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Effect of irradiated sodium alginate and phosphorus on biomass and artemisinin production in *Artemisia annua*

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ABSTRACT

It is now being realized that irradiation products of natural bioactive agents can also be beneficially utilized to impart value addition in agriculture by converting these bioactive agents into more useful form. Polysaccharides, such as sodium alginate, have proven to be wonderful growth promoting substances in their depolymerized form for various plants. Artemisinin has been increasingly popular as an effective and safe alternative therapy against malaria; also proved effective against the highly adaptable malaria parasite, which has already become resistant to many other drugs. The drug artemisinin can be extracted from the leafy tissues of *Artemisia annua*. Therefore, experiments were conducted with an aim to evaluate artemisinin production and overall plant development though depolymerized sodium alginate application and nutrient supply. In the present study, sodium alginate, irradiated by Co-60 gamma rays together with various phosphorus doses, was used to study their effect on growth, physiological and biochemical processes and production of artemisinin in *A. annua*. Among various applied doses of phosphorus fertilizer, P_{40} (40 kg P ha^{-1}) together with ISA_{80} (80 mg L^{-1}) significantly improved all the parameters studied. Increase in plant height as well as weight was noted at this treatment. Dry leaf yield, artemisinin concentration in leaves and artemisinin yield was also significantly enhanced by the treatment.

1. Introduction

Sodium alginate is a natural polysaccharide, derived from brown algae, with a large quantity of it available in nature. Alginates have widespread applications in the industries of pharmaceutical and bioengineering products and those of foods and drinks (Gaseca, 1988; Hien et al., 2000). Oligomers obtained from depolymerization of sodium alginate have been reported to have novel features such as stimulation of growth, promotion of germination and shoot elongation in plants (Akiyama et al., 1992; Natsume, Kamao, Hirayan, & Adachi, 1994; Tomoda, Umemura, & Adachi, 1994). Further, these biologically active oligosaccharides have been known to act as signal molecules that might regulate the growth and development as well as defence reactions in plants by regulating gene expression (Albersheim & Darvill, 1985). As compared to the conventional techniques, like acid or base hydrolysis or enzymatic methods (Shimokawa, Toida, & Kawashima, 1996), radiation processing of alginates by Co-60 gamma rays (Nagasawa, Mitomo, Yoshii, & Kume, 2000; Lee et al., 2003) offers rapid, inexpensive, safe and a clean one step method for the formation of the low molecular weight oligomers. Hu, Jiang, Hwang, Liu, and Guan (2004) and Hegazy, Abdel-Rehim, Diaa, and El-Barbary (2009) investigated that irradiated sodium alginate (ISA), obtained by radiation processing, enhanced not only the growth but also increased the productivity of *Zea mays* plants. They postulated that irradiated sodium alginate (ISA) could be used for agriculture purposes in order to promote the growth of crop plants. Besides, the ISA has been reported to enhance the production of alkaloids and essential oil in opium poppy, fennel and lemongrass (Idrees et al., 2011, 2012; Khan, Khan, Aftab, Idrees, and Naeem, 2011; Sarfaraz et al., 2011).

Malaria is the world's most severe parasitic infection, causing more than a million deaths out of more than 500 million cases reported annually (WHO, 2002). Artemisinin, isolated from *Artemisia annua*, responsible for antimalarial and anti-cancer activities, contains an endoperoxide bridge, which is rarely found in other secondary metabolites. It is a promising drug as it lacks cross resistance with other anti-malarial drugs and is known to

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have no adverse effects on human health together with its ability to clear the blood more rapidly than the other available drugs of malaria (Meshnick, 2002). Complete chemical (de novo) synthesis of artemisinin has, so far, been achieved by several research groups (Avery, Chong, & Jennings-White, 1992; Ravindranathan, Kumar, Menon, & Hiremath, 1990; Schmid & Hofheinz, 1983; Xu, Zhu, Huang, & Zhou, 1986). The procedure requires several steps and can start with different raw materials. However, because of low yield, complexity and high manufacturing cost, the isolation of artemisinin from the *A. annua* plant is the most feasible and economic method for its commercial production. Since 2001, artemisinin-based combination therapies (ACTs) have been recommended by the World Health Organization (WHO, 2002). This resulted in the enhanced demand for artemisinin that ultimately led to its supply shortages in the pharmaceutical markets. Although the situation has recently been reported to be under control (Roll Back Malaria, 2004), it is the need of the hour to explore scientific methods to enhance the productivity of *A. annua* L. in conjunction with cutting short the manufacturing cost of artemisinin (Aftab, Khan, Idrees, Naeem, Singh, et al., 2010; Aftab et al., 2012, 2013).

Understanding the usefulness of the unique oligomers, obtained by irradiating the sodium alginate, in augmenting the plant growth and production and considering the well known role of macronutrients in the overall improvement of physiology and biochemistry of plants, field experiments were conducted to test the hypothesis whether ISA in combination with phosphorus can enhance the growth, productivity, photosynthesis, enzyme activities and the content and yield of artemisinin of *A. annua*.

2. Materials and methods

The field trials were conducted in 2009–2010 and 2010–2011. In these experiments, the effect of foliar spray of 80 mg L^{-1} irradiated sodium alginate (ISA_{80}) with different doses of soil-applied phosphorus was studied. The dose of ISA was selected on the basis of our previous study (Aftab, Khan, Idrees, et al., 2011). Initially, the seeds were sown in the seedbeds (1 m × 1 m) to obtain seedlings in the month of November for both years' experiments. Then, seedlings were transplanted to the field plots of 9 m^2 (3 m × 3 m) having row to row and plant to plant distance of 50 cm. The plants were irrigated two times a week throughout the study. The treatments were consisted of control (spray of double distilled water) and 80 mg L^{-1} ISA with graded levels of phosphorus viz. 10 kg ha^{-1}, 20 kg ha^{-1}, 30 kg ha^{-1}, 40 kg ha^{-1} and 50 kg ha^{-1}. The plants were sprayed five times at one week interval starting from 45 days after planting (DAP) using a sprayer while phosphorus was applied to the field before planting. There were four replicates of each treatment. The samplings were done at pre-flowering stage (90 DAP) and flowering stage (120 DAP). Before transplanting, organic manure at the rate of 10 tonnes ha^{-1} was applied to the field.

2.1. Irradiation of sodium alginate and gel permeation chromatography (GPC)

Sodium alginate (Sigma Aldrich, USA) was irradiated in a Gamma Chamber (Cobalt-60, GC-5000) made by BRIT, Mumbai, India. The samples were irradiated to 520 kGy gamma radiation dose at a dose rate of 2.4 kGy/h. GPC of sodium alginate samples were done on Hitachi EMerck HPLC/GPC system using RI detector. The experimental conditions were as follows: Mobile phase-water, flow rate – 1.5 mL/min, column PL-Aquagel, mixed bed column, 300 mm × 10 mm, 20 μL injection loop. The average molecular weight of the un-irradiated sodium alginate samples was estimated to be about 695,131. Polyvinyl alcohol polymers of known molecular weight were used as standards.

2.2. Analyses of growth and yield parameters

The plants from each treatment were uprooted carefully and then shoot and root lengths were recorded. Plants were washed with tap water to remove adhering foreign particles. Roots of the plants were removed and fresh mass of the shoots was recorded after surface drying the shoots. The shoots were dried in a hot air oven at 80 °C for 48 h, and then shoot dry mass was recorded. Total dry leaves of the plants were weighed to determine leaf yield.

2.3. Photosynthetic parameters

Net photosynthetic rate (P_N) was measured on sunny days at 1100 h using fully expanded leaves with the help of 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 the gas exchange chamber of IRGA for 60 s. All the photosynthetic attributes measured were recorded three times for each treatment.

Total chlorophyll content was estimated in the youngest fully expanded leaves by the method of Lichtenthaler and Buschmann (2001). The fresh tissue from the interveinal leaf-area was ground using a mortar and pestle with 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).

2.4. Assay of nitrate reductase and carbonic anhydrase activity

Nitrate reductase (NR; E.C. 1.6.6.1) activity in the leaves 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 that formed subsequently, was calorimetrically determined at 540 nm after azocoupling with sulphanilamide and naphthylene diamine dihydrochloride. 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). Two hundred mg of fresh leaf pieces were incubated in Petri dishes, containing 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}.

2.5. Estimation of leaf -N, -P and -K contents

Leaf N content was estimated according to method of Lindner (1944) with slight modification by Novozamsky, Houba, Eck, and Vark (1983). The leaf dried powder was digested in H_2SO_4 in a digestion tube. A 10 mL aliquot (peroxide-digested-material) 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, using the spectrophotometer.

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 digested material. The peroxide-digested material 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 using the spectrophotometer.

Potassium content in the leaves was analyzed flame-photometrically (Hald, 1946). It was estimated in the aliquot with the help of emission spectra using specific filters. 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 is drawn into a flame. Combustion of the element produces light of a particular wavelength [λ_{max} for K = 767 nm (violet)]. The light produced was passed through the appropriate filter to impinge upon a photoelectric cell that activates a galvanometer. Readings were recorded with the help of emission spectra using specific filter in a flame-photometer (Model, C150, AIMIL, India).

2.6. Determination of H_2O_2 content

The content of H_2O_2 in the leaves was determined according to the method of Mukherjee and Choudhuri (1983). The youngest fully developed leaves (0.5 g) were homogenized using a cooled mortar and pestle using pre-cooled acetone (5 mL). The homogenate was centrifuged at 12,000 × g for 5 min. One milliliter of the supernatant was mixed with 0.1 mL of 5% $Ti(SO_4)_2$ and 0.2 mL of 19% ammonia. After a precipitate was formed, the reaction mixture was centrifuged at 12,000 × g for 5 min. The resulting pellet was dissolved in 3 mL of 2 M H_2SO_4 and the absorbance was recorded at 415 nm using the spectrophotometer. The H_2O_2 concentration was calculated according to the standard curve of H_2O_2 was prepared by using known concentrations of H_2O_2 ranging from 0 to 10 μM.

2.7. Artemisinin extraction and estimation

Dry leaf material (1 g) was used for the estimation of artemisinin that was modified to a compound Q_{260} and quantified using an HPLC method (Zhao & Zeng, 1986). A standard curve was prepared using 1 mg of standard artemisinin (Sigma–Aldrich, St. Louis, USA) dissolved in 1 mL of HPLC-grade methanol to make the stock solution. It was extracted with 20 mL petroleum ether using a shaker maintained at 70 rpm for 24 h. After 24 h, solvent was decanted and pooled and 20 mL of petroleum ether was again added; 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 and the filtrate concentrated under reduced pressure. Residues were dissolved in 1 mL of methanol. A 100-μL aliquot of each sample of each treatment was added to 4 mL of 0.3% NaOH. The samples were incubated in a shaking water bath maintained at 50 °C for 30 min, thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% MeOH). The pH (6.8) of the solution was maintained. Derivatized artemisinin was analyzed and quantified through a reverse phase column (C18; 5 μm; 4.6 mm; 250 mm) using a premix of methanol: 10 mM K-Phosphate buffer (pH, 6.5) in a 60:40 ratio having 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.

2.8. Statistical analysis

Data presented in this paper is the mean of two year experiments. Experiments were conducted according to simple randomized complete block design. Data were analyzed statistically using SPSS statistical software v. 17 (SPSS Inc., Chicago, IL, USA). Mean values were compared statistically by Duncan's multiple range test (DMRT) at $p < 0.05$.

3. Results

Degradation scheme and molecular weight distribution of unirradiated and irradiated sodium alginate is presented in Fig. 1a and b. The distribution curve in the GPC profile shows the elution of different molecular weight fractions against time. The 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 695,131 was observed in the control and 595,000 for the irradiated samples. However, considering the molecular weight values in Fig. 1, it may be said that these value may fall in natural variation of SA. The lower molecular weight fraction (less than 100,000) which is coming at the end of the profile is very small.

The effect of ISA_{80} application combined with different levels of soil-applied phosphorus was found significant on the shoot length per plant. Treatment $ISA_{80} + P_{40}$ enhanced shoot length per plant by 35.0 and 40.4% as compared with that of control at pre-flowering and flowering stages, respectively (Table 1). An increase in root length per plant was noted when ISA was applied with different doses of phosphorus. $ISA_{80} + P_{40}$ improved root length per plant by 33.4 and 41.2% over the control at pre-flowering and flowering stages, respectively. The values recorded for all treatment differed from each other at both growth stages of sampling (Table 1). Regarding the combined effect of foliar ISA and soil-applied phosphorus, it was noted that $ISA_{80} + P_{40}$ increased shoot fresh weight per plant gradually over the control. Application of $ISA_{80} + P_{40}$ proved optimum at both the growth stages of sampling. $ISA_{80} + P_{40}$ showed an increase of 23.5 and 24.7% over the control at pre-flowering and flowering stages, respectively (Table 1). Application of foliar ISA with soil-applied phosphorus on shoot dry weight per plant was found effective over the control at both stages of sampling. $ISA_{80} + P_{40}$ enhanced shoot dry weight per plant by 22.1 and 25.3% at pre-flowering and flowering stages, respectively. However, the effect of $ISA_{80} + P_{30}$ and $ISA_{80} + P_{50}$ was significantly similar on this parameter (Table 1).

Net photosynthetic rate was affected significantly by combined application of foliar ISA and soil-applied phosphorus at both growth stages of sampling. $ISA_{80} + P_{40}$ showed maximum effect and significantly increased net photosynthetic rate by 29.6 and 32.6% at pre-flowering and flowering stages, respectively over the control (Table 2). An increase in stomatal conductance was noted when ISA was applied with different doses of phosphorus. $ISA_{80} + P_{40}$ improved stomatal conductance by 30.7 and 31.1% over the control at pre-flowering and flowering stages, respectively. The values recorded for all treatment differed from each other at flowering stage, while $ISA_{80} + P_{30}$ and $ISA_{80} + P_{50}$ showed same response at pre-flowering stage (Table 2). Concerning the combined effect of foliar ISA and soil-applied phosphorus, it was noted that $ISA_{80} + P_{40}$ increased internal CO_2 concentration gradually over the control. Application of $ISA_{80} + P_{40}$ proved optimum at both the growth stages of sampling. $ISA_{80} + P_{40}$ showed an increase of 27.5 and 28.4% over the control at pre-flowering and flowering stages, respectively (Table 2). A progressive increase in chlorophyll content by combined application of foliar ISA and soil-applied phosphorus was noted at both growth stages. $ISA_{80} + P_{40}$ treatment proved best and gave significantly maximum value at all three stages of sampling. The percent increase in chlorophyll content resulted from application of $ISA_{80} + P_{40}$ was 25.3 and 28.2% over the control at pre-flowering and flowering stages, respectively (Table 2).

Among various combined treatments of ISA and phosphorus, $ISA_{80} + P_{40}$ proved best and gave significantly maximum value for

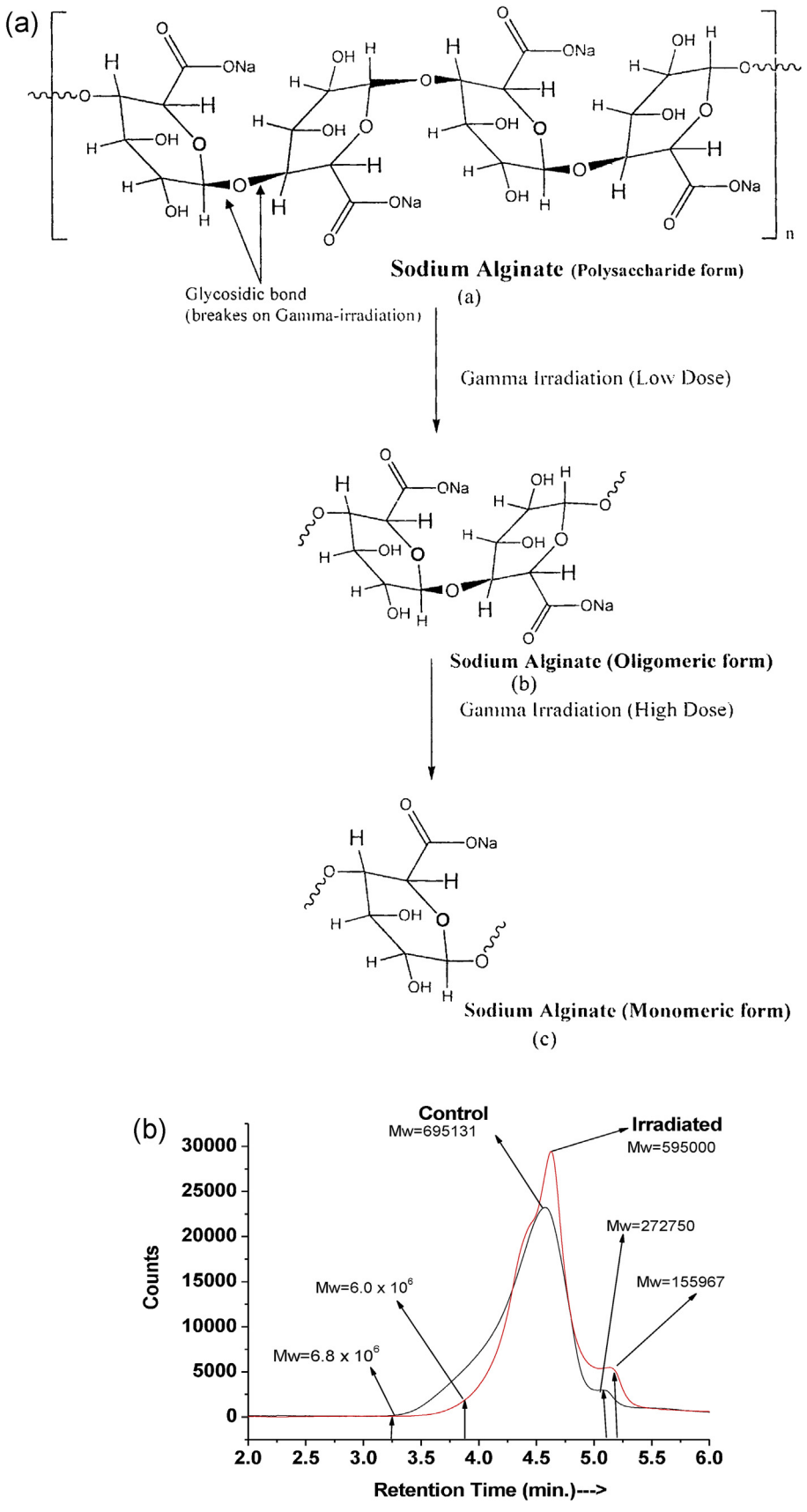

Fig. 1. (a) Degradation of sodium alginate upon irradiation, (b) molecular weight distribution of un-irradiated and irradiated sodium alginate.

Table 1
Effect of ISA along with different doses of phosphorus on shoot and root lengths; shoot fresh and dry weights of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

Treatments	Shoot length per plant (cm)		Root length per plant (cm)		Shoot fresh weight per plant (g)		Shoot dry weight per plant (g)	
	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage
Control	76.2 ± 2.32^{f}	89.3 ± 2.89^{f}	31.7 ± 1.38^{f}	36.4 ± 1.82^{f}	447.1 ± 5.54^{f}	509.4 ± 6.34^{e}	111.7 ± 2.44^{e}	135.5 ± 2.97^{e}
$ISA_{80} + P_{10}$	79.4 ± 2.42^{e}	98.5 ± 3.03^{e}	33.3 ± 1.39^{e}	38.2 ± 1.85^{e}	478.7 ± 5.69^{e}	531.5 ± 6.61^{d}	114.4 ± 2.52^{d}	139.4 ± 3.06^{d}
$ISA_{80} + P_{20}$	85.7 ± 2.53^{d}	107.9 ± 3.24^{d}	35.2 ± 1.43^{d}	43.8 ± 1.93^{d}	502.5 ± 5.92^{d}	567.6 ± 6.73^{c}	122.6 ± 2.64^{c}	146.3 ± 3.19^{c}
$ISA_{80} + P_{30}$	92.6 ± 2.78^{c}	116.8 ± 3.31^{c}	38.8 ± 1.49^{c}	46.7 ± 2.01^{c}	521.8 ± 6.24^{c}	589.7 ± 6.97^{b}	129.8 ± 2.78^{b}	154.8 ± 3.42^{b}
$ISA_{80} + P_{40}$	102.9 ± 3.02^{a}	125.4 ± 3.65^{a}	42.3 ± 1.54^{a}	51.4 ± 2.08^{a}	552.6 ± 6.48^{a}	635.3 ± 7.32^{a}	137.2 ± 2.91^{a}	169.8 ± 3.51^{a}
$ISA_{80} + P_{50}$	96.1 ± 2.96^{b}	120.6 ± 3.47^{b}	41.7 ± 1.63^{b}	49.1 ± 2.02^{b}	536.7 ± 6.07^{b}	612.5 ± 7.03^{b}	133.5 ± 2.72^{b}	161.9 ± 3.16^{b}

Table 2
Effect of ISA along with different doses of phosphorus on net photosynthetic rate, stomatal conductance, internal CO_2 concentration and total chlorophyll content of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

Treatments	Net photosynthetic rate ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)		Stomatal conductance ($mol\ m^{-2}\ s^{-1}$)		Internal CO_2 concentration ($\mu mol\ m^{-2}\ s^{-1}$)		Total chlorophyll content ($mg\ g^{-1}$ FW)	
	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage
Control	13.13 ± 0.69^{f}	14.45 ± 0.82^{f}	0.26 ± 0.003^{e}	0.29 ± 0.003^{f}	281.3 ± 1.38^{f}	313.5 ± 2.12^{f}	0.95 ± 0.007^{f}	1.10 ± 0.008^{e}
$ISA_{80} + P_{10}$	13.82 ± 0.73^{e}	15.52 ± 0.87^{e}	0.28 ± 0.003^{d}	0.30 ± 0.003^{e}	297.1 ± 1.42^{e}	324.2 ± 2.37^{e}	0.98 ± 0.007^{e}	1.16 ± 0.009^{d}
$ISA_{80} + P_{20}$	14.67 ± 0.78^{d}	16.76 ± 0.98^{d}	0.30 ± 0.003^{c}	0.32 ± 0.004^{d}	312.4 ± 1.51^{d}	347.7 ± 2.43^{d}	1.05 ± 0.008^{d}	1.23 ± 0.009^{c}
$ISA_{80} + P_{30}$	15.49 ± 0.82^{c}	17.92 ± 1.03^{c}	0.32 ± 0.004^{b}	0.35 ± 0.004^{c}	337.9 ± 1.67^{c}	373.8 ± 2.59^{c}	1.12 ± 0.008^{c}	1.30 ± 0.008^{b}
$ISA_{80} + P_{40}$	17.02 ± 0.85^{a}	19.16 ± 1.18^{a}	0.34 ± 0.004^{a}	0.38 ± 0.005^{a}	358.6 ± 1.92^{a}	402.6 ± 2.71^{a}	1.19 ± 0.009^{a}	1.41 ± 0.009^{a}
$ISA_{80} + P_{50}$	16.34 ± 0.79^{b}	18.01 ± 1.04^{b}	0.32 ± 0.004^{b}	0.37 ± 0.004^{b}	346.2 ± 1.81^{b}	385.1 ± 2.78^{b}	1.10 ± 0.009^{b}	1.35 ± 0.008^{b}

nitrate reductase activity at both the growth stages of sampling. An increase of 21.3 and 23.2% was observed in $ISA_{80} + P_{40}$ treated plants as compared to control at pre-flowering and flowering stages, respectively. The values recorded for all treatment differed from each other at both growth stages of sampling (Table 3). The effect of ISA_{80} application combined with different levels of soil-applied phosphorus was found significant on carbonic anhydrase activity. Treatment $ISA_{80} + P_{40}$ enhanced carbonic anhydrase activity by 21.0 and 23.3% as compared with that of control at pre-flowering and flowering stages, respectively. All the treatments were significantly different from each other at both stages (Table 3).

Application of foliar ISA combined with different levels of soil-applied phosphorus gave significantly maximum value for leaf-nitrogen content at both growth stages of sampling. $ISA_{80} + P_{40}$ recorded 13.1 and 11.6% higher value for leaf-nitrogen content per plant at pre-flowering and flowering stages, respectively, over the control. This was followed by $ISA_{80} + P_{50}$ at both growth stages (Table 4). Significant enhancement in leaf-phosphorus content was recorded when ISA was applied with different doses of phosphorus. $ISA_{80} + P_{40}$ improved leaf-phosphorus content by 20.7 and 20.0% over the control at pre-flowering and flowering stages, respectively. The values recorded for all treatment differed from each other at both growth stages of sampling (Table 4). Foliar application of ISA_{80} combined with different doses of phosphorus was found insignificant leaf-potassium content at both, pre-flowering and flowering stages (Table 5). The effect of ISA_{80} application combined with different levels of soil-applied phosphorus was found significant on H_2O_2 content in the leaves. Treatment $ISA_{80} + P_{40}$ enhanced H_2O_2 content by 21.9 and 32.4% as compared with that of control at pre-flowering and flowering stages, respectively (Table 5).

A gradual increase in dry leaf yield was observed by the foliar application of ISA together with soil-applied phosphorus at

Table 3
Effect of ISA along with different doses of phosphorus on nitrate reductase and carbonic anhydrase activities of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

Treatments	Nitrate reductase activity ($nmol\ NO_2^-\ g^{-1}\ FW\ h^{-1}$)		Carbonic anhydrase activity ($\mu mol\ CO_2\ kg^{-1}\ FW\ s^{-1}$)	
	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage
Control	287.7 ± 3.29^{f}	301.4 ± 3.54^{f}	207.7 ± 2.34^{f}	228.6 ± 2.47^{f}
$ISA_{80} + P_{10}$	293.2 ± 3.33^{e}	311.6 ± 3.64^{e}	218.4 ± 2.43^{e}	238.4 ± 2.51^{e}
$ISA_{80} + P_{20}$	307.9 ± 3.42^{d}	327.2 ± 3.81^{d}	227.1 ± 2.52^{d}	249.2 ± 2.66^{d}
$ISA_{80} + P_{30}$	327.3 ± 3.54^{c}	346.8 ± 3.89^{c}	239.8 ± 2.69^{c}	259.7 ± 2.95^{c}
$ISA_{80} + P_{40}$	349.1 ± 3.59^{a}	371.5 ± 4.05^{a}	251.4 ± 2.79^{a}	281.8 ± 3.13^{a}
$ISA_{80} + P_{50}$	336.6 ± 3.62^{b}	354.1 ± 3.79^{b}	232.7 ± 2.61^{b}	272.6 ± 2.98^{b}

Table 4
Effect of ISA along with different doses of phosphorus on leaf nitrogen and phosphorus contents of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

Treatments	Leaf nitrogen content (%)		Leaf phosphorus content (%)	
	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage
Control	3.29 ± 0.78^{d}	3.71 ± 0.93^{e}	0.29 ± 0.002^{e}	0.30 ± 0.003^{d}
$ISA_{80} + P_{10}$	3.32 ± 0.80^{d}	3.76 ± 1.07^{e}	0.30 ± 0.003^{d}	0.31 ± 0.003^{c}
$ISA_{80} + P_{20}$	3.43 ± 0.93^{c}	3.88 ± 1.16^{d}	0.31 ± 0.003^{c}	0.32 ± 0.004^{bc}
$ISA_{80} + P_{30}$	3.59 ± 1.01^{b}	3.95 ± 1.31^{c}	0.33 ± 0.004^{b}	0.34 ± 0.005^{b}
$ISA_{80} + P_{40}$	3.72 ± 1.07^{a}	4.14 ± 1.42^{a}	0.35 ± 0.005^{a}	0.36 ± 0.006^{a}
$ISA_{80} + P_{50}$	3.61 ± 0.98^{b}	4.04 ± 1.32^{b}	0.33 ± 0.004^{b}	0.34 ± 0.004^{b}

Table 5
Effect of ISA along with different doses of phosphorus on leaf potassium and H_2O_2 content of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

Treatments	Leaf potassium content (%)		H_2O_2 content (nmol g^{-1} FW)	
	Pre-flowering stage	Flowering stage	Pre-flowering stage	Flowering stage
Control	2.22 ± 0.82[a]	2.35 ± 0.81[a]	34.24 ± 1.28[e]	39.21 ± 1.37[f]
$ISA_{80} + P_{10}$	2.23 ± 0.83[a]	2.36 ± 0.84[a]	35.12 ± 1.30[d]	41.72 ± 1.45[e]
$ISA_{80} + P_{20}$	2.22 ± 0.82[a]	2.37 ± 0.84[a]	36.73 ± 1.36[c]	44.54 ± 1.51[d]
$ISA_{80} + P_{30}$	2.24 ± 0.83[a]	2.38 ± 0.82[a]	38.43 ± 1.49[bc]	47.25 ± 1.63[c]
$ISA_{80} + P_{40}$	2.24 ± 0.82[a]	2.38 ± 0.84[a]	41.74 ± 1.62[a]	51.93 ± 1.82[a]
$ISA_{80} + P_{50}$	2.23 ± 0.82[a]	2.36 ± 0.83[a]	39.15 ± 1.53[b]	49.16 ± 1.62[b]

flowering stage. $ISA_{80} + P_{40}$ proved optimum and enhanced dry leaf yield by 21.4% over the control at this stage. All the treatments were significantly different from each other (Fig. 2). All treatments of ISA_{80} along with different levels of phosphorus showed positive response and increased the artemisinin content over the control at flowering stage. Application of $ISA_{80} + P_{40}$ significantly enhanced artemisinin content by 32.2% as compared to control estimated at flowering stage (Fig. 3). Application of ISA along with phosphorus was found effective over the control in increasing artemisinin yield. Treatment $ISA_{80} + P_{40}$ enhanced artemisinin yield by 61.7% over the control at flowering stage (Fig. 4).

4. Discussion

The main processes that determine the quality and quantity of plant growth are cell division, enlargement and differentiation. These processes are affected by various internal and external factors including supply and absorption of nutrients, which have critical importance in cell metabolism and involvement of phytohormones/plant growth regulators for maintaining a healthy source–sink relationship (Patel and Golakia, 1988). Plant growth regulating substances get involved through the modification of transcription, translation and/or differential sensitivity of the tissue. As observed in present study, ISA together with soil-applied phosphorus improved growth attributes of *A. annua* significantly ($p < 0.05$) studied at pre-flowering and flowering stages.

Idrees et al. (2011, 2012) and Khan et al. (2011) reported significant improvement in plant growth attributes by the application of radiation-derived oligosaccharides of alginate. Also, the important role of irradiated sodium alginate in enhancing the biological activity of the plants has been shown by Kume, Nagasawa, and Yoshii (2002) and Luan et al. (2003). Albersheim and Darvill (1985) suggested that biologically active oligosaccharides act as signal molecules that regulate growth and development of the plant as well as defense reactions by regulating gene expression. In fact, polysaccharides, such as sodium alginate (SA), undergo chain scission by irradiation. The irradiation of SA by gamma rays affects the overall polymer cross-linking process. Consequently, its application influences the biological properties of the plant cells (El-Rehim, 2006). Aftab, Khan, Idrees, et al. (2011), Idrees et al. (2012) and Naeem, Idrees, Aftab, Khan, and Varshney (2012) also confirmed the growth promoting effect of irradiated carbohydrate polymers in *A. annua*, *Cymbopogon flexuosus* and *Mentha arvensis*. The observed positive effects of soil-applied phosphorus with foliar ISA_{80} may be ascribed to the roles of phosphorus various physiological processes. It is indispensable for all forms of life because of its role in energy transfer via ATP (Samuel, Werner, James, & John, 1993). Moreover, it plays an important role in various metabolic processes as it is an integral part of several compounds such as co-enzymes, nucleic acid, nucleotides, phosphoric acid and phosphorylated sugars (Marschner, 2002). Among essential nutrients, phosphorus is often limiting due to low availability, therefore its supply by any means may improve directly or indirectly cell division, cell enlargement and tissue and organ development. Beneficial effects of phosphorus either alone or in combination with other nutrients/growth regulators were reported by many workers in *A. annua* (Davies, Atkinson, Burns, Arroo, & Woolley, 2011; Ferreira, 2007; Singh, 2000; Weathers, Bunk, & Mccoy, 2005).

Net photosynthetic rate, stomatal conductance and internal CO_2 concentration, were progressively improved from pre-flowering to flowering stage. Hien et al. (2000) also observed significant enhancement in net-photosynthesis and CO_2 assimilation using ISA. A number of compounds involved in photosynthesis, such as chlorophylls, enzymes and coenzymes, not only depend upon this

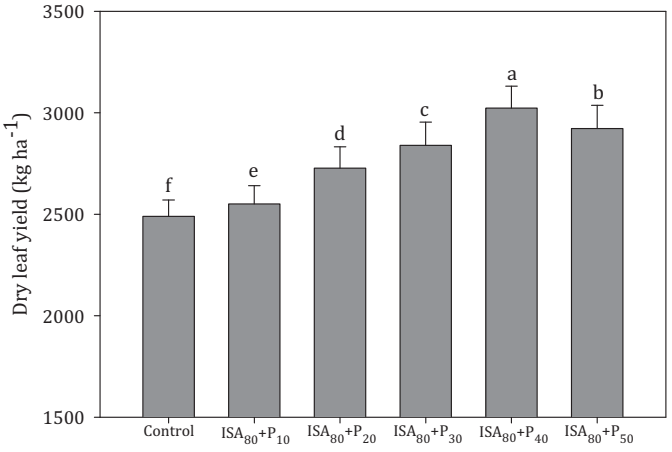

Fig. 2. Effect of ISA along with different doses of P on dry leaf yield of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (⊤) show SE.

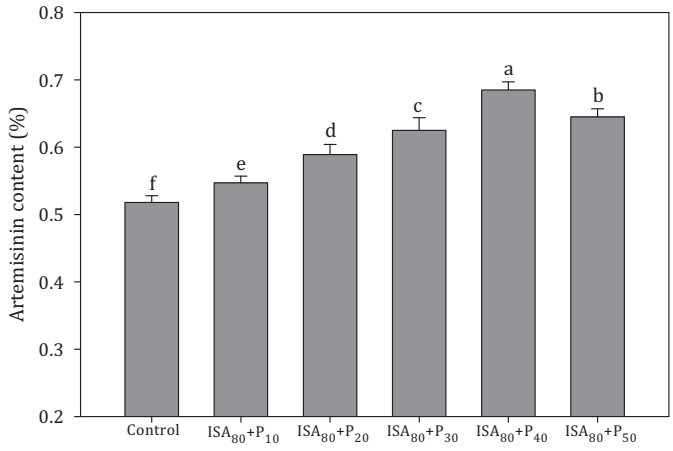

Fig. 3. Effect of ISA along with different doses of P on artemisinin content of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (⊤) show SE.

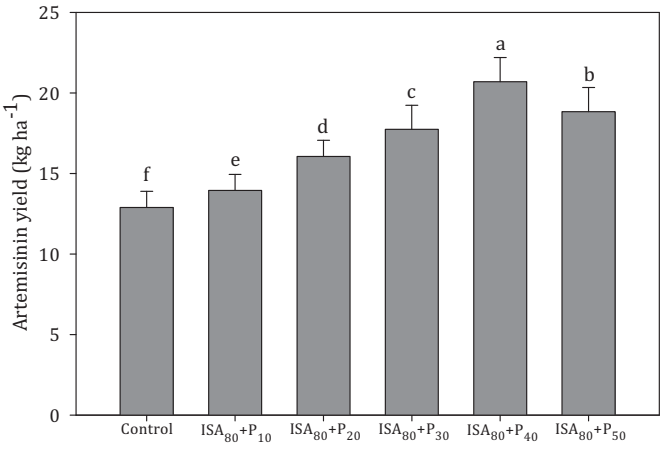

Fig. 4. Effect of ISA along with different doses of P on artemisinin yield of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (⊤) show SE.

essential nutrient element for their production but also show a linear increase to increasing quantities of added fertilizer within limits (Salisbury & Ross, 1992). The improved photosynthetic rates, as observed in this study, might be attributed to a high level of RuBisCO activity and CO_2 fixation together with increased cell expansion as a result of phosphorus application (Rao & Terry, 1989). An increase in phosphorus-mediated stomatal conductance and internal CO_2 concentration in this study could be considered as beneficial effects of phosphorus nutrition on photosynthesis as reported by Garg, Burman, and Kathju (2004) in the case of *Vigna aconitifolia*. Moreover, the present research indicates that photosynthetic rate may be limited by nutrient deficiencies but additional fertilizer supply may be more useful in improving the photosynthetic attributes as observed. As the oligosaccharides induce cell signalling that in turn, leads to stimulation of various physiological processes in various plants (John, Rohrig, Schmidt, Walden, & Schell, 1997), the application of ISA might stimulate the improvement in total chlorophyll content in this study. The enhancement in chlorophyll content as a result of phosphorus application together with ISA_{80} could possibly be attributed to the increase in number and size of chloroplast, the amount of chlorophyll per chloroplast and proper granna development in the chloroplast (Akimoto, Aoyagi, & Tanaka, 1999). Moreover, phosphorus is an integral part of many compounds of plant cells, including sugar-phosphates, intermediates of respiration and photosynthesis and that of phospholipids that constitute the plant membranes (Devlin & Witham, 1986). Therefore, the supplementation of soil-phosphorus might be helpful in enhancing chlorophyll content directly or indirectly.

Nitrate reductase (NR) is the rate-limiting enzyme in nitrogen assimilation and a key enzyme of metabolic regulation. The process of nitrate reduction is directly or indirectly dependent on the metabolic sensors and/or signal transducers (Campbell, 1999). The level of the enzyme is dependent on a number of factors, born within or outside the plants. One of the major regulatory factors, determining the activity of NR, is the level of endogenous phytohormones or those added from outside (Khan, Ansari, & Mobin, 1996). The increase in NR activity by ISA might be related to the increase in the uptake of nitrogen that might have increased the concentration of nitrate in the leaves to be acted upon by NR. Activity of NR was also increased by phosphorus application applied together with ISA_{80}, respectively. NR catalyze 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, Gazzarini, Gojon, & Frommer, 2000). Therefore, enriching soil-nutrient status must certainly have a positive impact on NR activity as observed in this study. Carbonic anhydrase (CA) is an enzyme that plays diverse roles in physiological processes such as ion exchange, acid–base balance, carboxylation/decarboxylation reactions and inorganic carbon diffusion between the cell and its environment as well as within the cell (Georgios et al., 2004). There are several other reports regarding the significant effect of ISA application on CA activity in the case of fennel, mentha, lemongrass and opium poppy (Idrees et al., 2011; Khan et al., 2011; Sarfaraz et al., 2011). The ability of ISA to promote RuBisCO activity and, thereby, to increase the yield has been reported by Idrees et al. (2011) for *Mentha arvensis*. The overall carbon fixation rate relates to the relative activities of carboxylase and oxygenase functions of RuBisCO. Therefore, 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 stimuli. In fact, the role of ISA in the activation of RuBisCO and PEP carboxylase has also been documented (Akimoto et al., 1999; Hien et al., 2000; Tomoda et al., 1994). In the present investigation, CA activity was improved over the control as a result of phosphorus application, together with ISA_{80}. Such an increase in RuBisCO activity might be expected in this study as in the presence of optimum nutrient supply, hydration of CO_2 is catalyzed rapidly that, in turn, increases the supply of RuBisCO at the site of its action, and probably it might be a strong reason behind the improvement in the rate of photosynthesis in the treated plants as reported by Siddiqui, Khan, Mohammad, and Khan (2008).

Application of ISA together with soil-applied phosphorus improved leaf-nitrogen and -phosphorus content of *A. annua* plants was enhanced significantly, while leaf-potassium content remained unaffected. There are limited reports regarding the effect of ISA on leaf-N, -P and -K contents (Idrees et al., 2011; Sarfaraz et al., 2011). An increase in membrane permeability, as suggested by Crozier and Turnbull (1984) facilitate the absorption and utilization of mineral nutrients and the transport of assimilates in plants. This would also contribute towards enhancing the capacity of biomass production by plants as reflected by the increase in fresh and dry weights of plants in the present investigation. An adequate supply of mineral nutrients at initial vegetative stage plays a pivotal role in growth and development of plants. Owing to the application fertilizers, the plants received an adequate supply of nutrients on the one hand; and on the other, the growth of plants was promoted by foliar sprays of ISA, leading to healthy vegetative growth and unhindered translocation of nutrients to the shoot that might be the reason behind the improved growth attributes together with enhanced leaf-nutrient contents in the treated plants.

Association of increased formation of H_2O_2 with some plant growth regulators has been reported by other workers. For example, Wallaart, Pras, Beekman, and Quax (2000), Ferreira (2007) and Pu et al. (2009) reported an increase in H_2O_2 content in consequence of night frost, soil-K deficiency and due to salicylic acid in *A. annua*. Additionally, Guo, Yang, Yang, and Zeng (2010) and Aftab, Khan, Idrees, Naeem, and Moinuddin (2010), Aftab, Khan, Idrees, Naeem, and Ram (2010) and Aftab, Khan, Idrees, Naeem, Moinuddin, et al. (2010) noticed enhanced formation of H_2O_2 in *A. annua* plants treated with boron, salicylic acid and methyl jasmonate. From these studies, it is quite clear that plant growth regulators may promote the formation of ROS up to an extent, which is useful for the plants too. There are reports in which antioxidant enzymes have been shown to be positively regulated by nutrient supply (Idrees et al., 2011). It is a common notion that antioxidant enzymes are generally increased when ROS are generated; therefore, it can be presumed that nutrient supply may regulate ROS status inside the cell.

Dry-leaf yield, calculated at flowering stage, was positively regulated by different treatments. As mentioned earlier, the biologically active oligosaccharides, derived from depolymerization of sodium alginate, may prove as signal molecules in order to regulate the growth, development and defense reactions in plants, following the expression of genes concerned (Albersheim & Darvill, 1985). As a result, the biological properties of the plant cells may get stimulated (El-Rehim, 2006), leading to overall improvement in the agricultural performance of the crop. The concentration per plant and yield of artemisinin, recorded at flowering stage, increased with increasing doses of phosphorus up to 40 kg ha^{-1} when applied with ISA_{80}. We speculate that ISA promoted the artemisinin synthesis by recuperating the level of H_2O_2, which converts dihydroartemisinic acid into artemisinin during artemisinin biosynthesis (Guo et al., 2010; Pu et al., 2009). As indicated by Wallaart et al. (1999), dihydroartemisinic acid might act as a scavenger of ROS (H_2O_2 in this case) that are released in plant cells due to various reasons. During the biochemical reaction, dihydroartemisinic acid hydroperoxide (DHAA-OOH) is generated that gets converted to artemisinin. Pu et al. (2009), Wallaart et al. (2000) and Ferreira (2007) advocated relatively high levels of ROS might be generated as a result of K-deficiency in the soil, foliar application of salicylic acid and occurrence of night frost that, in turn, could result in a greater conversion of dihydroartemisinic acid to artemisinin. There has been noticed a positive relationship between H_2O_2 and artemisinin contents by Aftab, Khan, Idrees, Naeem, and Ram (2010), Aftab, Khan, Idrees, Naeem, Moinuddin, et al. (2010), Aftab, Khan, Idrees, et al. (2011) and Aftab, Khan, Teixeira da Silva, et al. (2011) in the net-house and field conditions. Kapoor, Chaudhary, and Bhatnagar (2007) and Davies et al. (2011) are some of workers, who reported increased content and yield of artemisinin by the application of phosphorus.

5. Conclusion

The results showed that ISA together with phosphorus improved overall growth of the plants. Application of ISA together with soil-applied phosphorus improved NR and CA activity as well as enhanced leaf-nutrient content. Net photosynthetic rate, stomatal conductance and internal CO_2 concentration were progressively improved from pre-flowering to flowering stage by the treatment. Further, this treatment also enhanced artemisinin concentration and yield. Therefore, this combination can be used to enhance biomass and artemisinin production in *A. annua* plants.

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