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Effects of Gibberellic Acid on Growth, Photosynthetic Efficiency and Artemisinin Content of *Artemisia annua* L.

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ABSTRACT

Plant-derived antimalarials have made and continue to make a significant contribution towards a cure of the world's most severe parasitic infection i.e. malaria. Artemisinin, isolated from *Artemisia annua* L., is potentially a drug that could be effective against multidrug-resistant strains of the malarial parasite. In the research presented here, gibberellic acid (GA_3) was used to assess the changes in growth and yield parameters, photosynthetic attributes, enzyme activities and most importantly the artemisinin content, the most effective antimalarial of recent times. Results revealed that GA_3 had a significant positive effect on growth, physiological and biochemical parameters as well as on artemisinin content. The net photosynthetic rate, stomatal conductance and internal CO_2 were 26.2, 17.7 and 13.1% higher respectively, than control plants when 10^{-6} M GA_3 was applied. At this concentration, total chlorophyll content, NR and CA activities were 11.2, 22.7 and 21.3% higher than the control. Also, overproduction of artemisinin content (23.9% more compared to control) was noted in plants grown with 10^{-6} M GA_3.

Keywords: antimalarial drugs, carbonic anhydrase, nitrate reductase, plant growth regulators, sesquiterpene lactone

INTRODUCTION

Artemisia annua L. is an aromatic plant which has been used for centuries in Chinese traditional medicine for the treatment of fever and malaria (Klayman 1985). Being the world's most severe parasitic infection, malaria threatens more than one-third of the global population, killing approximately two million people annually (Snow *et al.* 2005). Despite tremendous efforts to control malaria, global morbidity and mortality have not significantly changed in the last 50 years (WHO 2006). Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has become increasingly popular as an effective and safe alternative therapy against malaria (Abdin *et al.* 2003). This endoperoxide bridge rarely exists in natural products but is essential for the medical function of artemisinin (Woerdenbag *et al.* 1990; Balint 2001). Since the non-natural chemical synthesis of artemisinin is very costly, the intact plant remains the only viable source of artemisinin production, and the enhanced production of artemisinin content in the whole plant is highly desirable (Abdin *et al.* 2003; Aftab *et al.* 2010).

Plant growth regulators (PGRs) stimulate growth and terpenoid biosynthesis in various aromatic plants, which can result in beneficial changes in both the quality and quantity of terpenoids (Shukla *et al.* 1992). Biosynthesis of terpenoids is dependent on primary metabolism, e.g. photosynthesis and oxidative pathways for carbon and energy supply (Singh *et al.* 1990). Gibberellic acid (GA_3), a phytohormone, exhibits a broad spectrum of physiological effects in plants. It induces growth, photosynthesis, flowering and cell expansion (Yuan and Xu 2001; Taiz and Zeiger 2006). GA_3 also enhances the metabolic activity within pathways leading to stress (defence-related secondary metabolites) and anthocyanin biosynthesis (Ohlsson and Bjork 1988). Various advancements have been made in the past to stimulate artemisinin biosynthesis (Shukla *et al.* 1992; Ferreira 2007; Ozguven *et al.* 2008; Pu *et al.* 2009; Mannan *et al.* 2010), but scientific cultivation still remains the most effective tool as it requires less effort and resources. In the present study the effects of GA_3 on the growth, photosynthetic efficiency, enzyme activities and artemisinin production in *Artemisia annua* L. are assessed.

If the productivity and artemisinin level can be raised in this immensely important antimalarial plant through the application of PGRs, it would be a great achievement. Therefore, the present study was taken up to assess the effects of GA_3 growth, physiological and biochemical parameters, and changes in artemisinin level in *A. annua.*

MATERIALS AND METHODS

A pot culture experiment was designed to analyze the changes in physiological and biochemical attributes of *A. annua* using the different concentrations of GA_3: 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M applied as foliar sprays on the leaves of the plant, three times starting from the time when plants were 90 days old. Seeds of *A. annua* cv. 'Jeevanraksha' were initially surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with double distilled water before sowing. Prior to sowing seed, 5.0 kg homogenous mixture of soil and farmyard manure (4: 1) was filled into each pot and uniform doses of N, P and K at a rate of 80, 40 and 40 mg/kg soil were applied. The soil samples were tested at the Government Soil Testing Laboratory, Quarsi Farm, Aligarh. Physico-chemical characteristics of the soil were: texture-sandy loam, pH (1:2) – 8.0, E.C. (1:2) -0.48 m mhos/cm, available N, P and K – 47.46, 10.21 and 107.0 mg kg^{-1} soil, respectively. Then seeds were sown at a depth of 2 cm in earthen pots (25 cm diameter × 25 cm height) containing sandy-loam soil. Growth and biochemical attributes of *A. annua* were determined at the flowering stage i.e. 150 days after sowing (DAS). The experiment was conducted according to a simple randomized complete block design. Each treatment was replicated five times and each replicate had three plants. The pots were watered regularly as and when required and one healthy plant was maintained per pot. Plants were grown under naturally illuminated environmental conditions.

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Table 1 Effect of different concentrations of GA_3 on growth and yield attributes of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$). The data shown are means of five replicates ± SE.

Growth and yield attributes	GA_3 concentrations (M)				
	Control	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Shoot length (cm)	105.3 ± 1.03 e	116.7 ± 1.22 d	123.9 ± 1.21 c	141.3 ± 1.36 a	135.5 ± 1.14 b
Shoot FW (g)	403.2 ± 6.34 e	421.6 ± 6.45 d	447.3 ± 7.19 c	574.1 ± 7.78 a	571.4 ± 6.77 b
Shoot DW (g)	173.3 ± 1.91 e	179.5 ± 2.44 d	201.6 ± 2.71 c	226.2 ± 2.53 a	215.4 ± 2.41 b
Fresh leaf yield (g)	301.6 ± 3.29 d	309.8 ± 3.33 c	337.3 ± 3.53 bc	379.4 ± 3.67 a	369.2 ± 3.66 b
Dry leaf yield (g)	113.8 ± 1.23 d	117.5 ± 1.25 c	129.2 ± 1.58 b	138.6 ± 2.06 a	135.3 ± 1.91 ab

Growth and yield characteristics

The plants from each treatment were carefully harvested with the roots and shoot height was recorded. Plants were washed with tap water to remove adhering foreign particles. Roots of the plant were removed and fresh mass of the shoots was recorded individually. The shoots were dried at 80°C for 48 h, and dry mass was then recorded. Total leaves of the plants were weighed to determine leaf yield.

Photosynthetic attributes and chlorophyll content

Net photosynthetic rate (P_N), stomatal conductance (gs) and internal CO_2 (c_i) were measured on sunny days at 11:00 a.m. using fully expanded leaves of *A. annua* with the help of an IRGA (Infra Red Gas Analyzer, LI-COR 6400 Portable Photosynthesis System, Lincoln, Nebraska, USA). Before recording the measurement, the IRGA was calibrated and zero was adjusted approximately every 30 min during the measurement period. Each leaf was enclosed in a gas exchange chamber for 60 s. All the attributes measured by IRGA were recorded three times for each treatment. Total chlorophyll content in fresh leaves was estimated by the method of Lichtenthaler and Buschmann (2001). The fresh tissue from interveinal leaf-area was ground using a mortar and pestle containing 80% acetone. The absorbance of the solution was recorded at 662 and 645 nm for chlorophyll estimation using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

Nitrate reductase and carbonic anhydrase activities

Nitrate reductase (NR; E.C. 1.6.6.1) activity in the leaf was determined by the intact tissue assay method of Jaworski (1971). Chopped leaf pieces (200 mg) were incubated for 2 h at 30°C in a 5.5 mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. The nitrite formed subsequently was colorometrically determined at 540 nm after azocoupling with sulphanilamide and naphthylene diamine dihydrochloride. The NR activity was expressed as nM NO_2 g^{-1} FW h^{-1}. Carbonic anhydrase (CA; E.C. 4.2.1.1) activity was measured in fresh leaves using the method as described by Dwivedi and Randhawa (1974). 200 mg of fresh leaf pieces were weighed and transferred to Petri dishes. 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 0.022% 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}.

Artemisinin extraction and estimation

Dry leaf material (1 g) was used for the estimation of artemisinin modified to a compound Q_{260} and quantified using HPLC (Zhao and Zeng 1986). A standard curve was prepared using 1 mg of standard artemisinin dissolved in 1 mL of HPLC-grade methanol to make the stock solution. It was extracted with 20 mL petroleum ether in shaker at 70 rpm for 24 h. After 24 h, solvent was decanted and pooled and 20 mL of petroleum ether added again and this step was repeated three times. Petroleum ether fractions were pooled and concentrated under reduced pressure and residues defatted with CH_3CN (10 mL × 3). Precipitated fat was filtered out and filtrate concentrated under reduced pressure. Residues were dissolved in 1 mL of methanol. 100 μL aliquot of each sample of each treatment was removed and to this 4 ml of 0.3% 1 N NaOH was added. The samples were incubated in a shaking water bath at 50°C for 30 min, thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% MeOH). The pH of the solution was maintained at 6.8. Derivatized artemisinin was analyzed and quantified through reverse phase column (C18; particle size, 5 μm; inner diameter, 4.6 mm; length, 250 mm) using premix methanol: 10 mM K-Phosphate buffer (pH, 6.5) in the ratio of 60: 40 as the mobile phase at a constant flow rate of 1 mL/min, with the detector set at 260 nm. Artemisinin was quantified against the standard curve of artemisinin (98% pure), obtained from Sigma–Aldrich, USA.

Statistical analysis

Each pot was treated as one replicate and all the treatments were replicated five times. The data was analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's Multiple Range Test (DMRT) at $P < 0.05$ following mean separation using ANOVA.

RESULTS

Shoot length of the treated plants increased at different GA_3 concentrations and maximum height was attained when plants were treated with 10^{-6} M. The increase in shoot height of 10^{-5} M-treated plants was 34.2% higher than control plants (**Table 1**). Shoot fresh weight (FW) and dry weight (DW) of the plant were significantly altered by different GA_3 treatments. At 10^{-5} M GA_3 the FW and DW of the shoots increased by 25.9 and 23.5%, respectively compared with the control (**Table 1**). Fresh and dry leaf yield were also determined in the present study and the most pronounced effect was noted at 10^{-5} M GA_3, 24.5 and 22.8% higher than that control plants, respectively (**Table 1**).

Photosynthetic efficiency was positively regulated by GA_3, but maximum photosynthetic efficiency was recorded at 10^{-5} M GA_3. At this treatment, net photosynthetic rate, stomatal conductance and internal CO_2 were 26.2, 17.7 and 13.1% higher, respectively than control plants (**Fig. 1A-C**). Total chlorophyll content was also 11.2% higher at this treatment than the control (**Fig. 1D**).

NR and CA activities were enhanced by different GA_3 treatments and a 22.7 and 21.3% increment in NR and CA activities was noticed when 10^{-5} M GA_3 was applied (**Fig. 2A, 2B**).

A significant upregulation in artemisinin biosynthesis by GA_3 application was observed in the present study. At 10^{-5} M GA_3 the artemisinin content was maximum, 23.9% more than that of untreated plants (**Fig. 3A**). Artemisinin yield was more pronounced at this concentration than the control (**Fig. 3B**), 50.2% more.

DISCUSSION

GA_3 significantly affected artemisia growth and yield attributes. 10^{-5} GA_3 enhanced shoot length significantly when applied to the foliage. It is well known that GA_3 promotes cell enlargement and cell division (Buchanan *et al.* 2000; Taiz and Zeiger 2006) and the effect was also proved effective in the studies of Khan *et al*. (2002) and Siddiqui *et al.* (2008) in mustard. Sadowska *et al.* (1984) reported that GA_3 as a

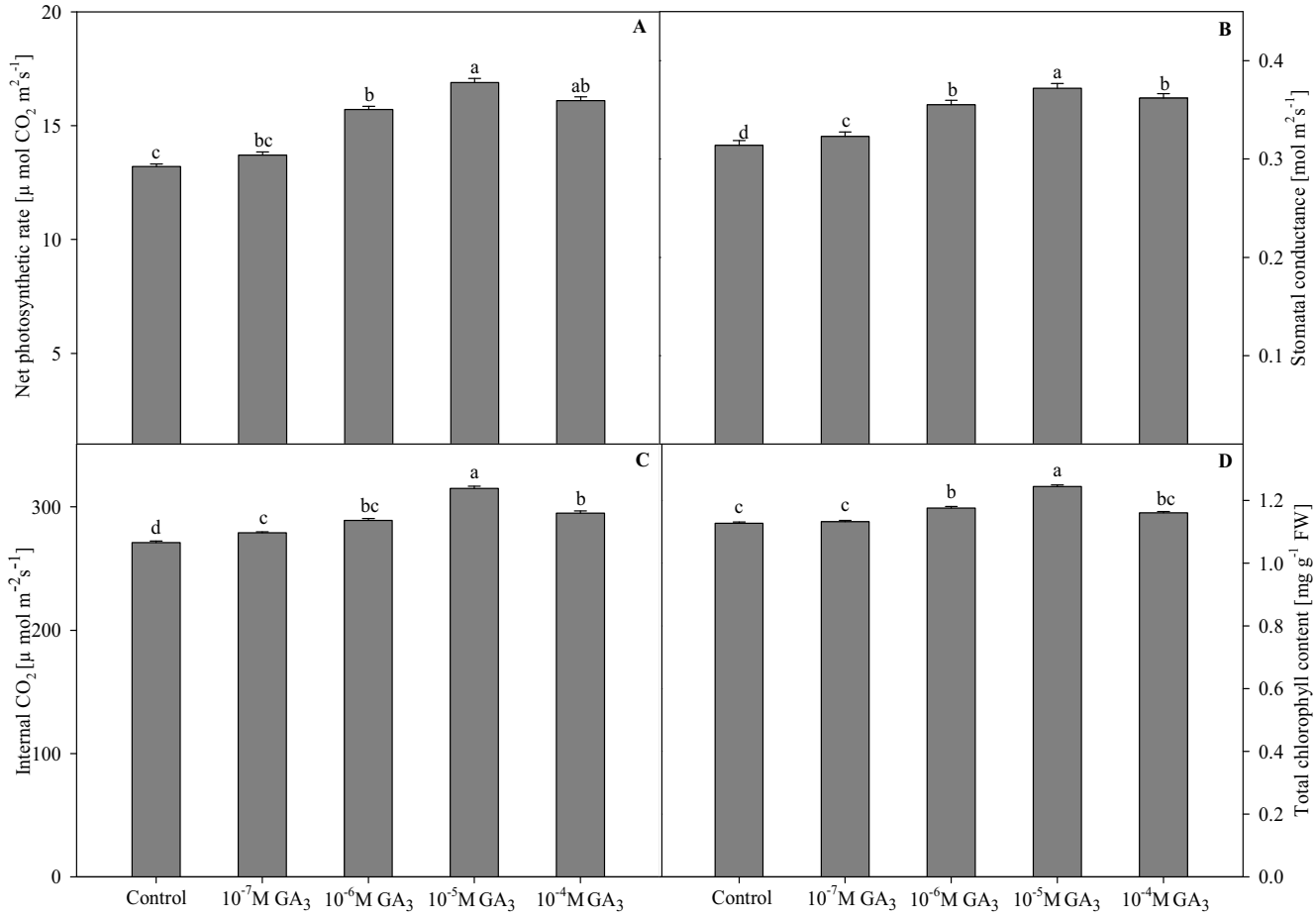

Fig. 1 Effect of different concentrations of GA_3 on photosynthetic rate (A), stomatal conductance (B), internal CO_2 (C) and total chlorophyll content (D) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars show SE.

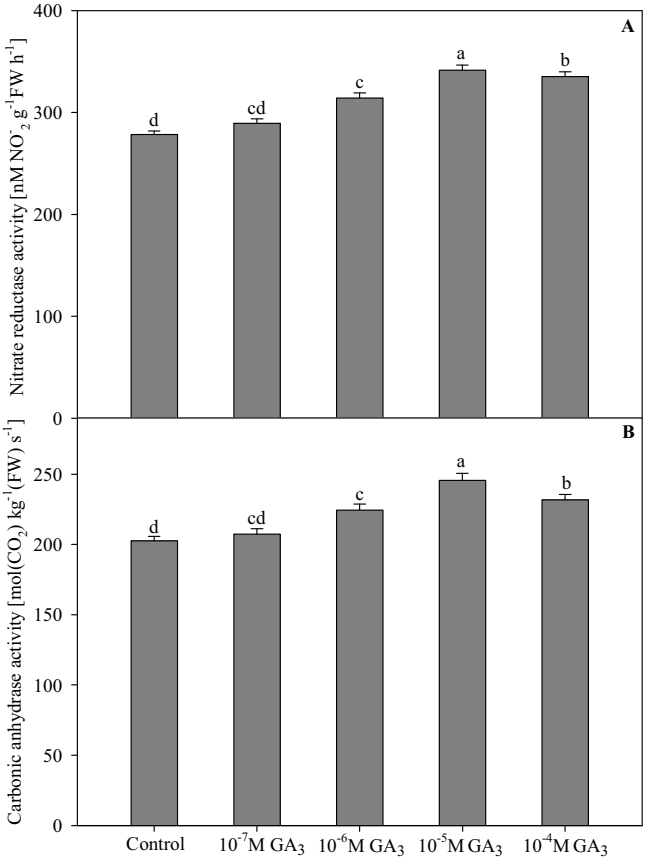

Fig. 2 Effect of different concentrations of GA_3 on nitrate reductase (A) and carbonic anhydrase (B) activities of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars show SE.

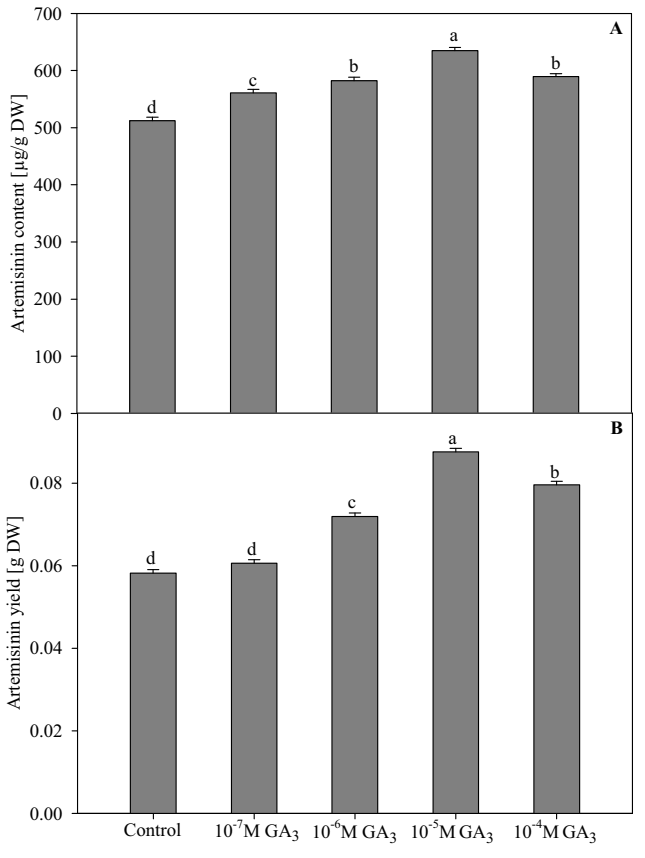

Fig. 3 Effect of different concentrations of GA_3 on artemisinin content (A) and artemisinin yield (B) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars show SE.

foliar spray on transplanted cuttings of *Catharanthus* increased plant height. Srivastava and Srivastava (2007) reported that a foliar spray of GA_3 increased plant height and leaf length of *Catharanthus* plants. Ferreira *et al.* (2005), Ritchey and Ferreira (2006) reported improved growth of *A. annua* plants by GA_3 applications. The FW and DW as well as fresh and dry leaf yields of the plant increased with GA_3 applications and most by 10^{-5} M GA_3. The present results are in accordance with a known fact that exogenous application of PGRs evokes the intrinsic genetic potential of the plant causing an increase in elongation of internodes as a consequence of cell division and cell wall extensibility (Moore 1989; Taiz and Zeiger 2006; Khan *et al.* 2007; Idrees *et al.* 2010). Shrivastava and Shrivastava (2007) and Shah *et al.* (2007) reported increased biomass production by GA_3 in *Catharanthus roseus* and *Nigella sativa*, respectively.

A significant improvement was found in photosynthetic attributes when varying GA_3 doses were applied, although 10^{-5} M GA_3 was optimum in enhancing photosynthetic attributes. An increase in total chlorophyll and photosynthetic CO_2 assimilation and specific activity of Rubisco by GA_3 (Taiz and Zeiger 2006) has already been reported. The increased content of photosynthetic pigments could be attributed to an increase in the number and size of chloroplasts and to the enhancement of ultrastructural morphogenesis of plastids by GA_3 (Arteca 1996). In fact, a number of studies have demonstrated an increased rate of CO_2 fixation in plants by the application of nanomolar concentrations GA_3 (Guoping 1997; Khan *et al.* 2009). A probable reason for the enhancement of CA activity, as observed in the present study, due to GA_3 might be the *de novo* synthesis of CA, which involves translation/transcription of the genes associated (Okabe *et al.* 1980).

NR is the key enzyme in nitrogen metabolism and is responsible for the initiation of nitrate assimilation and hence protein synthesis. An increase in NR activity by application of GA_3 may have exerted a pivotal role in enhancement of photosynthetic rate. The ultimate culmination of enhancement of NR activity increased the overall growth and yield of treated plants. NR catalyzes one of the most controlled reactions in plants, receiving inputs from light photosynthesis, CO_2, oxygen availability and nutrient status at the transcriptional, post-transcriptional and post-translational levels (von Wiren *et al.* 2000). Roth-Bejerano and Lips (1970) suggested that the NR activity is highly variable and depends on the presence of hormones such as gibberellins and/or cytokinin.

CA has many diverse roles in physiological processes such as ion-exchange, carboxylation/decarboxylation reactions and inorganic carbon diffusion between the cell and its environment as well as within the cell (Georgios 2004). In the present study CA activity improved over the control in GA_3-treated plants. The enhanced CA activity compared to control by GA_3 was observed in mustard and black cumin (Khan *et al.* 1998; Shah *et al.* 2007; Siddiqui *et al.* 2008; Khan *et al.* 2009). This increase in enzyme activity can be attributed to the fact that in the presence of an optimum nutrient supply hydration of CO_2 is catalyzed more rapidly, thereby increasing the supply of Rubisco and probably it may be a strong reason behind the improved photosynthesis in treated plants (Siddiqui *et al.* 2008). 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 treated plants. These findings are in accordance with those of Santos *et al.* (1998) and Shah *et al.* (2007), who reported improved productivity by 10^{-5} GA_3 in treated basil and black cumin plants. An increase in membrane permeability would facilitate absorption and utilization of mineral nutrients (Khan *et al.* 1998) and also transport of assimilate. Moreover, it contributes towards enhancing the capacity of the treated plants for biomass production as reflected by FW and DW of the shoots.

This increase in the above-mentioned parameters of treated plants is expected to culminate in the maximization of artemisinin content and yield. The concentration of artemisinin in the dry leaves was significantly higher in all treated plants than in the control. The artemisinin concentration was highest at 10^{-5} M GA_3-treated plants than in other treatments. It is well known that artemisinin production maximally produced at flowering or just before flowering and GA_3, being recognised as a hormone which plays an important role in flowering, might contribute to some stimulatory effect on artemisinin biosynthesis. Terpenoid biosynthesis through the mevalonate-isoprenoid pathway occurs in oil glands which are present in the leaves of the many aromatic plants. The substances which can improve leaf development and herbage have been used to increase terpenoid yield (Ries and Wert 1977). In fact, the leaves are the major site of trichomes in which biosynthesis of artemisinin occurs and having a well known role of GA_3 in the vegetative growth the increase in artemisinin content by application of GA_3 is obvious. But how GA_3 influences trichome development and density is still unclear. Now it is unequivocally proved that two distinct and independent biosynthetic routes exist to isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), the two building blocks for isoprenoids in plants. Artemisinin is synthesized from farnesyl pyrophosphate (FPP) via amorpha-4,11-diene and dihydroartemisinic acid involving many steps, it was tempting to suppose that the addition of GA_3 and N might contribute to some mechanism for stimulating artemisinin biosynthetic pathway (Akhila 2007). Terpenoid biosynthesis is an integration of several metabolic pathways which require linking of several steps such as continuous production of precursors, their transport and translocation to the active site of synthesis (Singh *et al*. 1999). The plant may adopt its metabolic pathway in response to nutrient imbalance, hormone application, etc. The exact mechanism involving GA_3 on the stimulation of artemisinin biosynthesis, however, needs further investigation.

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