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Arsenic, chromium and NaCl induced artemisinin biosynthesis in *Artemisia annua* L.: A valuable antimalarial plant

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ABSTRACT

Effect of As(III), Cr(VI) and NaCl on plant growth, antioxidant enzymes, SOD, TBRAS, protein, cDNA amplification of key genes of artemisinin pathway and artemisinin biosynthesis have been investigated to explore the actual changes in total herb and artemisinin yield in a crop cycle of *Artemisia annua*. Enhanced TBARS and SOD activity (4 U mg^{-1}), decreased catalase activity and total cholorophyll content were observed under metal(loid) and NaCl stress. Accumulation of As (III; $\mu g\ mg^{-1}$ DW) was higher in roots (10.75 ± 0.00) than shoot (0.43 ± 0.00) at 10 $\mu g\ ml^{-1}$. While Cr(VI; $\mu g\ ml^{-1}$ DW) accumulated more in shoots (37 ± 9.6, 41.1 ± 7.2 and 52.71 ± 19.6). cDNA template of these treated plants along with control were amplified with HMGR, ADS and CYP71AV1 genes (artemisinin biosynthetic pathway genes); showed very low expression with Cr(VI) while As(III) (5 and 7.5 $\mu g\ ml^{-1}$) showed higher expression than control. The results obtained from this study suggest that *A. annua* can grow well with favoring artemisinin biosynthesis with treatment of As(III) 5, 7.5 $\mu g\ ml^{-1}$ and NaCl, while 10 $\mu g\ ml^{-1}$ As(III) and all doses of Cr(VI) affect artemisinin synthesis. Finally some evidence also suggests that As(III), Cr(VI) and NaCl induces stress affects on total herb yield of plant.

1. Introduction

Artemisinin (a sesqueterpene lactone containing natural endoperoxide), is an effective antimalarial drug extracted from leaves of Chinese medicinal herb *Artemisia annua* L. (Asteraceae; Lui et al., 1979). Because of increasing resistance of *Plasmodium falciparum* to traditional antimalarial drugs (quinine and chloroquine), artemisinin and its derivatives have become the most important agents in the treatment of cerebral malaria. It is a single drug which can be used against sensitive and resistance malaria particularly in the form of artemisinin-based combination therapies (ACTs). *A. annua* is the only source of artemisinin but low content (0.01–1.0 percent) of artemisinin makes it a relatively expensive drug and also received attention for enhancement by using different breeding (Paul et al., 2010) and biotechnological tools. Artemisinin is also effective against a variety of other diseases, such as hepatitis B (Romero et al., 2005), parasites that cause schistosomiasis (Borrmann et al., 2001), and a range of cancer cell lines (Efferth et al., 2001; Singh and Lai, 2001).

A number of reports on food crops, vegetables, medicinal plants and agricultural/fertile lands showing gradual and persistent effect of heavy metal toxicity. Higher or excess uptake of heavy metals may affect the natural resistance of plants to disease and secondary metabolite synthesis (Nasim and Dhir, 2010). Moreover, some research results suggest that heavy metals may play an important role in triggering plant genes to alter the nature of secondary metabolites, although the exact mechanism by which this happens is still unclear. Toxic effects of heavy metal on plant growth, development and metabolism were reported by many workers, which may affect total dry mass production and yield (Nasim and Dhir, 2010; Manara, 2012). Heavy metals like As, Cr, Pb etc. causes deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition. Metabolic alterations due to Cr exposure have also been described in plants by a direct effect on enzymes or other metabolites or by its ability to generate reactive oxygen species which may cause oxidative stress. Formation of stress proteins is induced by any stress factors including toxic metals (Manara, 2012). Alternatively there are also reports of increase of secondary metabolites in plant under stress conditions (Ramakrishna and Ravishankar, 2011).

Abbreviations: As(III), arsenic; Cr(VI), chromium; HNS, Hoagland's nutrient solution; NaCl, sodium choloride; TBARS, 2-thiobarbituric acid reactive substances; MDA, malondialdehyde; TCA, trichloroacetic acid; TBA, thiobarbituric acid; SOD, superoxide dismutase; NBT, nitroblue tetrazolium salt; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase gene; ADS, amorpha-4, 11-diene synthase gene; CYP71AV1, amorpha-4, 11-diene 12-hydroxylase gene; DMRT, Duncan's multiple range test; DW, dry weight; FW, fresh weight

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African and some Asian countries are prone to malaria and heavy metal contaminations are also reported from these areas especially Pb, As, Cr and Cd etc. It is reported that secondary metabolite synthesis is affected when plant is growing under different biotic, abiotic (including heavy metal) and environmental stress. A dose of arsenic may enhance artemisinin biosynthesis (Rai et al., 2011) but higher dose may affect plant growth and metabolism. Similarly under salinity stress (NaCl) artemisinin content has also been enhanced (Qian et al., 2007) but there were no reports on the effect of these metals in total herb and artemisinin yield in a crop cycle which was grown in such contaminated areas. Hence, an attempt has been made with the following objectives. (1) To determine the impact of As(III), Cr(VI) and NaCl on the growth and overall physiological changes in plants, (2) effect of these compounds (at different doses) in artemisinin and total herb yield in a crop cycle and (3) study on comparative cDNA amplification with artemisinin biosynthetic pathway genes in treated and untreated plants.

2. Materials and methods

2.1. Plant material and experimental procedure and design

The plant material was collected from institute's experimental field where population of *A. annua* have been maintained and material of parent plants were taken from arboretum of the Institute (GBPIHED Kosi-Katarmal, Almora, Uttarakhand, India) and analyzed for artemisinin content. The experiments were carried out at poly house of Institute (1150 m amsl 29°38'15" N and 79°38'10" E). Based on artemisinin content, seeds of high yielding (0.3–0.5 percent) *A. annua* plants were disinfected with 0.1 percent $HgCl_2$ for 1 min and washed thoroughly for 5 times. Seeds were transferred in a tray between moist filter paper for 15–20 d in dark with 25 ± 2 °C. Then seedlings were transferred in acid treated sand with continuous supply of 30 percent Hoagland's nutrient solution (HNS) for 2 months before treatment. Three arsenic {As (III)}, chromium {Cr(VI)} and two NaCl treatments were made using $K_2Cr_2O_7$, As_2O_3 (5.0, 7.5, and 10 μg ml^{-1}) and NaCl (2 and 4 g l^{-1}) along with control were used, respectively. The experiment was conducted with five replicates for 180 d. Fresh leaves were used for biochemical analysis.

2.2. Plant growth parameters

Quantitative characters in the form of plant height (cm), number of primary and secondary branches, leaf length (cm), leaf width (cm), fresh weight (mg) and root length (cm) were recorded at the time of harvesting of each replicates. The harvested root and shoot biomass were subjected to different biochemical and molecular analysis and air shade dried leaf (approximately 15–20 percent moisture) material was subjected for chemical analysis (artemisinin).

2.3. Chlorophyll content

Total chlorophyll content in fresh leaves was estimated following method of Lichtenthaler and Buschmann (2001). The fresh tissue of leaf was ground using a mortar and pestle containing 2 ml of 80 percent acetone. The absorbance of solution was recorded at 662 and 645 nm for chlorophyll estimation using spectrophotometer (Amersham Biosciences, Ultrospec 2100 pro, USA).

2.4. Lipid peroxidation (TBARS) and protein content

Oxidative damage in leaf lipids was estimated by content of total 2-thiobarbituric acid reactive substances (TBARS; nmol g^{-1}) expressed as equivalents of malondialdehyde (MDA). TBARS content (fresh weight) was estimated by method of Cakmak and Horst (1991). TBARS was extracted from 0.5 g fresh leaves, ground in 5 ml of 0.1 percent (w/v) trichloroacetic acid (TCA). Ground material was centrifuged at 12000*g* for 5 min, 1 ml from supernatant was taken and added to 4 ml of 0.5 percent (w/v) TBA in 20 percent (w/v) TCA. Samples were incubated at 90 °C for 30 min. The reaction was stopped in ice bath and centrifuged at 10000*g* for 5 min. Absorbance of the supernatant was taken at 532 nm on a spectrophotometer (Amersham Biosciences, Ultrospec 2100 pro, USA) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. Protein was isolated following the protocol of Ni et al. (1996) using QB buffer and estimated with Bradford (1976) method.

2.5. Antioxidant enzymes assay

Catalase activity was measured following method of Chandlee and Scandalios (1984) with small 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 enzyme extract. The decomposition of H_2O_2 was followed by decline in absorbance at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Superoxide dismutase activity was assayed as given by Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10^{-6} M riboflavin, 5.6×10^{-5} M nitroblue tetrazolium salt (NBT) dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8) and 3 ml of reaction medium was added to 1 ml of enzyme extract. The mixtures were kept under fluorescent light (Philips 40 W). The reaction was initiated at 30 °C for 1 h. Identical solutions that were kept under dark served as blanks. The absorbance was taken at 560 nm in spectrophotometer against the blank and the activity of SOD has been measured in U/mg FW.

2.6. Extraction of artemisinin

Artemisinin content was estimated at three different stages (before treatment, 7 d after treatment and at time of harvesting i.e., 180 d). Air shade dried plant material of all plants were powdered and 0.1 g each were extracted in 10 ml hexane by initial heating at 50 °C for 3 min and left overnight at room temperature. The extract was then filtered and evaporated on water bath at 50 °C. After evaporation, extract was dissolved in 1 ml acetonitrile and 20 μl was injected in HPLC (Kontron Instruments, Milan, Italy) using RP_{18} column (Lichrosort, 250×4.6 mm^2 id, 5 μm) and eluted isocratically with acetonitrile and water (70:30 v/v, flow rate 0.75 ml/min). Detection was carried out at 210 nm using an online UV detector, the results were compared and level of artemisinin was estimated using a dose response curve made with standard Artemisinin (Sigma, USA).

2.7. Metal accumulation

The oven-dried tissue samples were ground and acid digested and As(III) and Cr(VI) were estimated following the method of Sinha et al. (2010).

2.8. Isolation and amplification of RNA

Total RNA was isolated from leaf tissue of treated along with control plants by using total RNA isolation kit (Merck Biosciences Germany). First strand cDNA synthesis was done following the manufacturer's protocol (Biorad, USA). Primers of three key genes of artemisinin biosynthetic pathway (encoding HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase Chen et al., 2000 Gene bank no AF142473) of Mevalonate (MVA) pathway, ADS (amorpha-4,11-diene synthase; Mercke et al., 2000, Gene bank no EF197888), and CYP71AV1 (CYP71AV1, amorpha-4,11-diene 12-hydroxylase enzymes; Ro et al., 2005 and Teoh et al., 2006; Gene bank no DQ268763) were used for amplification. Polymerase chain reactions (PCR) were carried out in 25 μl volume. A reaction tube contained 1 μg of cDNA, 0.3 units of *Taq* DNA polymerase, 5 mM of each dNTPs, 1.5 mM $MgCl_2$ and 10 pmol of HMGR (F (forward)-5′GGTCAGGATCCGGCCCAAAACATT3′; R (reverse)-5′CCAGCCAACACCGAACCAGCAACT3′), ADS (F-5′ATACAACGGGCACTAAAGCAAC C3′; R-5′GAAAACTCTAGCCCGGGAATACTG3′) and CYP71AV1 (F-5′GGTCAGGATCCG GCCCAAAACATT3′; R-5′CCAGCCAACACCGAACCAGCAACT3′) primers. The amplification was carried out using 94 °C for 5 min, 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min and final extension was 72 °C for 8 min for 35 cycles in thermal cycler (Biometra, Germany). Further quantification of different genes was determined by comparing with equal amount of cDNA template on formaldehyde gel.

2.9. Statistical analysis

Each plant in pot was treated as one replication and all treatments were replicated five times. The data was analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's Multiple Range Test (DMRT) at $p < 0.05$ percent level using different letters.

3. Results

3.1. Plant growth parameters

The presence of As(III) and Cr(VI) in acid wash sand supplemented with Hoaglands solutions showed significantly lower plant height, branching pattern in terms of number of primary and secondary branches and leaf morphology than the control.