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Arsenic exposure modulates physiological attributes and artemisinin biosynthesis in *Artemisia annua* L.

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Abstract
Arsenic (As) is as noxious and toxic to medicinal plants as to all other plants. Artemisinin, extracted from the leaves of artemisia (*Artemisia annua* L.), is considered as a safe remedy against malaria. A randomized-design pot experiment was conducted with two varieties of *A. annua*, namely 'CIM-Arogya' and 'Jeevan Raksha', under As stress. Plants were exposed to increasing levels of soil-applied As (0, 15, 30 and 45 mg kg^{-1} soil) in order to impose As stress. All plant measurements were made on 90 and 120 days after planting (DAP) at pre-flowering and flowering stages, respectively. As per results, variety 'CIM-Arogya' was more As tolerant than the variety 'Jeevan Raksha'. Irrespective of varieties, the peak As level (45 mg kg^{-1} soil) proved to be the most toxic one for *A. annua* plants. It significantly reduced the plant growth, rate of photosynthesis and leaf-chlorophyll content both at 90 and 120 DAP. It also significantly stimulated the activities of various antioxidant enzymes (CAT, POX and APX) at both the stages, indicating the As-mediated oxidative stress in *A. annua* plants. Interestingly, content as well as yield of artemisinin was significantly increased with increasing As levels regardless of varieties. At 45 mg of As kg^{-1} of soil, both varieties of *A. annua* registered the highest artemisinin values, increasing the artemisinin content of 'CIM-Arogya' by 38.0 and 42.6% and of 'Jeevan Raksha' by 32.6 and 35.7% at 90 and 120 DAP, respectively. Likewise, it increased the artemisinin yield of 'CIM-Arogya' by 42.9 and 45.7% and of 'Jeevan Raksha' by 37.5 and 40.5% at the corresponding stages. As stress increased the production of cellular H_2O_2 consistently regardless of the varieties used, representing the role of H_2O_2 in artemisinin biosynthesis in *A. annua*.

Keywords: *Artemisia annua* L., artemisinin, photosynthesis, antioxidant enzymes, as

1. Introduction
Exposure to As causes considerable stress in plants. As-stress leads to inhibition of growth and physiological imbalance [61, 62] and eventually the death of the plants. In fact, exposure to As may trigger a sequence of reactions leading to growth inhibition, disruption of photosynthetic and respiratory machinery, and stimulation of secondary metabolism [31, 45, 47]. Arsenic occurrence in the soil changes the normal functioning of the plants thereby reducing the crop productivity and leading to the stunted growth of the plants [18, 49]. Plants fight the As toxicity by a variety of mechanisms, including hyper accumulation, stimulation of antioxidant defense system and that of phytochelation. To counter the problem of food chain contamination by As, it is important to understand the mechanism as to how plants take up and metabolize the As.

Artemisinin and its derivatives are administered to restrain cancer and tumor activity [63, 75] besides their effective use in the treatment of malaria [25]. However, artemisinin-derived drugs are not available to millions of the world's poorest people because of the low yield (0.1 to 0.5 % of dry weight) of artemisinin in naturally-grown artemisia plants [65]. Genetic improvement of natural artemisinin varieties has been attempted, but the maximum artemisinin-yield achieved so far is 2% of leaf dry-weight [23]. Although artemisinin can be obtained by chemical synthesis, the method is complicated and is not economically feasible because of the poor yield of the drug. Since the artemisia plant is the only viable source of artemisinin production, the enhanced biosynthesis of artemisinin content in the plant is highly desirable [1-9, 28, 34, 66, 68, 69].

As per the World Health Organization (WHO), the toxin-free, artemisinin-based combination therapy (ACT) is most effective against the drug-resistant malaria parasite [19, 70]. Various scientific approaches have been used to enhance artemisinin production including chemical synthesis [10, 72, 73] and genetic engineering of the pathway genes involved in artemisinin biosynthesis in *A. annua* [17, 39, 48, 71]; but, not much success has been accomplished due to high cost of procedure or complex nature of regulation and expression of the genes responsible for artemisinin biosynthesis. Exposure of *A. annua* to abiotic stress-factors, such as light [33, 67], temperature [24], salinity [4, 43], heavy metals [40, 44, 45, 57] and UV light [46] have been reported to enhance ROS generation, which enhance artemisinin yield by facilitating rapid conversion of dihydroartemisinic acid to artemisinin [1-3, 42, 44, 65] during biosynthetic pathway.

In order to increase the desired production of artemisinin and utilize the land unsuitable for food crops (As-affected land), As-tolerant varieties need to be employed in addition to exploiting the scientific approaches with cheaper and more convenient strategies.

The present study was conducted to explore the As-stress mediated changes in the growth, photosynthesis, antioxidant defense system, and production of artemisinin in two *A. annua* varieties, differing in artemisinin yield.

2. Methodology

Before sowing, the seeds of *A. annua* L. (varieties 'CIM-Arogya' and 'Jeevan Raksha') were surface sterilized with 0.02% $HgCl_2$ solution for 5 min with frequent shaking and then washed with de-ionized water. Initially the seeds of both the varieties were sown in the seed beds (1m × 1m). After one month of sowing, the seedlings of uniform size were transplanted to earthen pots (one seedling per pot). The pots were watered a little after transplantation. Prior to transplantation, 5 kg homogenous mixture of soil and farmyard manure (4:1) was filled in each pot (25 cm diameter × 25 cm height). A uniform recommended basal dose of N, P and K was applied before sowing. Physico-chemical characteristics of the soil were: texture-sandy loam, pH (1:2) 7.5, E.C. (1:2) 0.46 m mhos cm^{-1}, and available N, P and K 102.0, 7.80 and 145.5 mg per kg of soil, respectively. Each treatment was replicated five times and each replicate had three plants. Thus, each treatment consisted of 15 pots, and each pot contained a single healthy plant. The pots were sufficiently watered as required.

The experiment was aimed at evaluating two varieties of *A. annua* viz. 'CIM-Arogya' and 'Jeevan Raksha', under As-stress conditions. Different levels of As (0, 15, 30, 45 mg kg^{-1} soil) were applied as sodium hydrogen arsenate heptahydrate ($Na_2HAsO_4.7H_2O$) to the soil after 30 days of transplantation. Arsenic-solution was added to the pots gradually until a final concentration of applied treatments of As was maintained. The experimental pots were irrigated (100-200 mL) daily with double distilled water (DDW) to keep the soil moist. Control plants were supplied with DDW only. Sampling for all the parameters studied was carried out at pre-flowering stage (90 DAP days after planting) and flowering stage (120 DAP).

2.1. Determination of growth attributes

Plants from each treatment were uprooted carefully and fresh and dry weights were recorded based on five replicates per treatment. The plants were dried in hot-air oven maintained at 80°C for 48 hours to record plant dry weight. Only 10% of the randomly selected total leaves of each sample (consisting of five plants) were used to determine the leaf area using graph paper sheet. The mean area per leaf, thus determined, was multiplied with the total number of leaves in order to measure the total leaf area per plant. Leaf-area index was determined according to Watson [67]. Total leaves of the plants were weighed to determine leaf yield.

2.2. Physiological attributes

2.2.1. Total contents of chlorophyll and carotenoids

Total contents of chlorophyll and carotenoids were estimated in the leaves using the method of Lichtenthaler and Buschmann [32]. The fresh tissue from the interveinal leaf-portion was grinded with 100% acetone using mortar-pestle. The optical density (OD) of the pigment solution was recorded at 662, 645 and 470 nm to determine chlorophyll *a*, chlorophyll *b* and total carotenoids content, respectively, using a spectrophotometer (Shimadzu UV-1700, Tokoyo, Japan). Total chlorophyll content was assessed by adding together the content of chlorophyll *a* and *b*. The content of each photosynthetic pigment was expressed as mg g^{-1} leaf FW.

2.2.2. Net photosynthetic rate and stomatal conductance

These parameters were determined employing the youngest fully expanded randomly selected leaves from the five replicates of each treatment. Measurements were made on sunny days at 1100 hours using the Infra-Red Gas Analyzer (IRGA, Li-Cor 6400 Portable Photosynthesis System Lincoln, Nebraska, USA) both at 100 and 120 DAP.

2.2.3. Carbonic anhydrase activity (CA)

The activity of carbonic anhydrase (E.C. 4.2.1.1) enzyme was measured in the fresh leaves selected randomly, using the method described by Dwivedi and Randhawa [21]. Two hundred mg of the leaves (chopped leaf-pieces) were transferred to Petri plates. The leaf pieces were dipped in 10 mL of of 0.2 M cystein hydrochloride solution for 20 minutes at 4°C. The solution adhering to leaf pieces was removed with the help of a blotting paper, followed by immediately transferring them to a test tube containing 4 mL of phosphate buffer (pH 6.8). To it, 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 activity was expressed as µmol CO_2 kg^{-1} leaf FW s^{-1}.

2.2.4. Estimation of proline (PRO) content

The PRO content (mg g^{-1} FW) was estimated using method of Bates *et al*. [11]. Plant material was homogenized in 3% aqueous solution of sulfosalicylic acid, followed by centrifuging the homogenate at 10,000 rpm. The supernatant was employed for the estimation of PRO content. The reaction mixture was added with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid. The content was boiled at 100°C for 1 h. After reaction-termination using ice bath, the PRO content was extracted with 4 mL of toluene, followed by recording the absorbance at 520 nm.

2.2.5. Lipid peroxidation (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). The total content of TBARS was estimated using the method of Cakmak and Horst [14]. TBARS were extracted from 0.5 g of chopped fresh leaves, grinding the latter 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) of tetrabutylammonium (TBA) in 20% (w/v) TCA. Samples were incubated at 90°C for 30 min. Thereafter, the reaction was stopped using an ice bath. The content was centrifuged 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 nmol g^{-1} FW.

2.2.6. Determination of endogenous H_2O_2 content

The leaf H_2O_2 content was determined according to the method of Mukherjee and Choudhuri [37]. 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 mL 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 (nmol g^{-1} FW) was calculated according to the standard curve of H_2O_2 that was prepared by using known concentrations of H_2O_2 ranging from 0 to 10 µM.

2.2.7. Catalase activity (CAT)

The activity of catalase (CAT) was measured according to the method proposed by Chandlee and Scandalios [15] 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_2O_2 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_2O_2 and 40 µL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units per milligram protein (U = 1 mmol of H_2O_2 reduced per minute per mg protein).

2.2.8. Peroxidase activity (POX)
The activity of peroxidase (Unit mg^{-1} protein) was measured according the method of Kumar and Khan [30]. 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_2O_2 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_2SO_4. 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_2SO_4 at zero time. The activity was expressed in units mg^{-1} 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^{-1} mg^{-1} protein.

2.2.9. Superoxide dismutase activity (SOD)
The activity of superoxide dismutase (SOD) was assayed as described by Beauchamp and Fridovich [12]. The reaction mixture contained 1.17×10^{-6} M of riboflavin, 0.1 M of methionine, 2×10^{-5} M of potassium cyanide (KCN) and 5.6×10^{-5} 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, kept under dark, were used as blanks. The absorbance was measured at 560 nm against the blank using the spectrophotometer. SOD activity was expressed as Units mg^{-1} protein. One unit is defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein.

2.2.10. Ascorbate peroxidase activity (APX)
The activity of ascorbate peroxidase (Unit mg^{-1} protein) was estimated by the method used by Nakano and Asada [38]. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mm EDTA, 0.5 mm ascorbate, 0.1 mm H_2O_2 and enzyme extract. The reduction in the absorbance of ascorbate at 290 nm was recorded. APX activity was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One unit of the enzyme is the amount necessary to decompose 1 µmol of substrate per minute at 25°C.

2.3. Yield and quality attributes
Herbage yield was recorded by weighing the total biomass per plant excluding the roots.

2.3.1. High Performance Liquid Chromatography (HPLC) analysis of artemisinin content
Dried leaf-material (1000 mg) was used for the estimation of artemisinin, which was modified to a compound Q260 and quantified using HPLC method [75]. Briefly, 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). Derivatized artemisinin was analyzed and quantified through a reverse phase column (C18; 5 m; 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 (Sigma-Aldrich, USA).

3. Results
Soil-applied As at higher concentrations exerted adverse effects on growth, yield and physiological attributes of *A. annua* at 90 as well as 120 DAP (Tables 1-5). Of the various As levels, 45 mg kg^{-1} soil proved most toxic level as compared to other concentrations. However, at lower As-level (15 mg As kg^{-1} soil), the growth and yield of the crop were slightly improved. Variety 'CIM-Arogya' proved to be more As-tolerant than 'Jeevan Raksha' in terms of above studied attributes. It appeared that variety 'CIM-Arogya' had As-tolerant behavior and could be adapted to As-stress conditions.

3.1. Growth attributes
The effect of different concentrations of As (0, 15, 30 and 45 mg of As kg^{-1} of soil) on morphological characteristics of *A. annua* varieties was significant both at 90 and 120 DAP. The presence of high As-level in the soil inhibited the growth of *A. annua* plants, the most toxic effects being noted at 45 mg of As kg^{-1} of soil., Under the highest As-stress, 'CIM-Arogya' showed a decrease of leaf-area index (15.2 and 16.8%), leaf-yield (15.6 and 10.6%), plant fresh weight (23.5 and 25.6%) and plant dry weight (28.5 and 30.5%) at 90 and 120 DAP, respectively over the water-treated control (Table 1). The percent reduction in the above mentioned growth parameters was higher in 'Jeevan Raksha' than in 'CIM-Arogya'; it was 17.2 and 17.8% for leaf-area index, 16.1 and 18.9% for leaf-yield, 23.6 and 25.0% for plant fresh weight, and 28.8 and 31.7% for plant dry weight, respectively (Table 1). However, the plant fresh and dry weights and leaf yield were slightly increased at 15 mg As kg^{-1} soil over the water-treated control at both the stages (Table 1).

3.2. Physiological parameters
As treatment significantly reduced the rate of photosynthesis and stomatal conductance in both the artemisia varieties over the control (Table 2). The highest As-level (45 mg kg^{-1} soil) proved most deleterious. In 'CIM-Arogya', it reduced the rate of photosynthesis by 14.5 and 15.5% and stomatal conductance by 28.0 and 28.6%, while in 'Jeevan Raksha' the reduction in rate of photosynthesis was 15.3 and 16.5% and that in stomatal conductance 28.6 and 29.2% at 90 and 120 DAP, respectively. Compared to the control, the reduction in chlorophyll was more severe in 'Jeevan Raksha' (26.2 and 28.3%) than in 'CIM-Arogya' (24.3 and 25.4%) at the corresponding stages (Table 2). Treatment 45 mg of As kg^{-1} of soil reduced the carotenoids content by 13.2 and 15.2% for 'CIM-Arogya' and by 15.8 and 17.6% for 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 2). However, at low As-level (15 mg As kg^{-1} soil) carotenoids content was significantly improved in both the varieties at both stages. The activity of carbonic anhydrase (CA) was significantly inhibited by increasing the levels of As in both the varieties at both the growth stages. The peak As-level decreased the CA activity of 'CIM-Arogya' by 14.2 and 15.6% and that of 'Jeevan Raksha' by 16.9 and 18.8% over the control at 90 and 120 DAP (Table 3). As treated plants of both the varieties produced the maximum level of proline in comparison to the control (Table 3). All As-levels significantly increased the leaf-proline content. However, the level of proline was higher in 'CIM-Arogya' than that in 'Jeevan Raksha'. Treatment 45 mg of As kg^{-1} of soil increased the proline content by 21.5 and 21.8% in 'CIM Arogya' and by 20.5 and 24.0% in 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 3). As-treated plants exhibited a higher level of lipid-peroxidation in the leaves, indicating

significant oxidative stress. Compared to the control, the peak As-level resulted in significant ascent in lipid-peroxidation (TBARS content). It increased leaf-TBARS content by 154.2 and 160.3% in 'CIM-Arogya' and by 151.7 and 156.9% in 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 3). The level of endogenous H_2O_2 was measured in order to determine the internal ROS generation caused by soil-applied As. H_2O_2 generation rate was increased sharply at 30 mg of As kg^{-1} of soil. In comparison to the control, it increased the endogenous H_2O_2 generation rate by 25.3 and 32.5% in 'CIM-Arogya' and by 26.7 and 30.0% in 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 3). Thereafter, H_2O_2 level was decreased in both the varieties (Table 3). Excess of As in the soil medium accelerated the activities of several antioxidant enzymes in the treated plants of both the varieties; however, the acceleration was maximally observed in 'CIM-Arogya' (Table 3). The activities of antioxidant enzymes (CAT, POX and APX) were rapidly stimulated at 45 mg of As kg^{-1} of soil. The activities of these enzymes were comparatively lower at lower As-levels. Comparing the control, the peak As-level increased the CAT activity by 20.7 and 24.5%, POX activity by 44.8 and 46.0% and APX activity by 120.0 and 125.3% in 'CIM-Arogya' at 90 and 120 DAP, respectively. As regards Jeevan Raksha, the peak As-level enhanced the CAT activity by 23.5 and 24.8%, POX activity by 45.8 and 48.2% and APX activity by 111.9 and 120.8% at 90 and 120 DAP, respectively (Table 4). However, in both the varieties, SOD activity reached the highest extent at 30 mg of As kg^{-1} of soil. At this As-level, the SOD activity of 'CIM-Arogya' was increased by 80.1 and 84.5%, and of 'Jeevan Raksha' by 76.9 and 79.3% at 90 and 120 DAP, respectively (Table 4). The SOD activity was decline at further higher As-levels in both the varieties (Table 4).

3.3. Yield and quality attributes

Crop herbage yield was noticeably increased at lower As-level (15 mg kg^{-1} soil) irrespective of the stages (Table 5). However, it decreased with increasing As-levels at both stages regardless of *A. annua* varieties. The maximum damage was noticed in 'Jeevan Raksha' at both the stages. Compared with the control, 45 mg of As kg^{-1} of soil (peak As-level) decreased the 'Jeevan Raksha' herbage-yield by 12.6 and 14.8%, while 'CIM-Arogya' herbage-yield was decreased by 10.4 and 8.1% at 90 and 120 DAP, respectively (Table 5). On the other hand, increasing As-levels resulted in higher artemisinin content in treated plants compared to the control regardless of varieties and stages. Variety 'CIM-Arogya' contained the highest artemisinin content under the peak As-level; it increased the artemisinin content by 38.0 and 42.4% in 'CIM-Arogya' and by 32.0 and 35.7% in 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 5). The As-treated plants of the two varieties also produced the highest yield of artemisinin in comparison to the control (Table 5). Though all As-levels significantly increased the production of artemisinin, the leaf-artemisinin content was higher in 'CIM-Arogya' than in 'Jeevan Raksha'. The peak As-level also increased the yield of artemisinin by 42.9 and 45.7% in 'CIM-Arogya' and by 37.5 and 40.5% in 'Jeevan Raksha' at 90 and 120 DAP, respectively (Table 5).

4. Discussion

Higher As-levels adversely affected the overall growth, leaf- and herbage yield and physiological attributes, while they augmented the activity of antioxidant enzymes as well as the content and yield of artemisinin. Surprisingly, low As-level (15 mg As kg^{-1} soil) improved the leaf yield as well as fresh and dry weight of the plant of both the varieties ('CIM-Arogya' and 'Jeevan Raksha') irrespective of stages. Presence of higher As-levels in the soil inhibited the growth of *A. annua* plants, the most toxic effects being noted at 45 mg of As kg^{-1} of soil. Percent reduction in the values was more in 'Jeevan Raksha' than in 'CIM-Arogya' for all the growth attributes studied (Table 1). Excess of As in the soil negatively affects plant growth and crop yield. Plants have developed a series of strategies to limit As toxicity [63]. Over the past decade, exploration of As-transporter proteins has led to the understanding of critical role in As metabolism in plants, indicating that As-moduled signal transduction pathways may lead to fast growth-reduction processes [74, 53]. As per reports, As-treated soils may causes several adverse morphological and reproductive changes in plants such as the loss of fresh and dry biomass of roots and shoots, loss of yield and fruit production [13, 51]. Miteva *et al*. [36] reported significant decrease in shoot and root growth of tomato plants at higher As-levels. According to Shaibur and Kawai [50] and Rai *et al*. [46, 47], a reduction in plant biomass at higher As-level (4,500 µg L^{-1}) might be due to the inhibition of enzyme activity or As-induced oxidative-stress both in *A. annua* and mustard spinach. They suggested that the reduction in *A. annua* biomass could be due to high As-absorption in artemisia plant. The peak As-level (45 mg As kg^{-1} soil) reduced the rate of photosynthesis and stomatal conductance in both the varieties compared to the control. Reduction in leaf-chlorophyll content was more in 'Jeevan Raksha' than in 'CIM-Arogya' (Table 2). On the other hand, As application at 15 mg As kg^{-1} soil significantly improved the level of carotenoids content in both the varieties regardless of the stages. According to Rai *et al*. [46, 47], significant reduction in synthesis of photosynthetic pigments could be due to the lack of adaptive modifications of pigment synthesis at high As-levels. This was supported by several workers [61, 62], who noted that the rate of CO_2 fixation and functional activity of Photosystem II were reduced in plants under As-stress. Results agree with the earlier findings that reveal decline in contents of photosynthetic pigments as a result of As exposure [35, 47, 58, 59]. Factually, As interferes with the functioning of enzymes of carbohydrate and nitrogen metabolism, which may result in impaired growth and reduced biomass [16, 26, 54]. Plant exposure to various As-levels reduced the CA activity in the present study. The decrease in CA activity was also noted previously in response to B as well as Al application to *A. annua* plants [2, 6]. As-treated plants of the two varieties resulted in maximum level of leaf-proline content in comparison to the control (Table 3). In support to our results, an AS-mediated increase in proline level has earlier been noticed regarding *Hydrilla verticillata* [55], *Spinacea oleracea* [41], *Vigna mungo* [56], *Oryza sativa* [29] and *Artemisia annua* [47]. As-treated plants exhibited a higher rate of lipid-peroxidation (TBARS content) in the leaves, indicating significant oxidative stress. At the peak As-level, the extent of lipid-peroxidation in plants was also of maximal extent regardless of varieties as well as stages (Table 3).

The activities of antioxidant enzymes and proline content in the leaves were increased with increasing As-levels, indicating ROS production and oxidative stress in AS-treated plants. As per Foyer and Noctor [22], an important pathway involved in combating ROS production at cellular and organellar (chloroplast and mitochondria) level in plants is the ascorbate-glutathione pathway, with APX and GR (glutathione reductase) being the parts of this pathway.

The present study reveals significant increase in leaf-TBARS content in As-treated plants, suggesting the As-induced production of superoxide radicals, which led to increased lipid peroxidation. It may be agreed that enhanced activities of ROS scavenging enzymes (CAT, POX and SOD) in As-treated plants might be due to increased generation of ROS as a result of oxidative stress. An observed increase in the activity of SOD, the major O_2^- scavenger, finds support from the previous data regarding Indian mustard [20, 27].

The activities of CAT, POX and APX were rapidly stimulated due to the highest concentrations of As (45 mg kg^{-1} soil)

treatment (Table 4). According to Rai *et al*. [45] As-increased activities of the antioxidant enzymes led to the conclusion that scavenging of H_2O_2 in *A. annua* was achieved. As-increased SOD activity in this study finds support from earlier reports [45, 52, 57]. Hyperactivities of these enzymes in *A. annua* may be a strategy adapted by the plant to combat As-toxicity [45, 47]. Herbage yield significantly increased at lower concentration of As (15 mg kg^{-1} soil) regardless of stages (Table 5); however, it decreased with increasing As-levels irrespective of stages and varieties. Maximum damage in herbage yield was noticed in 'Jeevan Raksha' as compared to 'CIM-Arogya' at both the stages. In this study, high As-levels enhanced the content and yield of artemisinin in *A. annua* (Table 5; Fig. 1). In this regard, Rai *et al*. [45, 47] emphasized about significant over-expression of the artemisinin-synthesis genes leading to the transcription of the enzymes such as 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR), amorpha-4, 11-diene synthase (ADS), cytochrome P450 monooxgenase (CPYA171) and farnesyl diphosphate (FDS); that might suggest that As-stress regulates transcription of genes of artemisinin biosynthetic pathway and thus increases artemisinin content in *A. annua*. Significant increase in the size of trichome cells further offer anatomical (site of artemisinin biosynthesis) evidence for increase in artemisinin biosynthesis due to abiotic stresses [65]. It has been reported that exposure of *A. annua* to abiotic stresses might lead to generation of ROS, which in turn facilitate the rapid conversion of dihydroartemisinic acid to artemisinin [64]. An increase in artemisinin content following As treatment could be due to a high conversion rate of its immediate precursor (dihydroartemisinic acid) into artemisinin. Similar observation was made by Rai *et al*. [47], Qureshi *et al*. [44] and by Pu *et al*. [42] under As, lead and salt, salicylic acid stresses, respectively in case of *A. annua*.

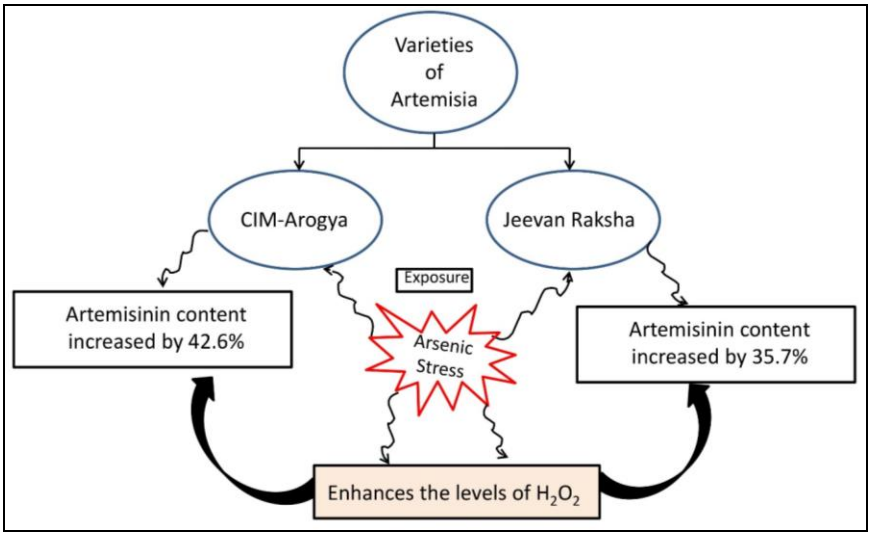

Fig 1: Graphical model represents production of artemisinin in two varieties of *Artemisia annua* L. under arsenic stress

Table 1: Effect of four concentrations of arsenic (0, 15, 30 and 45 mg of As kg^{-1} soil) on growth attributes of two varieties of *Artemisia annua* L. (CIM-Arogya and Jeevan Raksha) recorded at 90 and 120 DAP. The data shown are means of five replicates ± SE.

Parameters	DAP	Arsenic concentrations (mg kg^{-1} soil)							
		CIM-Arogya				Jeevan Raksha			
		0	15	30	45	0	15	30	45
Leaf area Index	90	4.35±0.12^{a}	4.15±0.10^{b}	3.80±0.13^{d}	3.69±0.14^{de}	4.28±0.10^{a}	4.10±0.20^{bc}	3.76±0.14^{d}	3.54±0.10^{f}
	120	4.64±0.10^{a}	4.50±0.12^{b}	4.10±0.14^{d}	3.86±0.11^{de}	4.50±0.14^{a}	4.35±0.12^{c}	3.99±0.15^{d}	3.70±0.13^{f}
Leaf-yield per plant(g)	90	65.40±0.90^{b}	70.62±0.85^{a}	58.40±1.20^{d}	55.20±1.42^{f}	62.42±0.65^{c}	65.20±0.85^{b}	56.10±1.32^{e}	52.40±1.10^{g}
	120	71.00±1.14^{c}	78.30±1.13^{a}	64.25±1.17^{e}	58.50±1.20^{g}	68.54±1.15^{d}	73.53±1.06^{b}	61.62±1.12^{f}	55.56±1.06^{h}
Fresh weight per plant(g)	90	445.0±2.15^{b}	453.5±2.25^{a}	396.0±2.14^{e}	340.4±2.30^{g}	438.6±2.32^{d}	442.0±2.40^{c}	385.0±2.25^{f}	335.0±2.10^{h}
	120	490.6±2.12^{b}	500.0±2.16^{a}	420.6±2.34^{e}	364.9±1.90^{g}	480.4±3.40^{d}	485.2±2.36^{c}	395.0±2.27^{f}	360.3±2.24^{h}
Dry weight per plant(g)	90	74.50±1.10^{b}	76.30±1.21^{a}	60.40±1.24^{e}	53.26±1.12^{g}	70.40±1.18^{d}	72.50±1.30^{c}	56.26±1.21^{f}	50.10±1.14^{h}
	120	87.62±1.06^{b}	90.34±1.10^{a}	70.14±1.21^{e}	60.94±1.15^{g}	80.24±1.20^{d}	82.41±1.31^{c}	64.42±1.20^{f}	54.80±1.12^{h}

Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Table 2: Effect of four concentrations of arsenic (0, 15, 30 and 45 mg of As kg^{-1} soil) on biochemical attributes of two varieties of *Artemisia annua* L. (CIM-Arogya and Jeevan Raksha) recorded at 90 and 120 DAP. The data shown are means of five replicates ± SE.

Parameters	DAP	Arsenic concentrations (mg kg^{-1} soil)							
		CIM-Arogya				Jeevan Raksha			
		0	15	30	45	0	15	30	45
Net photosynthetic rate (µmol m^{-2} s^{-1})	90	14.20±0.04^{a}	13.50±0.04^{d}	13.10±0.03^{e}	12.14±0.04^{g}	13.85±0.04^{b}	13.70±0.04^{c}	12.40±0.05^{f}	11.73±0.05^{h}
	120	16.10±0.03^{a}	15.21±0.05^{c}	14.80±0.04^{e}	13.60±0.02^{g}	15.40±0.02^{b}	15.14±0.03^{d}	14.26±0.03^{f}	12.86±0.04^{h}
Stomatal conductance (m mol m^{-2} s^{-1})	90	0.25±0.01^{a}	0.23±0.01^{b}	0.21±0.02^{b}	0.18±0.01^{c}	0.21±0.01^{b}	0.18±0.01^{c}	0.16±0.01^{d}	0.15±0.01^{d}
	120	0.28±0.01^{a}	0.26±0.01^{b}	0.24±0.01^{c}	0.19±0.02^{e}	0.24±0.01^{c}	0.21±0.01^{d}	0.19±0.02^{e}	0.17±0.02^{e}
Total chlorophyll content (mg g^{-1})	90	1.03±0.005^{a}	0.96±0.006^{b}	0.84±0.004^{d}	0.78±0.007^{f}	1.03±0.005^{a}	0.93±0.006^{c}	0.80±0.006^{e}	0.76±0.004^{g}
	120	1.14±0.006^{a}	1.03±0.004^{b}	0.92±0.004^{d}	0.85±0.005^{f}	1.13±0.004^{a}	1.02±0.005^{c}	0.88±0.005^{e}	0.80±0.006^{g}
Total carotenoids content (mg g^{-1})	90	0.440±0.001^{c}	0.460±0.002^{a}	0.410±0.002^{e}	0.382±0.001^{g}	0.430±0.003^{d}	0.452±0.002^{b}	0.390±0.002^{f}	0.362±0.002^{h}
	120	0.460±0.002^{c}	0.480±0.002^{a}	0.435±0.002^{f}	0.390±0.001^{g}	0.448±0.002^{d}	0.475±0.002^{b}	0.443±0.002^{e}	0.369±0.002^{h}

Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Table 3: Effect of four concentrations of arsenic (0, 15, 30 and 45 mg of As kg^{-1} soil) on CA activity, Proline content, TBRAS content and H_2O_2 content of two varieties of *Artemisia annua* L. (CIM-Arogya and Jeevan Raksha) recorded at 90 and 120 DAP. The data shown are means of five replicates ± SE.

Parameters	DAP	Arsenic concentrations (mg kg^{-1} soil)							
		CIM-Arogya				Jeevan Raksha			
		0	15	30	45	0	15	30	45
CA activity (μM CO_2 kg^{-1} leaf FW s^{-1})	90	210.4±3.24^{a}	203.4±3.54^{b}	191.6±4.14^{d}	180.5±3.54^{f}	212.0±3.34^{a}	199.0±3.50^{c}	187.0±3.43^{e}	176.0±4.27^{g}
	120	230.6±3.20^{a}	215.3±3.46^{c}	206.4±3.54^{d}	194.6±3.35^{f}	222.5±3.29^{b}	212.2±3.42^{c}	198.8±3.49^{e}	180.5±3.53^{g}
Proline content (mg g^{-1} FW)	90	9.30±0.30^{de}	9.70±0.51^{c}	10.46±0.43^{b}	11.30±0.43^{a}	9.13±0.30^{de}	9.45±0.40^{cd}	10.62±0.40^{b}	11.00±0.40^{a}
	120	10.50±0.45^{d}	10.80±0.48^{c}	11.50±0.42^{b}	12.79±0.54^{a}	10.00±0.40^{d}	10.40±0.40^{cd}	11.10±0.40^{bc}	12.40±0.40^{a}
TBRAS Content (nmol g^{-1} FW)	90	6.02±0.05^{g}	9.76±0.06^{e}	11.36±0.08^{c}	15.30±0.09^{a}	6.00±0.04^{g}	9.40±0.06^{f}	10.96±0.08^{d}	15.10±0.12^{b}
	120	6.14±0.06^{g}	9.87±0.06^{e}	12.24±0.09^{d}	15.98±0.09^{a}	6.12±0.08^{g}	9.45±0.09^{f}	12.70±0.09^{c}	15.72±0.10^{b}
H_2O_2 content (nmol g^{-1} FW)	90	36.20±0.46^{e}	38.16±0.43^{d}	45.36±0.54^{a}	43.24±0.50^{b}	35.62±0.43^{f}	37.78±0.52^{d}	45.12±0.64^{a}	42.78±0.65^{c}
	120	38.86±0.52^{g}	42.20±0.40^{e}	51.48±0.60^{a}	48.36±0.48^{b}	36.90±0.52^{g}	40.26±0.56^{f}	47.97±0.60^{c}	46.20±0.52^{d}

Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Table 4: Effect of four concentrations of arsenic (0, 15, 30 and 45 mg of As kg^{-1} soil) on antioxidant enzymes of two varieties of two varieties of *Artemisia annua* L. (CIM-Arogya and Jeevan Raksha) recorded at 90 and 120 DAP. The data shown are means of five replicates ± SE.

Parameters	DAP	Arsenic concentrations (mg kg^{-1} soil)							
		CIM-Arogya				Jeevan Raksha			
		0	15	30	45	0	15	30	45
CAT activity (U mg^{-1} protein)	90	9.40±0.12^{f}	9.76±0.12^{e}	10.24±0.12^{d}	11.35±0.13^{a}	9.10±0.10^{g}	9.50±0.12^{f}	10.64±0.15^{c}	11.24±0.15^{b}
	120	10.39±0.13^{g}	10.68±0.14^{e}	11.10±0.14^{d}	12.94±0.15^{a}	10.22±0.13^{g}	10.42±0.13^{f}	11.46±0.15^{c}	12.75±0.16^{b}
POX activity (U mg^{-1} protein)	90	44.6±0.12^{g}	50.4±0.12^{e}	58.0±0.12^{c}	64.6±0.12^{a}	43.2±0.12^{h}	48.0±0.12^{f}	54.8±0.12^{d}	63.0±0.12^{b}
	120	46.3±0.12^{g}	52.7±0.12^{e}	56.4±0.12^{c}	67.6±0.12^{a}	44.8±0.12^{h}	50.4±0.12^{f}	54.0±0.12^{d}	66.4±0.12^{b}
SOD activity (Unit mg^{-1} protein)	90	1.36±0.05^{g}	1.60±0.04^{e}	2.45±0.06^{a}	2.38±0.05^{b}	1.30±0.05^{h}	1.56±0.06^{f}	2.30±0.07^{c}	2.24±0.04^{d}
	120	1.42±0.04^{g}	1.64±0.05^{e}	2.62±0.08^{a}	2.56±0.04^{b}	1.35±0.05^{g}	1.60±0.06^{f}	2.42±0.06^{c}	2.32±0.04^{d}
APX activity (Unit mg^{-1} protein)	90	15.0±0.08^{g}	20.4±0.10^{e}	28.4±0.14^{c}	33.0±0.16^{a}	15.1±0.08^{g}	18.0±0.12^{f}	26.0±0.13^{d}	32.0±0.14^{b}
	120	15.8±0.09^{g}	21.2±0.12^{e}	30.4±0.16^{c}	35.6±0.18^{a}	15.4±0.09^{g}	19.4±0.10^{f}	27.8±0.15^{d}	34.0±0.15^{b}

Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

Table 5: Effect of four concentrations of arsenic (0, 15, 30 and 45 mg of As kg^{-1} soil) on content and yield of artemisinin of two varieties of *Artemisia annua* L. (CIM-Arogya and Jeevan Raksha) recorded at 90 and 120 DAP. The data shown are means of five replicates ± SE.

Parameters	DAP	Arsenic concentrations (mg kg^{-1} soil)							
		CIM-Arogya				Jeevan Raksha			
		0	15	30	45	0	15	30	45
Herbage yield (g)	90	315.0±3.20^{c}	328.8±3.49^{a}	294.0±3.56^{e}	282.4±3.50^{g}	310.0±3.18^{d}	322.0±2.40^{b}	286.5±3.32^{f}	270.8±3.20^{h}
	120	330.6±3.42^{c}	346.0±3.62^{a}	300.2±3.68^{e}	289.6±3.54^{g}	324.5±3.29^{d}	344.5±3.00^{ab}	293.0±3.46^{f}	276.6±3.41^{h}
Artemisinin content (μg g^{-1} DW)	90	526.4±0.20^{g}	570.0±0.24^{e}	640.4±0.24^{c}	726.4±0.27^{a}	510.0±0.18^{h}	545.0±0.26^{f}	600.0±0.29^{d}	676.4±0.30^{b}
	120	540.0±0.14^{g}	590.2±0.14^{e}	650.6±0.16^{c}	770.2±0.25^{a}	526.9±0.16^{h}	560.4±0.29^{f}	615.8±0.30^{d}	715.2±0.32^{b}
Artemisinin yield (g $plant^{-1}$ DW)	90	0.035±0.001^{d}	0.040±0.001^{c}	0.044±0.001^{b}	0.050±0.002^{a}	0.032±0.001^{e}	0.034±0.001^{d}	0.040±0.002^{c}	0.044±0.002^{b}
	120	0.046±0.001^{d}	0.052±0.002^{c}	0.058±0.002^{b}	0.067±0.002^{a}	0.042±0.001^{e}	0.045±0.001^{d}	0.051±0.002^{c}	0.059±0.002^{b}

Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

5. Conclusions

Two varieties of *A. annua* viz. 'CIM-Arogya' and 'Jeevan Raksha' were pot-grown under As stress for the first time in order to assess the As-induced changes on the basis of growth, physiological attributes and production of artemisinin. Of the applied As levels, 45 mg kg^{-1} of soil proved to be the most toxic one for growth, yield and quality of *A. annua*. Variety 'CIM-Arogya' proved more As-tolerant than variety 'Jeevan Raksha' at both the growth stages. Variety 'CIM-Arogya' registered more artemisinin production than 'Jeevan Raksha' under the highest As-stress. This concludes that *A. annua* variety 'CIM-Arogya' could be good alternative for cultivation in As-contaminated soils for enhanced artemisinin production.

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