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UV-B and UV-C pre-treatments induce physiological changes and artemisinin biosynthesis in *Artemisia annua* L. – An antimalarial plant

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ABSTRACT

Present study was undertaken to investigate if short-term UV-B (4.2 kJ m^{-2} day^{-1}) and UV-C (5.7 kJ m^{-2} day^{-1}), pre-treatments can induce artemisinin biosynthesis in *Artemisia annua*. Twenty-one day old *Artemisia* seedlings were subjected to short-term (14 days) UV pre-treatment in an environmentally controlled growth chamber and then transplanted to the field under natural conditions. Treatment of *A. annua* with artificial UV-B and UV-C radiation not only altered the growth responses, biomass, pigment content and antioxidant enzyme activity but enhanced the secondary metabolites (artemisinin and flavonoid) content at all developmental stages as compared to non-irradiated plants. The extent of oxidative damage was measured in terms of the activities of enzymes such as catalase, superoxide dismutase and ascorbate peroxidase. Reinforcement in the antioxidative defense system seems to be a positive response of plants in ameliorating the negative effects of UV-B and UV-C radiations. While the carotenoid content was elevated, the chlorophyll content decreased under UV-B and UV-C pre-treatments. The reverse transcription PCR analysis of the genes associated in artemisinin/isoprenoid biosynthesis like 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), cytochrome P450 oxidoreductase (CPR) and amorpha-4,11-diene synthase (ADS) genes at different growth stages revealed UV induced significant over-expression of the above protein genes. UV-B and UV-C pre-treatments, led to an increase in the concentrations of artemisinin at full bloom stage by 10.5% and 15.7% than that of the control respectively. Thus, the result of our study suggests that short term UV-B pre-treatment of seedlings in greenhouse prior to transplantation into the field enhances artemisinin production with lesser yield related damages as compared to UV-C radiation in *A. annua*.

1. Introduction

Depletion of stratospheric ozone due to anthropogenic sources such as chlorofluorocarbons has led to an increase in solar ultraviolet-B radiation (315–280 nm) reaching the Earth's surface, which has potentially deleterious consequences for agricultural production and natural plant ecosystems [1–3]. Increased UV-B radiation causes direct and indirect effects including degradation and conformational changes in DNA, protein and lipids and alterations in photosynthesis, growth and morphology of plants [4–6].

Studies conducted on impact of UV-B radiation on crops and herbaceous plants have revealed a variety of morphological alterations such as plant stunting, leaf discoloration, reductions in leaf area, biomass and plant productivity [7–9]. In order to protect from UV stress plants may produce UV-absorbing compounds such as flavonoids in the leaf epidermis [5,10]. Furthermore, plants activate enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), which mitigate UV-induced damage due to the production of active oxygen species (AOS). In contrast to the above, studies on the impact of UV-B on medicinal plants and their secondary metabolites are still very few [11,12] except some on essential oil products [13–15].

Artemisia annua L. chosen for the present study is an important medicinal plant, native to China and belonging to family Asteraceae. It has evoked wide interest for its artemisinin content, a secondary metabolite that has become increasingly popular as a promising drug against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. The drug has been shown to be effective against other infectious diseases such as schistosomiasis, leishmaniasis, and hepatitis B. More recently, it has also been demonstrated to be effective against numerous types of tumors [16]. It can also be used in treatment of a range of cancer cell lines, including breast cancer, human leukemia, colon, and

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small-cell lung carcinomas [17]. However, the low concentrations of artemisinin in *A. annua*, which range from less than 0.01% to 1% of the plant dry weight (DW) [18], makes artemisinin relatively expensive and difficult to meet the demand of over 100 million courses of artemisinin combination therapies (ACTs) each year [19]. Various approaches including the chemical synthesis [20,21] and genetic engineering of the pathway genes involved in artemisinin biosynthesis in *A. annua* [22–25], have been attempted to increase artemisinin production but none have been successful because of high cost and complexity. So, extraction of artemisinin from the source plant remains the most economically viable strategy, thus far.

Earlier reports have shown that exposure of *A. annua* to abiotic factors such as light [26,27], temperature [28], salinity [29] and heavy metal [30] significantly enhances the artemisinin yield in the plants. It is suggested that abiotic stresses such as heavy metals, low temperature, drought and salinity trigger the generation of AOS, which in turn, facilitate rapid conversion of dihydroartemisinic acid to artemisinin [31], thereby increasing artemisinin content in the plant. Despite being potent inducer of AOS, UV-B and UV-C radiations have never been tested to ascertain if they can enhance artemisinin biosynthesis in *A. annua.* Having known that UV-C is more detrimental than UV-B to plants, it has been chosen for this study, because we presume that its short pre-treatment may generate more AOS vis-à-vis more artemisinin content in the plants.

Keeping in mind the reports of UV induced secondary metabolites synthesis in *Glycyrrhiza* [32], *Catharanthus* [33] *Tropaeolum majus* [34], and *Taxus* [35] we hypothesized that short term UV-B or UV-C pre-treatments of *Artemisia* seedlings in the greenhouse may generate free radicals thereby converting its immediate precursors viz. dihydroartemisinic acid, artemisinic acid and arteannuin B into artemisinin leading to its higher production in pretreated than the control plants following transfer to the field conditions. While it is pertinent to examine that UV pre-treatment induces artemisinin production on transferring the plants outdoors on one hand, it should not severely damage the morphological, physiological attributes of the plant on the other. Furthermore, it is also relevant to know if the secondary metabolite production is a transient metabolic adjustment or a process involving genes of the biosynthetic pathway.

Taking recourse to the above, the present study was designed to compare the effect of short term treatment of UV-B and UV-C on (a) morphological characteristics, (b) markers of oxidative stress such as proline, TBARS and H_2O_2 contents, (c) antioxidative defense system, (d) protein genes of artemisinin biosynthetic pathway and finally (e) artemisinin production by the test plants. The study will provide first hand information to recommend as which of the two (UV-B or UV-C) could be a potential stressor for short term pretreatment of seedlings in greenhouse prior to transplantation into the field for continued artemisinin production.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of *Artemisia annua* L. (Family: Asteraceae) were collected from CIMAP, Lucknow and grown in Botanical garden of Banaras Hindu University, Varanasi, India. Seeds were surface-sterilized with 2% sodium hypochlorite for 15 min, and then rinsed several times with distilled water. Germination was performed in the dark at 25 ± 1 °C in plastic pots containing vermiculite and sand in 2:1 (vol./vol.) ratio. Plants were watered every 1–2 day with 10% Hoagland nutrient solution. After 7 day, germinated seeds were transferred to a growth chamber with a 65–75% relative humidity, 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 14/10 h photoperiod (8:00–22:00) and 20/16 °C air temperature in the day and night, respectively. The photosynthetically active radiation (PAR) was supplied with Philips HPI PLUS 250 W BU E40 lamps. Pots were maintained in growth chambers for 21 days.

2.2. Experimental design and UV treatments

Twenty-one days after germination all seedlings were randomized into three groups. One group of plants was exposed to PAR (control), whereas the other two groups were exposed to PAR + UV-B and PAR + UV-C respectively. Each group of plants had three replicates and each replicated consisted of four plants. UV-B and UV-C exposures were provided daily for 30 min from 11:30 to 12:00 (4.5 h after the beginning of the photoperiod) for 14 days in growth chambers. Control plants, were deprived of the UV-B and UV-C radiation through the interposition of a clear polyester film. (Folanorm 0.1 mm, Folex, Dreieich, Munich, Germany). UV-B was artificially provided by three Q panel UV-B 313 40 W, 120 cm fluorescent lamps (Q panel Inc Clevland, OH, USA). The lamps were held in a movable frame over the seedling canopy with the distance between adjacent lamps about 35 cm. Lamps were wrapped with 0.125 mm thick cellulose diacetate film (Lucky Films Co. Ltd., Baoding, China) to filter out the UV-C (<280 nm) irradiation in the UV-B treatments. The UV-B irradiance at the top of the plant canopy under the lamps was measured with an ultra violet intensity meter (UVP Inc, San Gabriel, USA). The spectral irradiance was weighted according to the generalized plant action spectrum [36] and normalized at 300 nm to obtain the biological effective (BE) UV-B irradiation (UV-BBE). The low (*L*UVB) biologically effective dose employed was 4.2 kJ m^{-2} day^{-1}. UV-B irradiation was maintained at the specified levels (measured at the top of the plant canopies) throughout the experiment by adjusting the lamp-to-plant canopy distance every 3 days. Another group of plants were exposed to UV-C irradiations provided by a germicidal lamp (STYLO STY 115, GE Lighting, Milan, Italy λ_{max}, 254 nm). Filters were changed every 3 days to avoid aging effects on the spectral transmission of UV radiations. 14 days after treatment (DAT), one set of UV-B and UV-C treated plants and one set of control plants were immediately harvested for biochemical and pigment analysis. Another two sets of treated plants along with the control were transferred to the outdoors in natural field conditions. Plants were sampled in triplicates at 45 (pre-flowering) 75 (full-bloom) and 95 (post-flowering) days after germination to analyze the impacts of UV-B and UV-C on secondary metabolite/artemisinin biosynthesis and at growth in plants.

2.3. Growth and biomass analysis

Growth analysis was performed at the full-bloom stage. Plant height, main stem diameter, number of branches, the angle of primary branch to main stem was recorded. Leaf area was measured with a portable leaf area meter (model LI-3000, LICOR Inc., USA). The above ground part of the biomass was partitioned into different organs (stem and leaves) and dry weights were obtained after oven-drying at 65 °C for 48 h.

2.4. Photosynthetic pigments analysis

Chlorophyll and carotenoid contents were determined by the method of Arnon [37]. 0.2 g leaf samples of control and treated plants were placed in 10 mL of chilled 80% acetone overnight in a refrigerator at 4 °C. The extract was homogenized and centrifuged at 12,000*g* for 15 min. The supernatant was collected and absorbance was recorded at 645 nm and 8663 nm to determine the amounts of chlorophyll a and b respectively. The amount of carotenoids (mg g^{-1} FW) was determined by recording the absorbance at 480 and 510 nm. All the spectrophotometeric assays were carried out using Hitachi U-2910 spectrophotometer.