

# Methyl jasmonate counteracts boron toxicity by preventing oxidative stress and regulating antioxidant enzyme activities and artemisinin biosynthesis in *Artemisia annua* L.

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Received: 5 August 2010 / Accepted: 4 October 2010 / Published online: 19 October 2010  
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**Abstract** Boron is an essential plant micronutrient, but it is phytotoxic if present in excessive amounts in soil for certain plants such as *Artemisia annua* L. that contains artemisinin (an important antimalarial drug) in its areal parts. Artemisinin is a sesquiterpene lactone with an endoperoxide bridge. It is quite expensive compound because the only commercial source available is *A. annua* and the compound present in the plant is in very low concentration. Since *A. annua* is a major source of the antimalarial drug and B stress is a deadly threat to its cultivation, the present research was conducted to determine whether the exogenous application of methyl jasmonate (MeJA) could combat the ill effects of excessive B present in the soil. According to the results obtained, the B toxicity induced oxidative stress and reduced the stem height as well as fresh and dry masses of the plant remarkably. The excessive amounts of soil B also lowered the net photosynthetic rate, stomatal conductance, internal CO<sub>2</sub> concentration and total chlorophyll content in the leaves. In contrast, the foliar application of MeJA enhanced the growth and photosynthetic efficiency both in the stressed and non-stressed plants. The excessive B levels also increased the activities of antioxidant enzymes, such as catalase, peroxidase and superoxide dismutase. Endogenous H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> levels were also high in the stressed plants. However, the MeJA application to the stressed plants reduced the amount of lipid peroxidation and stimulated the synthesis

of antioxidant enzymes, enhancing the content and yield of artemisinin as well. Thus, it was concluded that MeJA might be utilized in mitigating the B toxicity and improving the content and yield of artemisinin in *A. annua* plant.

**Keyword** *Artemisia annua* L. · Artemisinin · Boron · Methyl jasmonate (MeJA) · Reactive oxygen species (ROS)

## Introduction

*Artemisia annua* L. (family Asteraceae) is an antimalarial drug plant that has been used for centuries in Chinese traditional medicines 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 regarding the control of malaria, the global morbidity and mortality have not significantly been changed for the last 50 years (WHO 2006). Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has been increasingly popular as an effective and safe alternative therapy against malaria (Abdin et al. 2003). Since the chemical synthesis of artemisinin is very costly, the plant remains the only viable source of the artemisinin production. Hence, the enhanced production of artemisinin in the plants is highly desirable (Abdin et al. 2003; Aftab et al. 2010a, c).

B is an essential element required for normal growth of higher plants. It is unique among micronutrients in that the threshold between deficiency and toxicity is narrow (Yau and Ryan 2008). High B concentration might occur naturally in the soil and ground waters or could be added to the soil through mining, fertilizers and irrigation water (Nable et al. 1997). In the recent years, B toxicity has

Handling Editor: Peter Nick

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attracted increasing interest owing to the greater demand for desalinated water, in which the B concentrations might be too high for healthy irrigation (Parks and Edwards 2005). In fact, B-rich soils have decreased plant growth and crop yield in different regions of the world (Papadakis et al. 2004). The typical symptoms shown by plants, exposed to high B concentrations in the substrate, are reduced vigour, delayed development, leaf burn and decreased number and size of the fruits (Paull et al. 1992; Nable et al. 1997). Accumulation of reactive oxygen species (ROS), due to B toxicity, has been reported in apple root stock (Molassiotis et al. 2006), wheat (Gunes et al. 2007), barley (Inal et al. 2009) and tomato (Cervilla et al. 2007). Further, lipid peroxidation and hydrogen peroxide ( $H_2O_2$ ) accumulation in the cells and tissues caused by high concentration of B has also been reported (Karabal et al. 2003; Gunes et al. 2006; Molassiotis et al. 2006). In addition, altered activities of the antioxidant enzymes due to high B concentration have been used as indicators of oxidative stress in crop plants (Mittler 2002).

Methyl jasmonate (MeJA), a methyl ester of jasmonic acid, is a phytohormone with ubiquitous distribution among the plants. Generally, it is considered to modulate several physiological events in higher plants such as defence responses, flowering and senescence (Cheong and Choi 2003). Also, MeJA mediates plant responses to various biotic and abiotic stresses by triggering a transcriptional reprogramming that allows cells to cope with pathogens and stresses (Wolucka et al. 2005). MeJA has been shown to induce the production of antioxidant defence enzymes and secondary metabolites in some plants and cell cultures (Hampel et al. 2005; Kim et al. 2007). Recently, enhanced artemisinin production has been observed in the whole plant due to MeJA application (Wang et al. 2009; Guo et al. 2010). Such an enhancement in the plant artemisinin content has also been reported as a result of biotic or abiotic stresses like low temperature and mineral deficiency as well as toxicity (Wallaart et al. 2000; Ferreira 2007; Aftab et al. 2010b).

In previous paper, we reported that a mild stress of B accelerated the production of artemisinin content per plant, but it adversely affected the growth of the plants (Aftab et al. 2010b). The main reason behind the poor artemisinin yield was the morphological damage to the stressed plants due to excessive B application. The present research is the outcome of the hypothesis we postulated regarding the effect of exogenous supply of MeJA that led us to think if MeJA supply could combat B toxicity and whether it is involved in the upregulation of artemisinin biosynthesis.

## Materials and methods

A pot culture experiment was conducted to analyse the effects of two concentrations of B viz. 1.00 and 2.00 mM

applied with or without exogenous MeJA (300  $\mu$ M) on *A. annua*. The seeds were initially surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with double distilled water before sowing. Prior to seed sowing, pots were filled with 5.0 kg homogenous mixture of soil and farmyard manure (4:1). Physicochemical characteristics of the soil were texture-sandy loam, pH (1:2) 8.0, E.C. (1:2) 0.48 m mhos/cm and available N, P and K 97.46, 10.21 and 147.0 mg  $kg^{-1}$  soil, respectively. Soil B concentration was 0.05 mM, which is a safe limit for the cultivation of agricultural crops. Seeds were sown at a depth of 2 cm in the earthen pots (25 cm diameter  $\times$  25 cm height). B was applied to the soil at the rate of 0, 1.00 and 2.00 mM in the form of boric acid ( $H_3BO_3$ ) 60 days after sowing (AS), while MeJA was applied thrice on the foliage of the plants at an interval of 10 days. The first treatment of MeJA was given when the plants were 60 days old, and subsequently, the second and third treatments were applied. The experiment was conducted according to simple randomised block design, and the plants were sampled 120 days AS for various parameters studied.

### Analyses of growth and yield parameters

The plants from pots of each treatment were uprooted carefully and then shoot height was 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.

### Photosynthetic parameters

Net photosynthetic rate ( $P_N$ ) was measured on sunny days at 1100 hours using fully expanded leaves with the help of IRGA (Infra Red Gas Analyzer, LI-COR 6400 Portable Photosynthesis System, Lincoln, NE, USA). Before recording the measurements, 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 extracted chlorophyll solution was recorded at 662 and 645 nm for chlorophyll estimation using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

### Carbonic anhydrase activity

Carbonic anhydrase (CA; E.C. 4.2.1.1) activity was measured in the youngest fully expanded leaves using the method as described by Dwivedi and Randhawa (1974). Two hundred milligrams of fresh leaf pieces was transferred to Petri plates. The leaf pieces were dipped in 10 mL of 0.2 M cysteine 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 micromolar CO<sub>2</sub> per kilogram leaf FW per second.

### Estimation of B

B contents in the roots and leaves were determined by azomethine-H method (Wolf 1971). The plant samples were reduced to dry ash in a muffle furnace at 500°C for 6 h, and the carbon-free residue was then dissolved in 0.1 M HCl. Root and leaf B contents were determined in terms of milligrams per kilogram.

### Lipid peroxidation rate (TBARS content)

Oxidative damage to leaf lipids was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst (1991). TBARS were extracted from 0.5 g chopped fresh leaves, ground with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). Following the centrifugation at 12,000×g for 5 min, an aliquot of 1 mL of the supernatant was added to 4 mL of 0.5% (w/v) TBA in 20% (w/v) TCA. Samples were incubated at 90°C for 30 min. Thereafter, the reaction was stopped using an ice bath. Centrifugation was performed at 10,000×g for 5 min, and the absorbance of the supernatant was recorded at 532 nm with the help of a spectrophotometer and the values were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. TBARS content was expressed as nanomoles per gram FW.

### Antioxidant enzymes assay

Catalase (CAT) activity was measured according to the method given by Chandlee and Scandalios (1984) with a minor modification. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mM H<sub>2</sub>O<sub>2</sub> and 0.04 mL of the enzyme extract. The extract was centrifuged at 4°C for 20 min at 12,500×g. The supernatant was used for enzyme assay. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer

(pH 7.0), 400 µL of 15 mM H<sub>2</sub>O<sub>2</sub> and 40 µL of enzyme extract. The decomposition of H<sub>2</sub>O<sub>2</sub> was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units per milligram protein (U=1 mM of H<sub>2</sub>O<sub>2</sub> reduced per minute per milligram protein).

Peroxidase (POX) activity was assayed by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H<sub>2</sub>O<sub>2</sub> and 0.5 mL of the enzyme extract. The solution was incubated for 5 min at 25°C, after which the reaction was terminated by adding 1 mL of 2.5 N H<sub>2</sub>SO<sub>4</sub>. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2.5 N H<sub>2</sub>SO<sub>4</sub> at zero time. The activity was expressed in units per milligram protein. One unit of the enzyme activity corresponded to an amount of the enzyme that caused an increase in the absorbance by 0.1 min<sup>-1</sup> mg<sup>-1</sup> protein.

Superoxide dismutase (SOD) activity was assayed as described by Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10<sup>-6</sup> M of riboflavin, 0.1 of M methionine, 2×10<sup>-5</sup> M of KCN and 5.6×10<sup>-5</sup> M of nitroblue tetrazolium salt dissolved in 3 mL of 0.05 M of sodium phosphate buffer (pH 7.8). A 3-mL volume of the reaction medium was added to 1 mL of the enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes arranged in a single row. Illumination of the reaction mixture initiated the reaction that was maintained at 30°C for 1 h. Identical solutions were kept under dark served as blanks. The absorbance was measured at 560 nm against the blank using the spectrophotometer. SOD activity was expressed as U mg<sup>-1</sup> protein. One unit is defined as the amount of change in the absorbance by 0.1 h<sup>-1</sup> mg<sup>-1</sup> protein.

### Determination of ROS

ROS were estimated as the total content of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in the leaves. The leaf H<sub>2</sub>O<sub>2</sub> content 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 millilitre of the supernatant was mixed with 0.1 mL of 5% Ti(SO<sub>4</sub>)<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>, and the absorbance was recorded at 415 nm using the spectrophotometer. The H<sub>2</sub>O<sub>2</sub> concentration was calculated according to the standard curve of H<sub>2</sub>O<sub>2</sub> that was prepared by using known concentrations of H<sub>2</sub>O<sub>2</sub> ranging from 0 to 10 µM.

The production of singlet oxygen ( $O_2^-$ ) was measured in the fresh leaf tissue according to the method of Able et al. (1998) employing slight modifications. Accordingly, the reduction of XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide] was monitored in the presence of  $O_2^-$ . The leaf samples (0.5 g, FW) were homogenized with 1 mL of 50 mM Tris-HCl buffer (pH 7.5), and then the content was centrifuged at  $12,000\times g$  for 15 min. The reaction mixture contained 50 mM of Tris-HCl buffer (pH 7.5), 100  $\mu$ L of supernatant and 0.5 mM of XTT. The reduction of XTT was determined by measuring the absorbance at 470 nm using the spectrophotometer. The quantity of  $O_2^-$  was determined using the molar extinction coefficient ( $\epsilon$ )— $2.16\times 10^4 M^{-1} cm^{-1}$ .

#### Extraction and estimation of artemisinin

Dry leaf material (1 g) was used for the estimation of artemisinin that was converted to  $Q_{260}$  ( $Q_{260}$  is a UV absorbing compound that can be detected on a UV detector), which was subsequently quantified using the HPLC according to the method of Zhao and Zeng (1986). Stock solution for the standard curve was prepared using 1 mg of standard artemisinin dissolved in 1 mL of HPLC-grade methanol. The artemisinin was extracted with 20 mL of petroleum ether using a shaker maintained at 70 rpm for 24 h. Thereafter, the solvent was decanted and pooled followed by addition of 20 mL of petroleum ether again. This step was repeated thrice. Petroleum ether fractions were pooled and concentrated under reduced pressure, and the residues were defatted with  $CH_3CN$  (10 mL $\times$ 3). The precipitated fat was filtered out and the filtrate was concentrated under reduced pressure. The residues were dissolved in 1 mL of methanol. To a 100- $\mu$ L aliquot of each sample, 4 mL of 0.3% NaOH was added. The samples were incubated in shaking water bath at 50°C for 30 min; thereafter, they were cooled and neutralized with glacial acetic acid (0.1 M dissolved in 20% MeOH). The pH of the solution was maintained at 6.8. The derivatized artemisinin was analysed and quantified through reverse-phase column (C18; 5  $\mu$ m; 4.6 mm; 250 mm) using premix methanol/10 mM potassium phosphate buffer (pH 6.5) in the ratio of 60:40 as 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, obtained from Sigma-Aldrich, USA.

#### Statistical analysis

Each pot was treated as one replicate, and all the treatments were replicated five times. The data were analysed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA) according to simple randomized block design. Mean

values were statistically compared by Duncan's multiple range test at  $P<0.05$  level using different letters.

## Results

### Growth and yield characteristics

Morphological examination of the treated plants showed that the B, applied to the soil either at 1.00 or 2.00 mM, inhibited the growth of *A. annua* plants; the most toxic effects were noted at 2.00 mM of B (Table 1). The plants supplied with 300  $\mu$ M of MeJA showed an improved plant growth compared to the B-treated plants supplied with no MeJA (Table 1). Shoot fresh and dry masses were significantly decreased by either of the concentrations of applied B. The decrease in shoot fresh and dry masses was comparatively higher when 2.00 mM of B was applied through soil, in comparison to the control (Table 1). The toxic effects, generated by excessive B application, were ameliorated when the plants were supplemented with the exogenous MeJA. The fresh and dry leaf yields were also significantly affected by B toxicity, with the MeJA application proving effective in reducing the inhibitory effects of B toxicity (Table 1).

### Photosynthetic parameters

Values of all the photosynthetic parameters were significantly reduced by the B concentrations applied. Application of both the levels of B, viz. 1.00 and 2.00 mM, resulted in significant reduction in net photosynthetic rate, stomatal conductance and internal  $CO_2$  concentration, the decrease in values being higher at 2.00 mM of B compared to that at 1.00 mM of B and the control (Fig. 1a–c). Contrarily, MeJA alleviated the adverse effect of B toxicity, with the MeJA-treated plants showing significantly improved values of photosynthetic parameters. The chlorophyll content, observed in 2.00 mM of B, was lower in comparison to the plants exposed to 1.00 mM of B and the control. However, when the plants were treated with 300  $\mu$ M of MeJA, the chlorophyll content in the leaves increased, counteracting the B toxicity at the both the B concentrations applied (Fig. 1d).

### Carbonic anhydrase activity

In the present study, there was noted a reduction in the activity of CA in the leaves on account of both the B treatments. At 2.00 mM of B, the activity of CA was the lowest compared to that recorded at 1.00 mM of B and in the control. However, when MeJA was given to the stressed plants, the inhibitory effect of B was nullified (Fig. 2a).

**Table 1** Effect of different concentrations of B and MeJA on growth and yield attributes of *A. annua* L.

Treatments	Shoot length (cm)	Shoot fresh mass (g)	Shoot dry mass (g)	Fresh leaf yield (g)	Dry leaf yield (g)
Control	90.3±1.21b	402.8±8.36c	91.3±2.54b	315.3±4.43b	67.4±1.94b
300 $\mu$ M MeJA	98.6±1.32a	441.3±8.87a	99.8±2.54a	357.1±4.43a	74.7±1.94a
1.00 mM B (B <sub>1</sub> )	78.2±1.05d	336.7±7.28e	77.6±2.14d	267.2±4.83d	56.4±1.65d
2.00 mM B (B <sub>2</sub> )	62.4±0.81e	273.3±4.75f	63.1±1.88e	215.9±3.49e	44.9±1.37e
B <sub>1</sub> +300 $\mu$ M MeJA	93.3±1.36ab	412.4±6.04b	92.2±2.53b	317.6±5.87b	68.2±1.88b
B <sub>2</sub> +300 $\mu$ M MeJA	83.7±1.14c	389.2±5.73d	85.7±2.01c	302.5±5.45c	63.9±1.72c

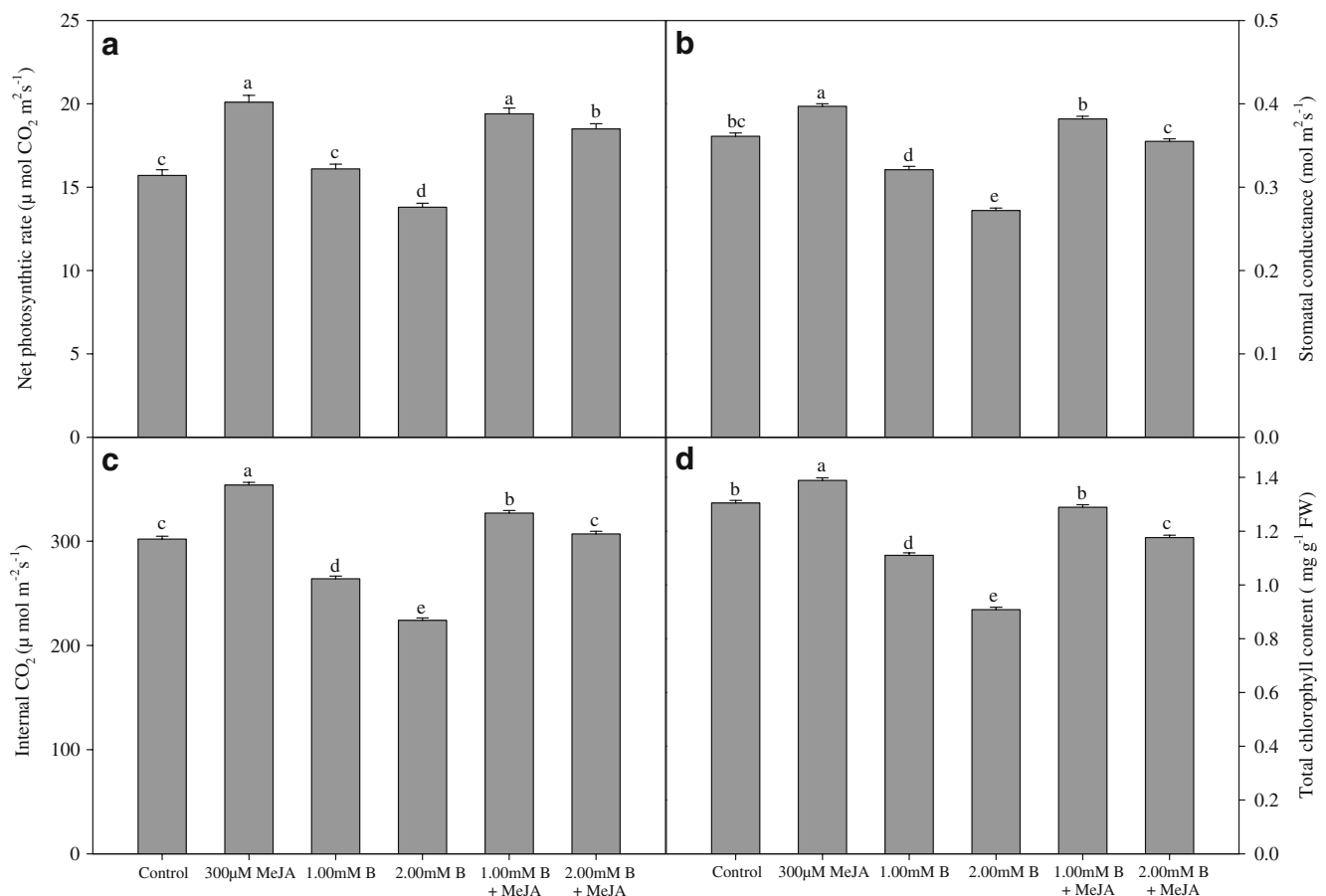
Means within a column followed by the same letter are not significantly different ( $p \leq 0.05$ ). The data shown are means of five replicates  $\pm$  SE

### B accumulation in plant tissue

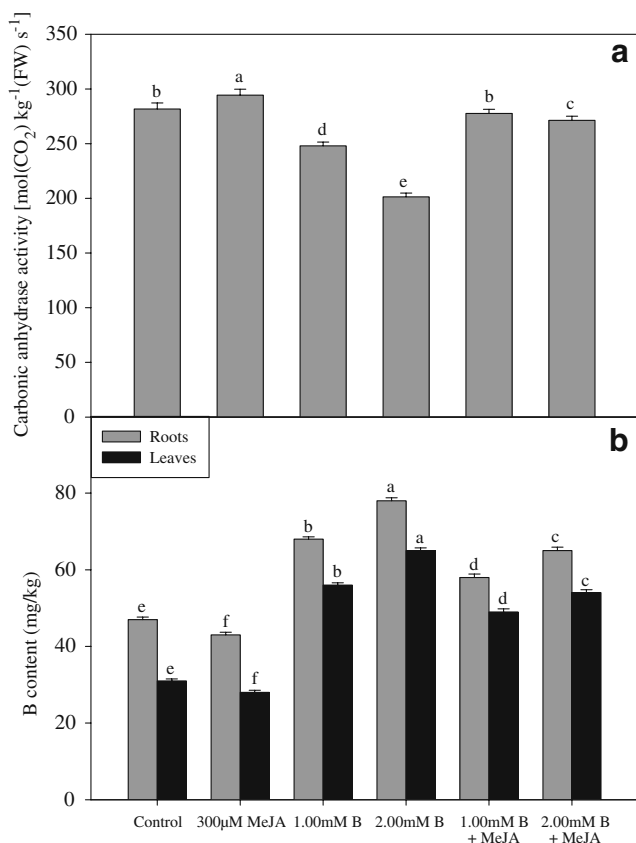
B concentration in plant tissues increased with increasing B concentration in the soil in concentration-dependent manner, with B concentration of root being higher compared to that of leaf. Exogenous application of MeJA, applied to B-stressed plants, also influenced the accumulation of B in roots and leaves by significantly preventing the accumulation of B in the tissues of roots and leaves (Fig. 2b).

### Oxidative stress (lipid peroxidation)

Lipid peroxidation was examined by estimating MDA content in the B-treated plants. Since the B is known to induce oxidative stress, the B-treated plants exhibited high rate of lipid peroxidation. At 2.00 mM of B, the lipid peroxidation rate in leaves was higher compared to that at 1.00 mM of B and the control treatment (no B application; Fig. 3a). However, MeJA-treated plants lowered the



**Fig. 1** Effect of B and MeJA on net photosynthetic rate (a), stomatal conductance (b), internal CO<sub>2</sub> (c) and total chlorophyll content (d) of *A. 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 ( $\tau$ ) show SE



**Fig. 2** Effect of B and MeJA on CA activity (a) and B accumulation in roots and leaves (b) of *A. 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 ( $\tau$ ) show SE

membrane destruction caused by B application both at 1.00 and 2.00 mM of B (Fig. 3a).

#### Antioxidant enzymes

The presence of B in the soil accelerated the activities of antioxidant enzymes (CAT, POX, and SOD) in the plants that are a natural mechanism of protection of plants against the B stress. The B applied at 2.00 mM resulted in a rapid stimulation of the activities of CAT, POX and SOD compared to that at 1.00 mM of B. The follow-up exogenous application of MeJA was effective in further inducing the activities of all the antioxidant enzymes measured, suggesting the role of the MeJA regarding protection against the B stress in *A. annua* plants (Fig. 3b–d).

#### Endogenous generation of ROS

In the present study, the level of endogenous  $H_2O_2$  and  $O_2^-$  was also measured in order to determine the internal status of ROS due to soil applied B, alone and in combination with MeJA. Both  $H_2O_2$  and  $O_2^-$  contents were higher in the

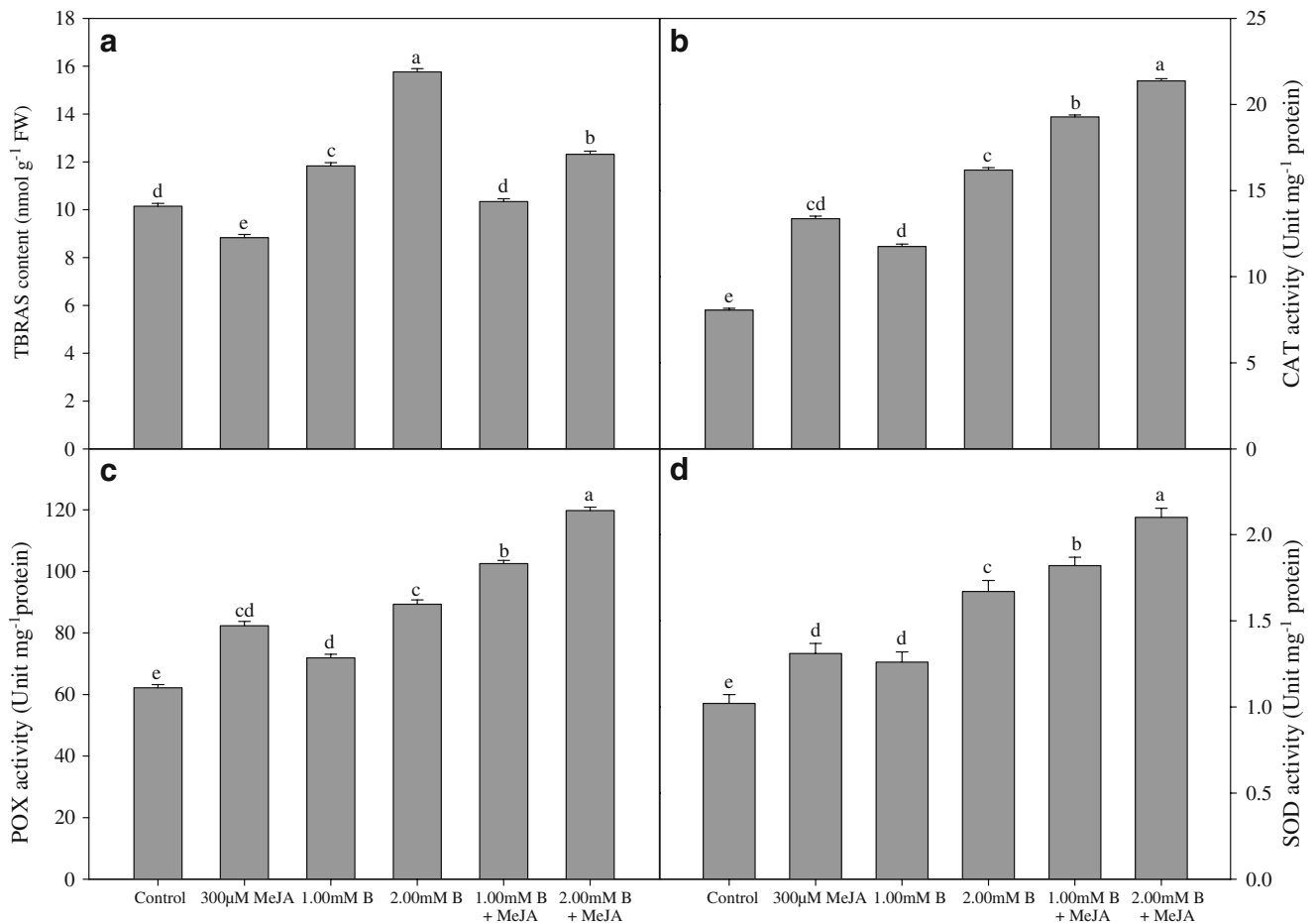
B-treated plants as compared to the control at both the concentrations of B. At 1.00 mM of B, the increase in internal  $H_2O_2$  and  $O_2^-$  was higher compared to that at 2.00 mM of B and in the control treatment (Fig. 4a, b). A slight increase in the levels of ROS was also noted when MeJA was supplied to the plants in combination with B (Fig. 4a, b).

#### Artemisinin content and yield

The objective behind the present study was to explore whether the exogenously applied MeJA in the presence of B stress can have positive impact on artemisinin production in *A. annua*. An increase of 58.8% in the artemisinin content was noted when the plants were grown at 1.00 mM concentration of B together with MeJA, while the increase was 31.2% when 2.00 mM of B was applied with MeJA (Figs. 5a and 6). Also, the artemisinin yield was recorded to be the highest when MeJA was applied along with 1.00 mM of B. A significant increase in artemisinin content was also noted when MeJA was applied to the plants alone (Fig. 5b).

#### Discussion

Plant hormones are the active members of signal cascades involved in the induction of plant stress responses (Pedranzani et al. 2003). Abiotic stresses negatively affect plant growth and development, and to combat with these stresses, the use of exogenous plant growth regulators could be of great importance (Jaleel et al. 2009). B toxicity significantly decreased the values of growth parameters in the present study (Table 1). According to Srivastava and Sharma (1990), *A. annua* plants, fertilized with either very high (2.50 mg/L) or very low (0.05 mg/L) B levels, were highly sensitive to B deficiency that significantly decreased the plant growth and tissue artemisinin content. Plants that received very low B concentrations exhibited necrosis in growing tips, resulting in delayed flowering (Srivastava and Sharma 1990). Increasing concentrations of B reduced the height of the plant as well as the fresh and dry masses per plant. The observed lower values for fresh and dry masses of the plant due to B treatments are in agreement with the results of several researchers investigating the response of various plant species to B stress (Asad et al. 2003; Molassiotis et al. 2006; Eraslan et al. 2007). B is directly involved in many physiological and biochemical processes during plant growth such as cell elongation and division, cell wall biosynthesis and membrane functions (Blevins and Lukaszewski 1998). Growth reduction under B toxicity is well documented in tomato (Alpaslan and Gunes 2001), cucumber (Cervilla et al. 2007) and barley (Karabal et al. 2003). Therefore, the observed reduction in growth param-



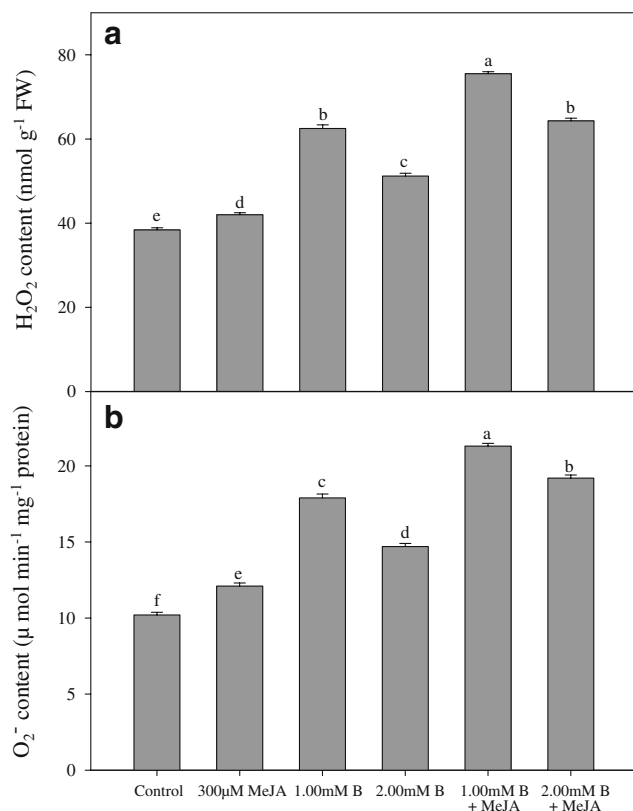
**Fig. 3** Effect of B and MeJA on TBARS content (a), CAT (b), POX (c) and SOD (d) activities in roots and leaves of *A. annua* L. Bars showing the same letter are not significantly different at  $p \leq 0.05$  as determined by Duncan's multiple range test. Error bars ( $\tau$ ) show SE

eters upon exposure of plants to high B levels was quite obvious. Moreover, when MeJA was applied exogenously to non-stressed and stressed plants, it was effective for improvement in plant growth. Also, it proved efficient in reversing the toxic effects of B toxicity. The MeJA is an important regulator of cellular events and is involved in diverse developmental processes such as germination, root growth, fertility and senescence (Wasternack and Hause 2002). In addition, MeJA activates the plant defence mechanism in response to various environmental stresses (Cheong and Choi 2003). In fact, Christov et al. (2001) and Maksymiec and Krupa (2006) showed that MeJA was capable to enhance tolerance in plants against various stresses. Thus, the improved growth of the B-stressed plants by MeJA could be expected in the present investigation.

The net photosynthetic rate in the leaves of B-treated plants decreased in a concentration-dependent manner, showing the lowest photosynthetic activity in the leaves of the plants applied with the highest B level (Fig. 1a). Since the B toxicity also decreased the internal CO<sub>2</sub> concentration, less of the photon energy, captured by the

light harvesting system, is expected to be used in electron transport system, leading to decrease in the photosynthesis. Further, the decrease in chlorophyll content, leading to reduction in net photosynthesis, could be attributed to oxidation of chlorophyll and chloroplastic membranes, which might be exacerbated by the excess of B (Lee 2006; Ardic et al. 2009). The present results suggested that application of 300 µM of MeJA had a beneficial effect on photosynthesis of control and B-treated *A. annua* plants. Fedina and Benderliev (2000) showed that the exogenous supply of MeJA improved the CO<sub>2</sub> fixation, which gives support to the MeJA-improved CA activity in our study (Fig. 2a). Further, this might also be responsible for the enhanced photosynthetic rate in the present study (Fig. 1a). Earlier reports also suggest that MeJA protects photosynthetic pigments under various stresses (Golovatskaya and Karnachuk 2008; Yoon et al. 2009), supporting the improved photosynthetic response of MeJA-treated plants in this study.

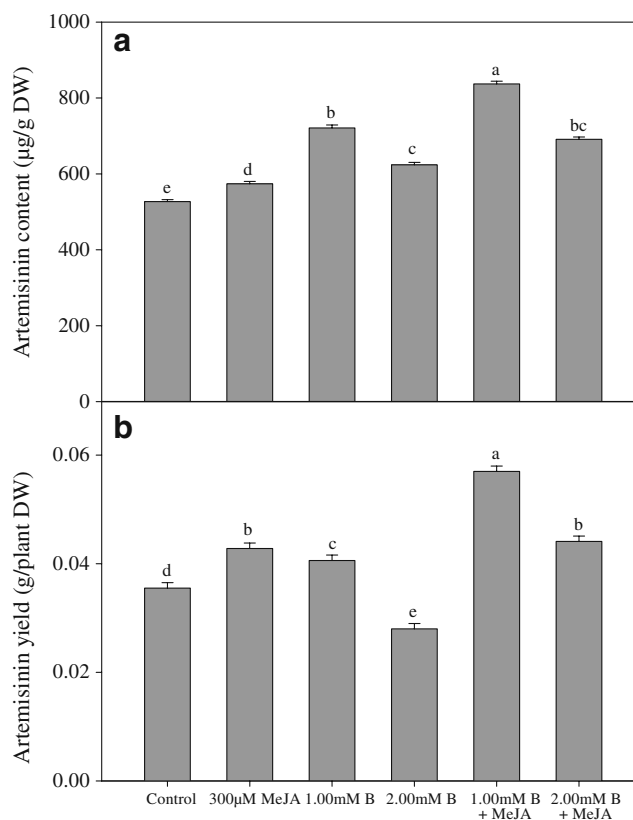
Roots play a number of important roles during plant growth and development and are typically the first part of



**Fig. 4** Effect of B and MeJA on  $H_2O_2$  (a) and  $O_2^-$  (b) contents of *A. 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 ( $\tau$ ) show SE

the plant to encounter several types of environmental stresses (Parra-Lobato et al. 2009). Our results revealed that there was a higher accumulation of B in roots compared to that in leaves in a concentration-dependent manner with increasing B concentrations (Fig. 2b). Exogenous application of MeJA had significant influence on the accumulation of B either in roots or leaves. In this context, our results corroborate the findings of Kaya et al. (2009), who reported comparatively a higher accumulation of B in roots of tomato than in other plant parts. In fact, the ability of the plants to tolerate high B could be due to the capacity of the plants to accumulate more B in roots and a lesser amount of the B being transported to shoots (Paull et al. 1992). However, B uptake responses of the crops might differ significantly with regard to the susceptibility of crops to various stresses (Hu and Brown 1997; Alpaslan and Gunes 2001).

In line with the present results, other researchers have found that excessive B might increase TBARS content in plants (Gunes et al. 2006; Molassiotis et al. 2006). Our results also demonstrated that MeJA was involved in the inhibition of formation of the lipid peroxides, as evident by lower TBARS content in *A. annua* plants exposed to B stress (Fig. 3a). In *Arabidopsis thaliana*, the expression of



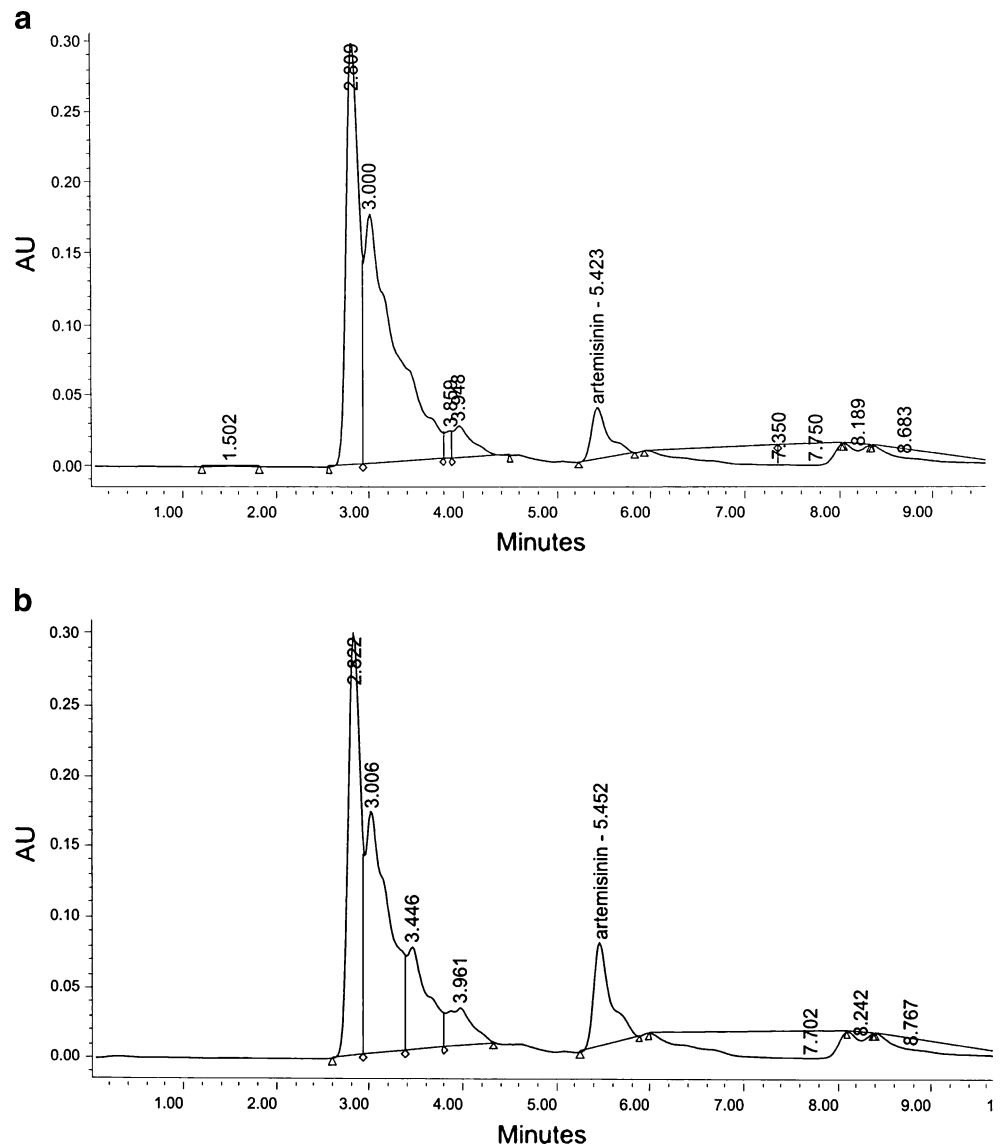
**Fig. 5** Effect of B and MeJA on artemisinin content (a) and artemisinin yield (b) of *A. annua* L. Bars showing the same letter are not significantly different at  $p \leq 0.05$  as determined by Duncan's multiple range test. Error bars ( $\tau$ ) show SE

oxidative stress-tolerance genes including those for peroxidases and oxidases has, in fact, been shown to be upregulated by the MeJA (Jung et al. 2007).

Abiotic stresses increase the formation of ROS that oxidize the membrane lipids, proteins and nucleic acids (Gong et al. 2005). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have great resistance to oxidative damage (Sudhakar et al. 2001). The activities of CAT, POX and SOD were increased in the leaves significantly on account of MeJA treatment applied with or without B in respect with the control (Fig. 3b–d) indicate that MeJA triggered this anti-ROS mechanism as well. Enhancement in the activities of antioxidant enzymes has also been documented by several researchers in response to the B toxicity in various plant species (Molassiotis et al. 2006; Cervilla et al. 2007; Gunes et al. 2007; Han et al. 2009; Jaleel et al. 2009). The present results, showing increased activity of antioxidant enzymes in the B-stressed plants, are in agreement with the ROS scavenging capability of *A. annua* plants (Jaleel et al. 2009). Since the enzymes involved in antioxidant metabolism are usually co-regulated and their activities increase upon exposure to stress, higher activity levels of antioxidant enzymes together with the increased lipid peroxidation



**Fig. 6** HPLC chromatogram showing peaks of artemisinin in control (a) and 1.00 mM B+ 300  $\mu$ M MeJA-treated plants (b) of *A. annua* L.



is quite justified. The application of MeJA further enhanced the activities of all antioxidant enzymes, both in non-stressed and stressed plants, supplementing the ROS scavenging mechanism in *A. annua* plants and, thereby, mitigating the B stress. Mitigation of ROS effects by the MeJA has been reported in the case of strawberry under water stress and in maize seedlings subjected to paraquat (Wang 1999; Norastehnia and Nojavan-Asghari 2006). Thus, under B stress, MeJA could induce antioxidant defence activity in plants to remove the possible toxic effects of free radicals, making the plants more resistant to B stress. In the present study,  $H_2O_2$  and  $O_2^-$  concentrations were increased significantly by applying 1.00 and 2.00 mM of B. According to our results, when MeJA was applied to the B-stressed plants, it further enhanced the ROS level in the plants, particularly in plants applied with 1.00 mM of B but the ROS level was reduced in plants applied with

2.00 mM of B (Fig. 4a). Thus, it appears that MeJA stimulated the production of ROS when it was applied alone or with mild concentration of B (1.00 mM of B). However, when it was applied with high B concentration (2.00 mM of B), it combated the ROS production more than that it did against the application of 1.00 mM of B by inducing more of the antioxidant enzymes as observed in this investigation (Fig. 3b–d). However, such considerations need further researches to be carried out in this regard.

Like that we observed in the present study (Fig. 5a), in our earlier study also, artemisinin content was enhanced significantly due to 1.00 mM of B (Aftab et al. 2010b). The artemisinin content was further increased due to the exogenous application of MeJA (Fig. 5a). Presumably, a mild B stress (1.00 mM of B) promoted the artemisinin biosynthesis by ROS mediation, which might play an important role of converting dihydroartemisinic acid to

artemisinin during artemisinin biosynthesis. In this regard, Wallaart et al. (1999) suggested that dihydroartemisinic acid could act as a scavenger of ROS ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  in this case) that might be released in plant cells upon exposure to oxidative stress. During the reaction, dihydroartemisinic acid hydroperoxide (DHAA-OOH) is generated that gets converted into artemisinin. Our results are in agreement with the observations recorded by Pu et al. (2009), Mannan et al. (2010) and Guo et al. (2010), who advocated that relatively high levels of ROS could result in the enhanced the conversion of dihydroartemisinic acid to artemisinin. Further, it has been reported that terpenoid biosynthesis could be significantly induced by MeJA treatment (Zhao et al. 2005). Terpenoids, especially sesquiterpenoids and their oxygenated derivatives, account for the majority of secondary metabolites in *A. annua* (Li et al. 2006). Studies of Wang et al. (2009) indicated that besides artemisinin, artemisinic acid and dihydroartemisinic acid and other sesquiterpenoids and triterpenoids in *A. annua* were induced by exogenous application of MeJA. Therefore, the co-regulated compounds, like methyl artemisinic acid, dihydroartemisinin and squalene, might be considered responsible for enhanced artemisinin production on account of MeJA application. In fact, methyl artemisinic acid is highly accumulated in *A. annua* plants treated by MeJA (Wang et al. 2009). The above discussion can put forth some insight to the overproduction of artemisinin exposed to MeJA under B toxicity; however, the relationship between methyl artemisinic acid, artemisinic acid and artemisinin needs further investigation.

In summary, our study revealed that the B had inhibitory effects on plant growth as evidenced by reduced shoot length and fresh and dry masses of the plants applied with B. The B stress also adversely affected the photosynthetic efficiency; however, a follow-up treatment of MeJA alleviated the toxic effects of B. The B-stressed plants also showed high lipid peroxidation rate and antioxidant enzyme activities in *A. annua* plants. A mild stress of B (1.00 mM of B), however, stimulated artemisinin biosynthesis. When MeJA was applied exogenously, it alleviated the toxicity of B, improved the plant growth, lowered lipid peroxidation rate and induced the synthesis of several antioxidant enzymes. In the present study, a higher amount of B was accumulated in the roots compared to that in the leaves, indicating the phytoremediation potential of *A. annua*, which needs serious consideration for removing toxic elements from the soil. Most importantly, artemisinin content and yield was higher in MeJA-treated plants under B toxicity. Therefore, it can be concluded that MeJA had protected *A. annua* from B toxicity and stimulated artemisinin biosynthesis in presence or absence of B.

**Acknowledgements** Authors wish to thank Mr. M. Ram (SRF-CSIR) of Jamia Hamdard (Hamdard University) for his kind help in HPLC analysis regarding artemisinin. The financial support to the first author in the form of Research Assistantship by Council of Science and Technology, UP, Lucknow (CST/D-3539) is also gratefully acknowledged.

**Conflict of interest** Authors declare that they have no conflict of interest.

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