 ± 0.82^{ab}	24.60 ± 0.81^{a}	22.10 ± 0.84^{ab}
Shoot fresh weight (g)	40.56 ± 1.36^{e}	42.12 ± 1.16^{de}	45.25 ± 1.26^{cd}	49.05 ± 1.26^{bc}	51.88 ± 1.54^{b}	56.19 ± 1.27^{a}	49.36 ± 1.14^{b}
Shoot dry weight (g)	9.72 ± 0.55^{d}	10.05 ± 0.46^{cd}	10.72 ± 0.60^{bcd}	11.86 ± 0.48^{abc}	12.84 ± 0.54^{a}	13.73 ± 0.63^{a}	12.25 ± 0.73^{ab}
Root fresh weight (g)	12.20 ± 0.25^{c}	12.60 ± 0.69^{c}	13.46 ± 0.36^{c}	15.00 ± 0.42^{b}	15.90 ± 0.36^{ab}	17.05 ± 0.84^{a}	15.37 ± 0.32^{b}
Root dry weight (g)	3.17 ± 0.32^{d}	3.30 ± 0.31^{d}	3.51 ± 0.26^{cd}	4.06 ± 0.32^{bcd}	4.60 ± 0.34^{ab}	5.14 ± 0.33^{a}	4.35 ± 0.26^{abc}
Number of leaves per plant	148 ± 2.39^{e}	152 ± 2.52^{e}	160 ± 2.19^{d}	174 ± 2.71^{c}	186 ± 2.31^{b}	207 ± 2.49^{a}	178 ± 2.21^{c}
Leaf area (cm^2)	2035.0 ± 18.13^{f}	2093.6 ± 13.78^{e}	2159.5 ± 14.90^{d}	2253.2 ± 15.01^{c}	2414.8 ± 14.76^{b}	2465.7 ± 18.52^{a}	2382.9 ± 13.40^{b}

Fig. 4. Structural formula of citronellal.

of shoot by 38.5% and 41.3% and fresh and dry weights of root by 39.8% and 62.2%, respectively, over the control (Table 1). Treatment T6 also increased the leaf area and number of leaves per plant by 21.2% and 39.9%, respectively, when compared with T1 (control) (Table 1).

3.2. Physiological and biochemical characteristics

3.2.1. Photosynthetic pigments

As far as the photosynthetic pigments are concerned, the application of depolymerized form of ISA at T6 (120 mg L^{-1} of ISA) significantly increased the total content of chlorophyll (23.9%) and carotenoids (23.5%) at 270 DAS as compared to the T1 control (Table 2).

3.2.2. Nitrate reductase (NR) activity

ISA, applied at 120 mg L^{-1} of ISA, increased the NR activity maximally. The percent increase over the T1 control was 27.3% higher (Table 2).

3.2.3. Carbonic anhydrase (CA) activity

The application of T6 proved optimum for the CA activity at 270 DAS. It registered 25.8% higher activity of the enzyme compared to the control (Table 2).

3.2.4. Leaf -N, -P and -K contents

Leaf -N, -P and -K contents were also significantly enhanced by ISA application, with T6 proving the best. It increased the leaf -N, -P and -K content by 15.9%, 10.5%, and 8.6%, respectively, over the control at 270 DAS (Table 2).

3.3. Yield of EO and citronellal contents

Application of ISA enhanced the values of yield attributes significantly. Among the concentration of ISA, T6 gave the highest values for EO content, oil yield, and content and yield of citronellal (Fig. 4).

3.3.1. Content and yield of EO

Among various concentrations of ISA, T6 gave the best results regarding yield and main components of EO. As compared to the control, it maximally augmented the EO content by 33.3% (Table 3) and the per-plant yield of EO by 86.7% (Table 3).

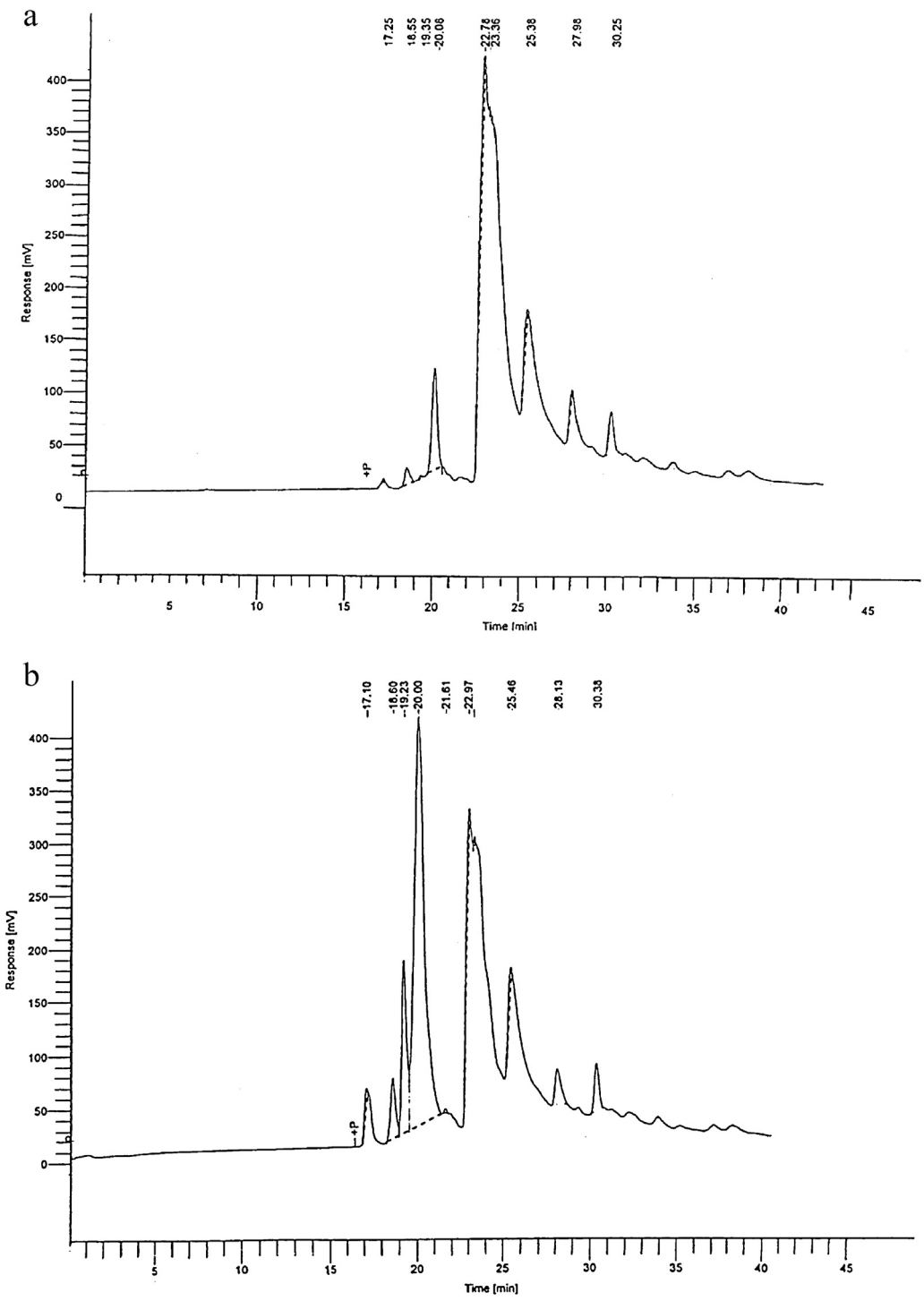

Fig. 5. (a) GC chromatogram showing peaks of citronellal in control of *Eucalyptus citriodora*. Hook. (b) GC chromatogram showing peaks of citronellal in 120 mg L^{-1} ISA treated plants of *Eucalyptus citriodora* Hook.

3.3.2. Content and yield of citronellal yield

The results of GC analyses indicated that ISA significantly promoted the active constituents of the EO of *Eucalyptus*. The ISA spray at T6 increased the contents of main EO constituent citronellal by 63.4% compared to the control (Table 3 and Fig. 5a and b). As compared to the control, T6 considerably increased the yield of citronellal content by 205.5% at 270 DAS (Table 3).

4. Discussion

4.1. Growth attributes

The data presented in table (1) indicated that ISA has promotive effect on all the growth attributes studied. Foliar spray of ISA (120 mg L^{-1}) exhibited significant response to improve the growth attributes as compared to the control.

Table 2
Effect of foliar application of different concentrations of irradiated sodium alginate on physiological and biochemical parameters of *Eucalyptus citriodora* Hook. (Means of five replicates ± SE). Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Treatments/parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
Total chlorophyll (mg g^{-1} FW)	1.772 ± 0.008^{f}	1.839 ± 0.012^{e}	1.858 ± 0.010^{e}	1.966 ± 0.007^{d}	2.043 ± 0.014^{b}	2.195 ± 0.003^{a}	2.010 ± 0.007^{c}
Total carotenoid (mg g^{-1} FW)	0.549 ± 0.003^{e}	0.555 ± 0.005^{e}	0.580 ± 0.004^{d}	0.611 ± 0.006^{c}	0.645 ± 0.008^{b}	0.678 ± 0.002^{a}	0.631 ± 0.006^{b}
Nitrate reductase activity ($nMNO_2$ g^{-1} FW h^{-1})	332.1 ± 3.60^{e}	336.4 ± 3.52^{e}	357.1 ± 3.32^{d}	386.6 ± 3.73^{c}	406.1 ± 2.96^{b}	422.7 ± 3.97^{a}	392.3 ± 3.37^{c}
Carbonic anhydrase activity [$\mu mol(CO_2)$ kg^{-1} FW s^{-1}]	240.6 ± 1.50^{f}	243.8 ± 1.60^{f}	250.6 ± 1.46^{e}	271.5 ± 1.83^{d}	283.2 ± 1.75^{b}	302.7 ± 1.60^{a}	276.9 ± 1.55^{c}
Leaf-nitrogen content (%)	1.95 ± 0.02^{d}	1.98 ± 0.03^{d}	2.01 ± 0.03^{cd}	2.08 ± 0.02^{bc}	2.15 ± 0.03^{b}	2.26 ± 0.04^{a}	2.11 ± 0.03^{b}
Leaf-phosphorus content (%)	0.082 ± 0.001^{c}	0.083 ± 0.002^{bc}	0.083 ± 0.001^{bc}	0.085 ± 0.002^{bc}	0.087 ± 0.001^{ab}	0.091 ± 0.001^{a}	0.086 ± 0.001^{bc}
Leaf-potassium content (%)	1.12 ± 0.04^{a}	1.13 ± 0.06^{a}	1.15 ± 0.03^{a}	1.18 ± 0.04^{a}	1.20 ± 0.06^{a}	1.22 ± 0.03^{a}	1.19 ± 0.05^{a}

Table 3
Effect of foliar application of different concentrations of irradiated sodium alginate on yield parameters of *Eucalyptus citriodora* Hook (Means of five replicates ± SE). Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Treatments/parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
Essential oil content (%)	0.750 ± 0.012^{e}	0.758 ± 0.014^{e}	0.808 ± 0.017^{d}	0.884 ± 0.017^{c}	0.936 ± 0.013^{b}	1.000 ± 0.010^{a}	0.895 ± 0.012^{bc}
Essential oil yield per plant (mL)	0.30 ± 0.010^{e}	0.31 ± 0.014^{de}	0.35 ± 0.015^{d}	0.42 ± 0.018^{c}	0.47 ± 0.019^{b}	0.56 ± 0.017^{a}	0.44 ± 0.014^{bc}
Citronellal content (%)	42.75 ± 0.02^{g}	43.20 ± 0.03^{f}	46.59 ± 0.02^{e}	51.23 ± 0.02^{d}	60.51 ± 0.02^{b}	69.83 ± 0.02^{a}	53.40 ± 0.03^{c}
Citronellal yield per plant (mL)	0.128 ± 0.002^{g}	0.134 ± 0.002^{f}	0.163 ± 0.001^{e}	0.215 ± 0.001^{d}	0.284 ± 0.002^{b}	0.391 ± 0.002^{a}	0.235 ± 0.002^{c}

Fig. 2 shows the molecular weight distribution of un-irradiated and irradiated sodium alginate. The distribution curve in the GPC profile shows the elution of SA fractions differing in molecular weight with respect to retention time. The profile shows shifting of the whole graph to higher retention time indicating radiation-mediated degradation of SA, leading to formation of lower molecular weight oligomers of SA. Average molecular weight of unirradiated SA (control) was 0.695 MDa, while it was 0.595 MDa for the irradiated samples of SA at the highest peak of fraction-counts. The lower molecular weight fractions (less than 100 MDa) which appeared at the end of the profile might be expected to elicit physiological activities in plants. However, it is difficult to say as to which fraction of the irradiated SA acted as a stimulant in this regard. In future studies, we will try to separate these fractions using column chromatography in order to study plant promoting activity of different fractions.

These results are in agreement with those of (Hien et al., 2000) who reported that gamma-irradiated SA, applied at a concentration of 20–100 ppm, promoted the productivity of tea, carrot, cabbage, rice, barley and peanut during field studies. Ruizhi et al. (2009); revealed that alginate-derived oligosaccharides promoted the biomass or dry weight of tomato seedlings significantly. According to El-Rehim (2006) and Mollah et al. (2009) that the oligomers derived from de-polymerization of alginate released from the copolymer were absorbed by plants. The absorption of oligomers acted as a growth promoter, which resulted in elongation of plant root and shoot of plants and, in turn, resulted in an increase in plant productivity and significant improvement in physiological parameters compared with the untreated plants. They maintained that ISA might have growth promoting activities like other plant growth promoters and could act as a bio-fertilizer. Exogenous factors include various plant growth promoters, which have direct or indirect influence on growth and development of the plants. The effect of irradiated natural polysaccharides, such as sodium alginate, carrageenan and chitosan has been reported positive for plant growth parameters in various earlier studies (Natsume, Kamao, Hirayan, & Adachi,1994; Tomoda, Umemura, & Adachi, 1994; Hien et al., 2000; Relleve et al., 2000; Kume et al., 2002; Luan et al., 2003; Hu, Jiang, Hwang, Liu, & Guan, 2004; Abad et al., 2009; Mollah et al., 2009; Jamsheer, 2010; Qureshi, 2010; Sarfaraz et al., 2011; Khan et al., 2011; Aftab et al., 2011, 2013; Naeem et al., 2011, 2012; Idrees et al., 2012).

4.2. Physiological and biochemical attributes

Increment in the chlorophyll content due to application of ISA (Table 2) might be ascribed to a favorable effect of ISA application on photosynthesis as well as on the overall growth of the plant. In fact, various workers have reported positive effect of ISA on the total content of chlorophyll and carotenoids in the leaves of artemisia (Aftab et al., 2013), lemongrass (Idrees et al., 2012), mentha (Naeem et al., 2011, 2012), fennel (Sarfaraz et al., 2011), red amaranth (Mollah et al., 2009) opium poppy (Khan et al., 2011). The ISA has also been reported to induce cell signaling, leading to stimulation of various physiological processes in various plants, including ISA-mediated improvement in the content of photosynthetic pigments and net photosynthetic rate (Farmer, Thomas, Michael, & Clarence, 1991).

4.3. NR activity

A significant increase in leaf-NR activity by ISA in this study might be related to the increase in leaf-N and -P content that might have possibly increased the nitrate concentration in leaves. In fact, the substrate (nitrate) concentration essentially induces functional NR activity (Hewitt & Afridi, 1959) by producing a nitrate sensing protein of unknown nature (Campbell, 2002). The results of the present study are concordant with those obtained regarding artemisia (Aftab et al., 2013), lemongrass (Idrees et al., 2012), and in the case of fennel (Sarfaraz et al., 2011), and those of regarding opium poppy (Khan et al., 2011). The positive effect of ISA application on NR activity has also been reported by Naeem et al., 2011; in the case of *Mentha arvensis* L.

4.4. CA activity

CA is one of the most abundant zinc containing proteins 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 (Badger & Price, 1994). Such a plant response to ISA application is expected because the deploymerized natural polysaccharides have been reported to increase the stomatal conductance significantly (Naeem et al., 2011), which might facilitate the diffusion of additional amount 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 ISA-mediated de novo synthesis of CA, which might involve transcription/translation of the genes associated as has been reported for other degraded natural polysaccharides (Knowles & Ries, 1981). Expectedly, the enhancement of CA activity in the ISA-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 (Table 1). Regarding ISA-mediated CA activity, our findings are similar to those that claim the synthesis of certain enzymes in the tissue culture as a result of application of irradiated natural polysaccharides (Patier et al., 1995; Akimoto, Aoyagi, & Tanaka, 1999). Although there is no direct evidence regarding the effect of ISA on leaf-CA activity of eucalyptus, there are some reports regarding the significant effect of application of ISA on leaf-CA activity of different crops such as artemisia, lemongrass, fennel, opium poppy, mint, artemisia (Khan et al., 2011; Sarfaraz et al., 2011; Idrees et al., 2012; Naeem et al., 2011; Aftab et al., 2013) Thus, the observed effect of ISA on CA activity might be a reflection of the combined effect of a number of underlying factors associated with ISA-mediated stimulation of physiological activities.

4.5. Leaf -N, -P and -K contents

In different studies, ISA appeared to facilitate the efficient absorption and subsequent utilization of mineral nutrients in plants (Sarfaraz et al., 2011; Aftab et al., 2011). There is no report of the effect of ISA on leaf-N, -P and -K content regarding eucalyptus. However, similar enhancement in leaf-N, -P and -K contents as a result of ISA application has earlier been reported in various other crops (Khan et al., 2011; Sarfaraz et al., 2011; Naeem et al., 2011, 2012; Idrees et al., 2012; Aftab et al., 2013). In addition, Khan et al. (2011) reported improvement in leaf-N content to the maximum extent on account of ISA application at 120 mg L^{-1} concentration. ISA-mediated increase in the membrane permeability has been reported to facilitate the absorption and utilization of mineral nutrients in addition to the improved transport of assimilates (Khan et al., 2011) Such an effect of ISA might have contributed toward the enhanced production of plant biomass in this study that is also reflected by the ISA-mediated increase in fresh weight of plants. We presume that increased uptake of nutrients enhanced the photosynthesis and improved the translocation of photosynthates and other metabolites to the sinks, leading to the improved yield of ISA treated plants. Like these results, Naeem et al. (2012); reported significant increase in the uptake of N and P at an IC concentration of 80 mg L^{-1} applied to *M. arvensis* L. Such an impact of

ISA application could be ascribed to the ISA-mediated increase in overall growth of plants (Table 2), which accordingly demanded for higher uptake of these nutrients from the soil, leading to their significant accumulation in the leaves.

4.6. Yield of EO and citronellal contents

As revealed by the data of leaf analysis, the absorption of nutrients was increased in treated plants, which might have led to better nutrient status, efficient assimilation and translocation of photosynthates, improved growth and yield of leaves, and greater yield of oil and active constituents. Hence, the enhanced values of yield attributes with ISA might be ascribed to the role of ISA in plant growth stimulation in general (Hien et al., 2000; Tham et al., 2001; Kume et al., 2002; Luan et al., 2005; Mollah et al., 2009; Khan et al., 2011; Aftab et al., 2011; Sarfaraz et al., 2011; Naeem et al., 2011, 2012; Idrees et al., 2012). The GC chromatograms of EO were compared with the standard chromatograms of citronellal (Fig. 5a and b), as reported by (Adams, 2007). The results of GC analyses indicated that ISA significantly promoted the active constituents of the EO of *Eucalyptus*. There is no scientific literature available till date regarding the effect of ISA as a plant growth regulator on the content and yield of EO of this medicinal crop; but, like ISA, various other plant growth regulators have been used to improve the yield and quality of EO of these crops. According to Idrees et al. (2012) application of ISA at 60 mg L^{-1} increased plant growth, physiological attributes, modulation of phosphoenolpyruvate carboxylase, production of EO and increase in citral content of lemongrass; while according to Naeem et al. (2012); observed that foliar spray of oligomers of irradiated carrageenan at 80 mg L^{-1} increased plant growth, physiological attributes, herbage yield and the content and yield of EO and those of EO components regarding *M. arvensis* L.

Increment in the yield of EO and that in the contents of its active constituents might be due to the ISA-stimulated vegetative growth, population of leaf oil glands, nutrient accumulation (N and P) and also due to the beneficial effect of ISA on plant metabolism and enzymes activities responsible for mono or sesqueterpene-biosynthesis, which could have been subsequently used to enhance the formation of metabolites with regard to oil formation. This conclusion is in accordance with the findings of, Sarfaraz et al. (2011), Hashmi et al. (2012), Idrees et al. (2012) and Naeem et al. (2012).

5. Conclusions

The application of irradiated sodium alginate resulted in significant improvement in growth attributes, physiological and biochemical parameters in addition to enhancing the main constituent i.e. citronellal and yield of EO in eucalyptus, with 120 mg L^{-1} proving the best ISA concentration. These findings corroborate the earlier findings carried out regarding the effect of other irradiated natural polysaccharides on growth, yield and quality of other medicinal and aromatic crops. However, such an ISA dependent enhancement of growth, yield and quality of eucalyptus has been worked out for the first time in this investigation. Further, this research may also instigate the scientists to find out the optimum concentration of ISA or other irradiated natural polysaccharides for different medicinal and aromatic plants in order to enhance the productivity, quality and production of EO and other active constituents. Furthermore, the technique is highly safe as we used the gamma radiations only to depolymerize the sodium alginate rather exposing the plants or seeds. In addition to that this is one-step processing method to obtain the oligosaccharides. Above that the application of ISA used is this study is very economical and ergonomical.

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