

Ameliorative Effect of Triacontanol on the Growth, Photosynthetic Pigments, Enzyme Activities and Active Constituents of Essential Oil of *Ocimum basilicum* L.

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ABSTRACT

Sweet basil (*Ocimum basilicum* L.) is an aromatic herb that has been used traditionally as a diverse medicinally herb for curing various diseases including common cold, headache, malaria, heart disease, stomach and kidney disorders. Its essential oil (EO) is used in the manufacturing of cosmetic and pharmaceutical products as well as aroma therapy. Keeping the medicinal as well as economical importance of this herb in mind, a simple pot experiment was conducted to determine whether foliar sprays of triacontanol (TRIA) could enhance growth, yield and EO production of basil. The plants grown in pots containing soil were sprayed twice with deionised water (control) and three concentrations of TRIA (10^{-8} , 10^{-6} , 10^{-4} M) at 50 and 60 days after sowing. A foliar spray of TRIA at 10^{-6} M significantly enhanced shoot and root lengths, leaf area, fresh and dry weights, chlorophyll and carotenoid contents, activities of nitrate reductase and carbonic anhydrase, leaf-protein and carbohydrate contents and EO content of basil. GLC analysis revealed that major components of *O. basilicum* EO were linalool (41.26%), methyl eugenol (35.30%), and eugenol (12.68%) as significantly improved by TRIA application.

Keywords: eugenol, linalool, methyl eugenol, sweet basil, triacontanol

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is an essential oil producing species of Labiatae family (Grayer *et al.* 1996). It is annual herb, 20 to 70 cm in height, cultivated in tropical and warm areas, such as India, Africa and southern Asia. Basil is used in food, perfumery and pharmaceutical industries. Basically, oils are the most valuable commercial forms of basil and contribute flavors and aromas to a variety of products in the food and cosmetic industries (Putievsky and Galambosi 1999). The leaves and flowering tops of sweet basil are used as carminative, galactagogue, stomachic and antispasmodic (Duke 1989). Triacontanol (TRIA) is a long chain primary alcohol [$\text{CH}_3(\text{CH}_2)_{28}\text{CH}_2\text{OH}$] known to be a potent plant growth-promoting substance for many agricultural and horticultural crops (Ries and Houtz 1983; Ries 1985; Naeem *et al.* 2009, 2010). Most profound effect of TRIA has also been studied to enhance the growth and yield of many important cereals, vegetables, horticultural (Ries and Wert 1977; Ries and Houtz 1983; Naeem *et al.* 2009, 2010) and oil crops (Setia *et al.* 1989). Furthermore, TRIA increases protein content, water uptake (Hangarter and Ries 1978), uptake of elements (Ramani and Kannan 1980) and photosynthetic CO_2 uptake (Erihsen *et al.* 1981).

Keeping the stimulatory effect of TRIA on various characteristics in view, the main objective of the present study was to find out the ameliorative effect of TRIA on the growth, yield and essential oil components of basil.

MATERIALS AND METHODS

Plant materials and growth conditions

Healthy seeds of basil were obtained from Indian Agricultural Research Institute, New Delhi, and surface sterilized with 0.02% HgCl_2 solution for 5 min with frequent shaking and then washed

with de-ionized water. Prior to sowing seed, 5 kg homogenous mixture of soil was filled in the earthen pots (25 cm diameter \times 25 cm height). The pot experiment was conducted in a net house at the Botany Department, A.M.U., Aligarh ($27^\circ 52'$ N latitude, $78^\circ 51'$ E longitude, and 187.45 m altitude).

Experimental design and growth analysis

Foliar spray of TRIA was applied when the plants were at the stage of 2-3 true leaves. Spray of TRIA was done using a hand sprayer at 40 and 60 days after sowing (DAS). The treatments were included as control (deionised water), and different concentrations of TRIA (10^{-8} , 10^{-6} and 10^{-4} M). Growth attributes of the herb were determined at 75 DAS. Four plants from each treatment were harvested at 75 DAS. Then, the plant fresh weight was recorded. The plants were dried at 80°C for 24 h using a hot air oven and the dry weights of the plants were recorded thereafter. Each treatment was replicated four times and each replicate had three plants. Plants were grown under naturally illuminated environmental conditions. The pots were watered as and when required.

Biochemical analysis

1. Estimation of total chlorophyll and carotenoid contents

Total chlorophyll (Chl) and carotenoids content in fresh leaves were estimated using the method of (Lichtenthaler and Buschmann 2001). The fresh tissues from interveinal leaf area were ground in mortar and pestle containing 80% acetone. The optical density (OD) of the solution was read at 662 and 645 nm (Chl *a* and *b*) and 470 nm (carotenoids) using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan). The photosynthetic pigments were expressed as mg g^{-1} FW.

2. Determination of nitrate reductase activity

Nitrate reductase (NR; E.C.1.6.6.1) activity was estimated in fresh leaves by the intact tissue assay method developed by Jaworski (1971). Fresh chopped leaves, weighing 200 mg, were transferred to a plastic vial. Each vial contained 2.5 mL phosphate buffer (pH 7.5), 0.5 mL potassium nitrate solution and 2.5 mL of 5% isopropanol. After incubation, 1% sulphanilamide and 0.02% *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED-HCL) were added. The test tubes were kept for 20 min at room temperature for maximum colour development. The OD of the content was recorded at 540 nm using the spectrophotometer. NR activity was expressed as $n \text{ M NO}_2^- \text{ g}^{-1} \text{ FW h}^{-1}$.

3. Determination of carbonic anhydrase activity

The carbonic anhydrase (CA; E.C. 4.2.1.1) activity in the fresh leaves was analyzed using the method described by Dwivedi and Randhawa (1974). 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 $\mu \text{ M CO}_2 \text{ kg}^{-1} \text{ leaf FW s}^{-1}$.

Estimation of leaf nutrients

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. Dried leaves were powdered using a mortar and pestle and the powder was passed through a 72 mesh. 100 mg of oven-dried leaf-powder was carefully transferred to a digestion tube where 2 mL of AR (analytical reagent) grade concentrated sulphuric acid was added also. This solution was heated on a temperature-controlled assembly at 80°C for about 2 h and then cooled for about 15 min at room temperature. Afterwards 0.5 mL of 30% hydrogen peroxide (H₂O₂) was added to the solution. The addition of H₂O₂ followed by heating was repeated until the contents of the tube became colourless. The prepared aliquot (H₂O₂-digested leaf-material) was used to estimate per cent N, P and K in the leaves on dry weight basis.

1. Estimation of leaf-nitrogen content

Leaf-nitrogen content was estimated according to method of Lindner (1944) with slight modification by (Novozamsky *et al.* 1983). The leaf-dried powder was digested in H₂SO₄ in a digestion tube. A 10 mL aliquot was poured into a 50 mL volumetric flask where 2 mL of a 2.5 N sodium hydroxide and 1 mL of 10% sodium silicate solutions were added to neutralize the acid excess and prevent turbidity. A 5 mL aliquot of this solution was poured into a 10 mL graduated test tube and a 0.5 mL Nessler's reagent was added. The OD of the solution was recorded at 525 nm.

2. Estimation of leaf-phosphorous content

The method of Fiske and Subba Row (1925) was used to estimate the leaf-P content in the digested material. The same aliquot was used to determine the leaf-P content. A 5 mL aliquot was poured into a 10 mL graduated test tube where 1 mL of molybdic acid (2.5%) was added, followed by addition of 0.4 mL 1-amino-2-naphthol-4-sulphonic acid. When the colour became blue, the volume was increased to 10 mL with the addition of double distilled water. The OD of the solution was recorded at 620 nm.

3. Estimation of leaf-potassium content

Potassium content in the leaves was analyzed flame-photometrically (Hald 1947). Potassium content was estimated in the same aliquot (H₂O₂-digested leaf-material) with the help of emission spectra using specific filter. In the flame-photometer (Model, C150, AIMIL, India), the solution (digested leaf-material) was discharged through an atomizer in the form of a fine mist into a chamber, where it is drawn into a flame. Combustion of the element pro-

duces light of a particular wavelength [λ_{max} for K = 767 nm (violet)]. The light produced was passed through the appropriate filters to impinge upon a photoelectric cell that activates a galvanometer. Leaf-K content was estimated in the aliquot and the readings were recorded with the help of emission spectra using specific filter in a flame-photometer.

4. Estimation of leaf-protein and -carbohydrate contents

The protein content in leaves was estimated by the method of Lowry *et al.* (1951). The leaf powder (50 mg) was transferred to a mortar and 5% cold trichloroacetic acid (TCA) was added. In addition, 0.5 mL of Folin phenol reagent was added rapidly with immediate mixing. The leaf powder containing solution was maintained at room temperature for thirty minutes. Extracted protein was recorded at 660 nm. The observed readings were compared with a calibration curve obtained by using a known dilution of standard egg albumin solution. The per cent leaf-protein content was calculated on a dry weight basis.

The carbohydrate content in the dried leaves was estimated as described by Sadasivam and Manickam (2008). One hundred mg of the leaves powder was transferred into a test tube containing boiling sulphuric acid. The content was centrifuged at 4,000 rpm. Later, 4 mL of anthrone reagent was added and the OD of the resultant dark green solution was recorded at 630 nm. The reading was compared with the calibration curve obtained by using the graded dilutions of glucose. The per cent leaf-carbohydrate content was calculated on a dry weight basis.

Isolation of essential oil

The essential oil (EO) content of basil herb was extracted by using a Clevenger apparatus. Plant material was hydro-distilled using a Clevenger's apparatus for 3 h. The EO was extracted and determined gravimetrically (Clevenger 1928) and was dried over anhydrous sodium sulphate and stored in sealed glass vials at 4°C for using analysis. The EO content was calculated on a dry weight basis.

Gas liquid chromatography (GLC) analysis

The EO components were analysed by gas liquid chromatography (GLC, Nucon 5700, New Delhi) equipped with an AT-1000 stainless steel column, a flame ionization detector and an integrator. Nitrogen was used as a carrier gas. The flow rates of nitrogen, hydrogen and oxygen were 0.5, 0.5 and 5 mL s⁻¹, respectively. Detector temperature, 250°C; oven temperature, 160°C; injector temperature, 250°C; sample size, 2 μ L. Identification of active constituents (linalool, methyl eugenol and eugenol) was based on the retention time and quantified comparing with the peaks from reference standards reported in the literature (Masada 1976; Adams 2007) and thus the content (%) of different constituents was calculated.

Statistical analysis

The data were analysed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's multiple range test (DMRT) at $P < 0.05\%$. Data were presented as mean \pm SE of four replicates.

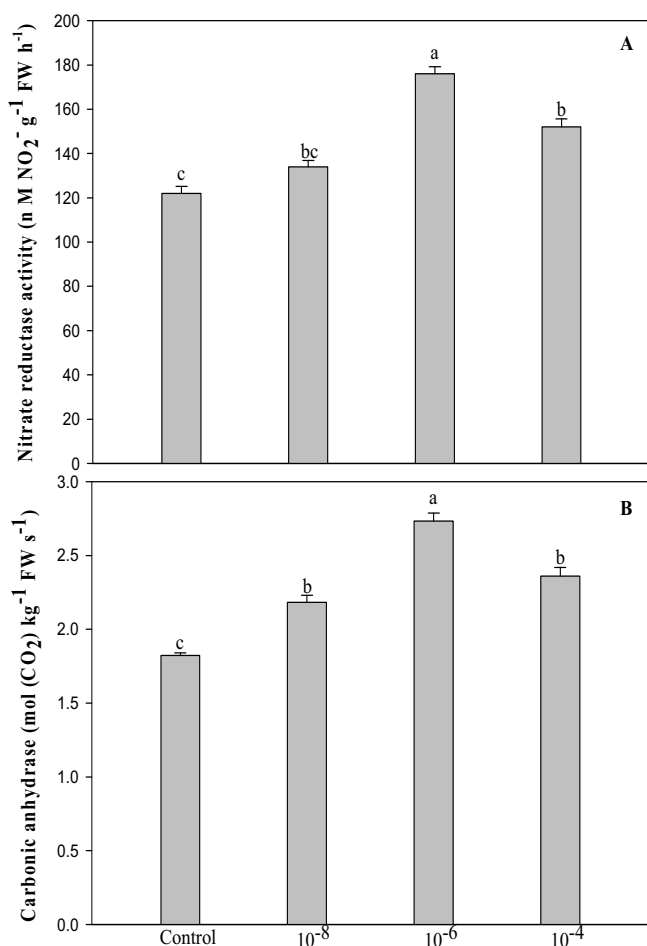
RESULTS AND DISCUSSION

Growth and yield attributes

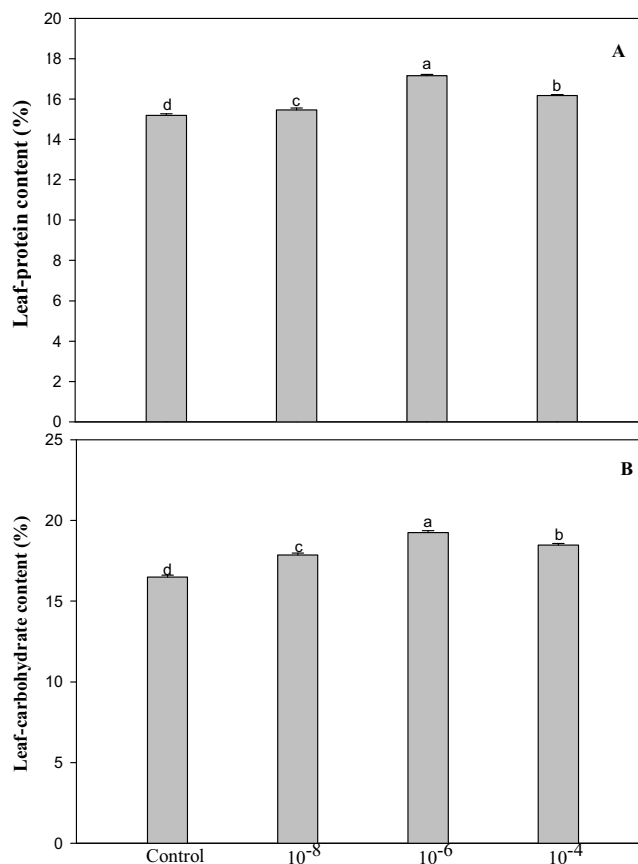
Foliar sprays of TRIA at different concentrations enhanced the overall growth and yield attributes at 75 DAS. 10⁻⁶ M TRIA proved better than the other concentrations. TRIA at 10⁻⁶ M significantly enhanced growth in terms of shoot and root lengths, number of leaves, number of spikes per plant, total leaf area, fresh and dry weights of plants by 26.7, 36.8, 68.0, 73.0, 33.3, 12.7 and 17.7% higher values for studied parameters, respectively over the control (Table 1). Our present findings are in accordance with the data of various workers, who reported that TRIA application increased

Table 1 Effect of foliar application of TRIA on growth attributes of *Ocimum basilicum* L. Means within a row followed by the same letter(s) are not significantly different ($P \leq 0.05$). The data shown are means of four replicates \pm SE.

Attributes	TRIA concentration (M)			
	Control	10^{-8}	10^{-6}	10^{-4}
Shoot length (cm)	30.00 \pm 0.112 d	32.50 \pm 0.115 c	38.00 \pm 0.121 a	33.00 \pm 0.121 b
Root length (cm)	19.00 \pm 0.062 c	22.50 \pm 0.072 b	26.00 \pm 0.081 a	22.00 \pm 0.077 b
Number of leaves	15.67 \pm 1.20 c	20.33 \pm 0.88 b	26.33 \pm 1.20 a	22.33 \pm 0.88 b
Leaf area per plant (cm ²)	9.00 \pm 0.32 d	11.50 \pm 0.36 b	13.56 \pm 0.38 a	10.80 \pm 0.37 c
Number of spikes per plant	12.33 \pm 0.88 b	14.66 \pm 1.72 b	21.33 \pm 1.20 a	16.33 \pm 0.88 ab
Fresh weight per plant (g)	10.26 \pm 0.027 d	10.48 \pm 0.040 c	11.56 \pm 0.066 a	10.66 \pm 0.030 b
Dry weight per plant (g)	4.18 \pm 0.027 d	4.40 \pm 0.046 c	4.92 \pm 0.038 a	4.69 \pm 0.054 b

**Fig. 1** Effect of four concentration of TRIA (10^{-0} , 10^{-8} , 10^{-6} , 10^{-4} M) on nitrate reductase activity (A) and carbonic anhydrase activity (B) of *Ocimum basilicum* L. studied at 75 DAS. Means within a column followed by the same letter(s) are not significantly different ($P \leq 0.05$). The data shown are means of four replicates. Error bars show SE.

yield and growth attributes in various crops (Bittenbender *et al.* 1978; Hangarter *et al.* 1978; Erihsen *et al.* 1981). The growth-promoting effects of TRIA on various growth and yield characteristics has been reported earlier by Misra and Srivastava (1991), Kumaravelu *et al.* (2000), Muthuchelian *et al.* (2003), Naeem and Khan (2005), Chaudhary *et al.* (2006), Khan *et al.* (2006), Sharma *et al.* (2006), Khan *et al.* (2007), Aftab *et al.* (2010), Idrees *et al.* (2010) and Naeem *et al.* (2009, 2010) in *Cymbopogon flexuosus*, *Vigna radiata*, *Erythrina variegata*, *Decalepis hamiltonii*, *Artemisia annua*, *Coriandrum sativum*, *Lablab purpureus*, *Capsicum annum*, *Lycopersicon esculentum*, *Glycine max*, *Papaver somniferum*, *Lablab purpureus* and *Senna occidentalis*, respectively.

**Fig. 2** Effect of four concentration of TRIA (10^{-0} , 10^{-8} , 10^{-6} , 10^{-4} M) on leaf-protein (A) and -carbohydrate contents (B) of *Ocimum basilicum* L. studied at 75 DAS. Means within a column followed by the same letter(s) are not significantly different ($P \leq 0.05$). The data shown are means of four replicates. Error bars show SE.

Biochemical parameters

1. Photosynthetic pigments

All biochemical parameters were significantly affected by the application of TRIA (Figs. 1, 2). Exogenous application of TRIA enhanced the content of photosynthetic pigments significantly. The spray of 10^{-6} M of TRIA increased Chl *a* (25.6%), Chl *b* (33.9%), total Chl (25.2%) and carotenoid contents (13.0%), respectively (Table 2). However, the values decreased when TRIA concentrations higher than 10^{-6} M were applied. The increased photosynthetic pigments in TRIA sprayed plants might be attributed to the increase in the number and size of chloroplasts as revealed by Ivano and Angelov (1997), Chen *et al.* (2003) and Muthuchelian *et al.* (2003).

2. Nitrate reductase activity

TRIA at 10^{-6} M proved optimum for NR activity and an increase of 44.6% over the control was observed (Fig 1A).

Table 2 Effect of foliar application of TRIA on chlorophyll *a* and *b* (mg g⁻¹), total chlorophyll and carotenoid content (mg g⁻¹), leaf-nitrogen (%), -phosphorous (%) and -potassium (%) contents of *Ocimum basilicum* L. Means within a row followed by the same letter(s) are not significantly different ($P \leq 0.05$). The data shown are means of four replicates \pm SE.

Attributes	TRIA concentration (M)			
	Control	10 ⁻⁸	10 ⁻⁶	10 ⁻⁴
Chlorophyll <i>a</i> content (mg g ⁻¹)	0.78 \pm 0.007 d	0.90 \pm 0.008 c	0.98 \pm 0.006 a	0.92 \pm 0.004 b
Chlorophyll <i>b</i> content (mg g ⁻¹)	0.56 \pm 0.003 c	0.67 \pm 0.004 b	0.75 \pm 0.003 a	0.68 \pm 0.003 b
Total chlorophyll content (mg g ⁻¹)	1.42 \pm 0.030 c	1.57 \pm 0.037 b	1.78 \pm 0.05 a	1.62 \pm 0.037 b
Total carotenoid content (mg g ⁻¹)	1.15 \pm 0.011 c	1.20 \pm 0.015 b	1.30 \pm 0.017 a	1.27 \pm 0.012 a
Leaf-nitrogen content (%)	2.28 \pm 0.041 c	2.55 \pm 0.070 b	3.35 \pm 0.165 a	3.35 \pm 0.165 a
Leaf-phosphorous content (%)	0.31 \pm 0.026 c	0.37 \pm 0.041 b	0.49 \pm 0.020 a	0.47 \pm 0.015 a
Leaf-potassium content (%)	2.44 \pm 0.036 c	2.59 \pm 0.030 b	2.71 \pm 0.027 a	2.69 \pm 0.020 a

Table 3 Effect of foliar application of TRIA on essential oil content and active constituents viz. eugenol, methyl eugenol and linalool contents of *Ocimum basilicum* L. Means within a row followed by the same letter(s) are not significantly different ($P \leq 0.05$). The data shown are means of four replicates \pm SE.

Attributes	TRIA concentration (M)			
	Control	10 ⁻⁸	10 ⁻⁶	10 ⁻⁴
Essential oil content (%)	0.52 \pm 0.029 c	0.56 \pm 0.030 c	0.78 \pm 0.030 a	0.66 \pm 0.017 b
Eugenol content (%)	10.56 \pm 0.030 d	10.66 \pm 0.024 c	11.90 \pm 0.032 a	10.82 \pm 0.035 b
Methyl eugenol content (%)	10.82 \pm 0.058 c	12.01 \pm 0.092 b	14.31 \pm 0.106 a	13.71 \pm 0.060 a
Linalool content (%)	46.65 \pm 1.21 c	50.31 \pm 1.20 c	65.90 \pm 1.64 a	56.70 \pm 1.00 b

However, 10⁻⁵ M TRIA exhibited a negative response compared with 10⁻⁶ M TRIA. The positive effect of TRIA application on NR activity has also been reported by Srivastava and Sharma (1990), Misra and Srivastava (1991), Kumaravelu *et al.* (2000), Muthuchelian *et al.* (2003), Aftab *et al.* (2010), Idrees *et al.* (2010) and Naeem *et al.* (2009, 2010) in case of *Papaver somniferum*, *Cymbopogon flexuosus*, *Vigna radiata*, *Erythrina variegata*, *Artemisia annua*, *Coriandrum sativum*, *Lablab purpureus* and *Senna occidentalis*. Moreover, TRIA concentration (10⁻⁶ M) significantly increased the leaf-N concentration and may have increased the capacity of leaves to assimilate more amounts of nitrogen with nitrate reductase activity.

3. Carbonic anhydrase activity

Foliar application of TRIA increased CA activity at 10⁻⁶ M proving to be the best treatment. In the present study, TRIA-treated leaves of plants possessed 29.67% higher CA activity than the control (**Fig. 1B**). A probable reason for the enhanced CA activity may be due to the influence of TRIA on the *de novo* synthesis of CA, which involves translation/transcription (Okabe *et al.* 1980).

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

TRIA significantly affected an increase in leaf-N, -P and -K contents of basil (**Table 2**). TRIA at 10⁻⁶ M increased the leaf-N, -P, and -K contents by 46.9, 58.1 and 11.1%, respectively. Control plants showed the minimum values. In fact, TRIA mediates activation of a number of membrane-bound enzymes (Ries and Houtz 1983; Ries 1991). The stimulation of these enzymes leads to dephosphorylation of L(+) forms of AMP, ADP and ATP, resulting in the formation of 9- β -L(+) adenosine, which activates a cascade of events leading to fast physiological responses (Ries 1991). Enhancement in leaf-nutrients, particularly N, due to TRIA application could be attributed to the compositional or chemical changes in plants leading to alterations in nitrogen concentration (Knowles and Ries 1981). Presumably, increased uptake of nutrients enhanced photosynthesis and improved translocation of photosynthates and other metabolites to the sinks that might have contributed to the improved yield of TRIA treated plants. Our results are in accordance with TRIA effects on the plant nutrient elements of *Oryza sativa*, *Dracaena fragrans*, *Zea mays*, *Erythrina variegata*, *Capsicum annuum*, *Lycopersicon esculentum*, *Papaver somniferum*, *Artemisia annua*, *Lablab purpureus* and *Senna occidentalis* (Knowles and Ries 1981; Ries and Houtz 1983; Kumaravelu *et al.* 2000; Chaudhary *et al.* 2006; Khan *et al.*

2006; Khan *et al.* 2007; Naeem *et al.* 2009; Aftab *et al.* 2010; Naeem *et al.* 2010).

5. Leaf-protein and carbohydrate contents

TRIA spray was effective in increasing leaf-protein content compared to the control. TRIA at 10⁻⁶ M was the best treatment and increased the protein content by 12.9% (**Fig. 2A**). The increase in protein content by TRIA application might be ascribed to increased N content in leaves that might have promoted amino acid synthesis leading to the improved protein content. The results coincide with those obtained by Knowles and Ries (1981), Ries and Houtz (1983), Kumaravelu *et al.* (2000), Muthuchelian *et al.* (2003) and Naeem *et al.* (2009, 2010) in various crops. Compared to the control, foliar spray of TRIA was also effective in increasing carbohydrate content in the dried leaves by 16.6% (**Fig. 2B**).

6. Essential oil content and its active constituents

A foliar spray of 10⁻⁶ M TRIA significantly improved EO content per plant by 50.0% over the control (**Table 3**). It also enhanced the major active constituents viz. linalool (41.3%), methyl eugenol (35.3%), and eugenol contents (12.7%) over no applied TRIA (**Table 3**). It has earlier been reported that the composition of the essential oil of essential oil bearing medicinal plants might be altered by addition of plant growth regulators (Sangwan *et al.* 2001; Swamy and Rao 2009). TRIA might trigger the intrinsic genetic potential of the plants to produce more essential oil. The increment in EO content might be due to the increase in growth attributes, nutrient accumulation or alterations in leaf oil gland population and monoterpene biosynthesis (Simon *et al.* 1990). Furthermore, the significant increase in the above mentioned parameters recorded in the treated plants could be ascribed to TRIA mediated activation of a number of membrane bound enzymes. This might have induced alterations in the biosynthetic pathways of EO constituents causing elicitation in the content of EO. The present results corroborate the findings of Misra and Srivastava (1991), Shukla and Farooqi (1992), Bhattacharya *et al.* (1996), Khan *et al.* (2007, 2009), Aftab *et al.* (2010) and Idrees *et al.* (2010) regarding various medicinal crops including *Cymbopogon flexuosus*, *Artemisia annua*, *Pelargonium graveolens*, *Papaver somniferum*, *Lycopersicon esculentum*, *Artemisia annua* and *Coriandrum sativum*. Ries and Houtz (1983) suggested that TRIA, like other plant hormones, may activate enzymes or alter a membrane, which triggers a cascading effect resulting in increased metabolism and the accumulation of various critical intermediate compounds. The

increase in the essential oil content in lavender and coriander by EBL and TRIA application has been reported earlier in this regard (Youssef and Talaat 1998; Swamy and Rao 2009 Idrees *et al.* 2010). However, no exact biosynthetic pathway linking the action of TRIA with EO production has been reported. Further research is thus required in this regard.

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