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Effect of high light intensity on photoinhibition, oxyradicals and artemisinin content in *Artemisia annua* L.

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Abstract

Artemisia annua L. produces a compound called artemisinin that is a potent anti-malarial compound. However concentration of artemisinin within the plant is typically low (less than 0.8% of dry mass) and currently supply of the drug by the plant does not meet world demand. This investigation was carried out to determine whether high intensity light treatment would increase production of artemisinin in leaves of *A. annua*. Photoinhibition (14%) was induced in leaves of *A. annua* when they were subjected to 6 h of high-intensity light [2,000 μmol(photon) m^{-2} s^{-1}]. Maximum photochemical efficiency of PSII showed a recovery of up to 95% within 24 h of light induced inhibition. During the light treatment, photochemical efficiency of PSII in leaves of the high-intensity light-treated plants was 38% lower than for those from leaves of plants subjected to a low-intensity-light treatment of 100 μmol(photon) m^{-2} s^{-1}. Nonphotochemical quenching of excess excitation energy was 2.7 times higher for leaves treated with high-intensity light than for those irradiated with low-intensity light. Elevation in oxidative stress in irradiated leaves increased presence of reactive oxygen species (ROS) including singlet oxygen, superoxide anions, and hydrogen peroxide. Importantly, the concentration of artemisinin in leaves was two-fold higher for leaves treated with high-intensity light, as compared to those treated with low-intensity light. These results indicate that *A. annua* responds to high irradiance through nonphotochemical dissipation of light energy yet is subject to photoinhibitory loss of photosynthetic capacity. It can be concluded that *A. annua* is capable of rapid recovery from photoinhibition caused by high light intensity. High light intensity also induced oxidative stress characterized by increased concentration of ROS which enhanced the content of artemisinin. Such a light treatment may be useful for the purpose of increasing artemisinin content in *A. annua* prior to harvest.

Additional key words: chlorophyll fluorescence; environmental stress; irradiation.

Introduction

Artemisia annua L. is an aromatic annual herb that is native of temperate Asia. It produces a compound called artemisinin, which is an endoperoxide-bridged sesquiterpene lactone synthesized in glandular trichomes (Pandey *et al.* 1999). Artemisinin is currently one of the most effective drugs for treatment of malaria and is recommended by the World Health Organization (WHO) in the form of artemisinin-based combination therapy (ACT). Artemisinin has also been shown to have activity against viral diseases including hepatitis B and C (Efferth *et al.* 2008) and nonmalarial parasitic infections including schistosomiasis (Krishna *et al.* 2008) and leishmaniasis (Sen *et al.* 2010). Because the concentration of artemisinin in *A. annua* tissues is extremely low [0.01–0.8% of dry mass (DM); van Agtmael *et al.* 1999] the availability of an adequate supply of the compound to meet world demand has traditionally been a problem (Abdin *et al.* 2003).

Recently, a protocol for efficient semisynthetic production of artemisinic acid, a precursor of artemisinin, and for the conversion of artemisinic acid to artemisinin using singlet oxygen has been reported (Paddon *et al.* 2013). This methodology of artemisinin synthesis has the potential to provide a stable, inexpensive source of artemisinin to meet the world demand for ACT. However, methods to increase artemisinin content in plants prior to extraction are still of great interest since there is evidence that other compounds found in *A. annua* work synergistically with artemisinin for enhanced efficacy

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Abbreviations: ACT – artemisinin-based combination therapy; APX – ascorbate peroxidase; FM – fresh mass; F_m – maximal fluorescence yield of the dark-adapted state; F_m' – maximal fluorescence yield of the light-adapted state; F_s – steady-state fluorescence yield; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; HPLC – high pressure liquid chromatography; NPQ – nonphotochemical quenching of PSII; ROS – reactive oxygen species; q_P – photochemical quenching coefficient; SD – standard deviation; UV-B – ultraviolet-B; UV-C– ultraviolet-C; Φ_{PSII} – quantum efficiency of PSII; WHO – World Health Organization.

against malaria (Ferreira *et al.* 2010) and cancer (Ivanescu and Corciova 2014). Further, it would be more economically feasible to produce artemisinin through plant extraction methods in certain locations where malaria is prevalent (Brisibe and Chuckwurah 2014).

There is great variability in the artemisinin content from different accessions of *A. annua* (Delabays *et al.* 2001). Increased leaf artemisinin content has been achieved using traditional plant breeding techniques (Delabays *et al.* 2001) and specific genetic-loci affecting the yield of artemisinin have been identified (Graham *et al.* 2010). Artemisinin content is dependent on the developmental stage of plants (Ferreira *et al.* 1995) and is variable for harvested plant tissue depending on post-harvest drying methods used (Ferreira and Luthria 2010). Soil nitrogen and potassium availability affects biomass and artemisinin production for *A. annua* in the field (Ferreira 2007, Davies *et al.* 2009) and preharvest treatment of plants with plant growth regulators such as GA_3 and triconatrol (Aftab *et al.* 2010a, Banyai *et al.* 2011), mechanical wounding of leaves (Liu *et al.* 2010), application of the fungicide, chitosan, (Lei *et al.* 2011) and water deficit (Charles *et al.* 1993, Marchese *et al.* 2010) lead to an increase in artemisinin production for plants harvested.

Treatment of *A. annua* with reactive oxygen species (ROS) or environmental conditions that lead to the production of ROS including lead and soil salinity (Qureshi *et al.* 2005) and dimethyl sulfoxide addition (Mannan *et al.* 2010) also tend to increase artemisinin production in leaves. Enhanced production of ROS that correlated with higher artemisinin contents were documented in response to environmental stresses such as chilling (Feng *et al.* 2009), exposure to boron (Aftab *et al.* 2010b) and cadmium (Li *et al.* 2012), and application of the antibiotic fosmidomycin (Zeng *et al.* 2011). Salicylic acid treatment also induced artemisinin production and alleviated salinity-induced oxidative stress and loss of photosynthetic capacity for *A. annua* (Pu *et al.* 2009, Aftab 2011).

The objective of this study was to determine whether short-term exposure to high-intensity light could be used to induce increase in artemisinin production for leaves of *A. annua.* Wang *et al.* (2008) determined that although *A. annua* can grow in lower intensity light (15% full sun), growth at high-intensity light (up to 100% full sunlight) leads to increased photosynthesis, growth, and artemisinin production. Rai *et al.* (2011) found that treatments of UV-B (ultraviolet-B) and UV-C (ultraviolet-C) radiation prior to harvest enhance artemisinin and flavonoid content in the plant. The effect of a high-intensity visible-light pretreatment on artemisinin production has not been investigated. Because light that exceeds photosynthetic capacity of plants is known to be damaging to their photosynthetic machinery (*e.g.*, Kok 1956) and most plants are able to avoid light-induced damage through nonphotochemical quenching (NPQ) energy dissipation pathways (*e.g.*, Müller *et al.* 2001, Logan 2005) we investigated the capacity that *A. annua* has to dissipate excess light energy. Given the potential for enhanced ROS production in *A. annua* under photoinhibitory light conditions (Asada and Takahashi 1987, Asada 2006), we hypothesized that exposure of such conditions prior to harvest might enhance ROS-mediated artemisinin production in *A. annua* plants.

Materials and methods

Plant material: Seeds of an ornamental strain of *A. annua* (*Horizon Herbs*, Oregon, USA) were sown in flats in a mixture of soil (*Sunshine Professional, Sun Gro Horticulture*, Bellevue, Washington, USA) and sand (1:1) and germinated in a chamber (*E-30B*, *Percival Scientific*, Iowa, USA) at 24°C. At four weeks, after the first true leaves were formed, plants were transplanted to 15 cm pots in the same medium and were grown in a greenhouse under a mix of natural and supplemental metal halide lamps to achieve 500 μmol(photon) m^{-2} s^{-1} of PAR. Plants were fertilized weekly and watered as needed. When plants were at least 90 but not more than 100 days old, a subset of plants was randomly selected and subjected to an irradiance treatment ranging between 100 (LL) and 2,000 μmol(photon) m^{-2} s^{-1} PAR (HL) for 6 h. Light was provided by 1,000 W metal halide lamps (*Aurora Innovations*, Eugene, Oregon, USA) and the intensity of the light was controlled using neutral density filters. Light intensity was monitored with a quantum sensor (*LI-250, LI-Cor Inc.*, Lincoln, Nebraska, USA) and temperature throughout the treatment remained between 24–28°C. Prior to and after the light treatment, *A. annua* plants were used for physiological measurements as described below.

Chlorophyll (Chl) fluorescence was measured using a pulse-modulated fluorometer (*Fluorescence Monitoring System-1, Hansatech*, England, UK). Maximum dark-adapted photochemical efficiency of PSII (F_v/F_m; Genty *et al.* 1989) was measured after a 30 min dark-adapt period. Steady-state fluorescence yield (F_s) was monitored under light-adapted conditions, and maximal fluorescence yield of the light-adapted state (F_m') was measured during a saturating pulse of white light. From these values photochemical efficiency of PSII [$\Phi_{PSII} = (F_m' - F_s)/F_m'$] the photochemical and nonphotochemical quenching coefficients of fluorescence were calculated according to Genty *et al.* (1989) at 1 min, 15 min, 30 min, 1 h, and hourly intervals until 6 h during the light treatment.

Superoxide ($O_2^{\bullet -}$): Superoxide production was measured according to the method of Able *et al.* (1998) as modified by Lei *et al.* (2011). Leaves of *A. annua* [500 mg of fresh

mass (FM)] were homogenized with 1.0 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 12,000 × *g* for 15 min at 4°C. The reaction mixture, in a final volume of 3.0 ml, contained 50 mM Tris–HCl (pH 7.5), 0.5 mM XTT (*Sigma-Aldrich*, St. Louis, Missouri, USA), and 100 µl of supernatant. The reduction of XTT was determined by measuring the absorbance at 470 nm in a spectrophotometer (*UV-2401 PC, Shimadzu*, Japan). The quantity of $O_2^{\bullet-}$ was determined using the molar extinction coefficient (ε) of 2.16×10^4 M^{-1} cm^{-1} (Lei *et al.* 2011).

Hydrogen peroxide (H_2O_2): The concentration of H_2O_2 in the *A. annua* leaf tissue was determined according to the method of Mukherjee and Choudhuri (1983) as modified by Pu *et al.* (2009). Leaves of *A. annua* leaves (500 mg FM) were homogenized with 5 ml of precooled acetone and the homogenate was centrifuged at 12,000×g for 5 min. A volume of 1 ml of the supernatant was mixed with 0.1 ml of 5% $TiOSO_4$ (*Sigma-Aldrich*, St. Louis, Missouri, USA) 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 2M H_2SO_4 and the absorbance was determined at 415 nm using a spectrophotometer (*UV-2401 PC, Shimadzu*, Japan). The H_2O_2 concentration (µM) was determined using a standard curve of H_2O_2 ranging from 0 to 10 µM.

Singlet oxygen ($^1O_2$): Singlet oxygen was quantified spectrophotometrically by percentage bleaching at 440 nm (A_{440}), of the $^1O_2$ scavenger, N,N-dimethyl-p-nitrosoaniline using the methods of Feng *et al.* (2009) and Guo *et al*. (2010) with modifications. *A. annua* leaf tissue of 450 mg (FM) was put into 10 ml of assay mixture containing 45 mM PBS buffer (pH 7.1), 10 mM histidine, and 50 µM N,N-dimethyl-p-nitrosoaniline (*Sigma-Aldrich*, St. Louis, Missouri, USA). The assay mixture was incubated at 30ºC under a 400 W metal halide lamp at 320 µmol(photon) m^{-2} s^{-1} in order to facilitate assay bleaching. Absorbance at 440 nm was determined spectrophotometrically (*UV-2401 PC, Shimadzu*, Japan) at intervals of 0.5 h for a 2.5 h duration of the bleaching assay. The relative strength of $^1O_2$ [%] was determined as:
$[(A_{440\ blank} - A_{440\ sample})/A_{440\ blank}] \times 100$.

Artemisinin concentration: Artemisinin was extracted and quantified as in Han *et al.* (2006). Treated and control leaves were collected and dried for 24 h in an oven at 50°C. Dried plant material was ground to fine powder and 100 mg of powder was extracted with 40 mL of petroleum ether for 12 h. The mixture was sonicated for 2 min and filtered. Filtrates were evaporated to dryness, dissolved in 10 mL of methanol and centrifuged at 12,000 × *g* for 5 min. Supernatant was collected and used as artemisinin extraction solution. One mL solution was mixed with 4 mL of 0.2% (w/v) NaOH and incubated in a 50°C water bath for 30 min. After cooling to room temperature with tap water, 5 mL of 0.05 mol/L acetic acid was added to the solution and mixed thoroughly. The mixture was filtered and then used for high pressure liquid chromatography (HPLC) assay. HPLC was performed under the following conditions: 3.9 × 150 mm C_{18} reverse phase column, mobile phase of 0.01 mol L^{-1} sodium phosphate buffer (pH 7.0): methanol (50:50), flow rate of 1 mL min^{-1}, and wavelength of 260 nm. The injection volume was 10 µL. The retention time of artemisinin was approximately 3.7 min. The calibration curve was obtained using an artemisinin standard (*Sigma-Aldrich*, St. Louis, Missouri, USA).

Statistical analysis: All experiments were repeated with plants grown at different times of approximately the same age (90–100 d old). Oxidative stress assays were replicated eight times and artemisinin concentrations and Chl fluorescence evaluations were replicated six times. *Student*'s *t*-tests were employed to determine statistically significant differences between treated and control plants.

Results

Chl fluorescence: Maximum quantum efficiency of PSII (F_v/F_m) decreased by 14% after 6 h treatment with HL of 2,000 µmol(photon) m^{-2} s^{-1} as compared to values measured prior to the treatment or for plants that were treated with LL of 100 µmol(photon) m^{-2} s^{-1} (Fig. 1). Leaves treated with 500 or 1,000 µmol(photon) m^{-2} s^{-1} for 6 h had F_v/F_m values similar to plants treated with LL (data not shown). After a 24-h low-light recovery period, photoinhibited plants recovered to 95% of their initial F_v/F_m value. Upon exposure to HL, Φ_{PSII} increased two times in value across the first 15 min of exposure, after which values stabilized throughout the remainder of the light treatment (Fig. 2). Over the course of the 6-h treatment, average NPQ values for plants treated with HL, were up to 20 times higher than those for plants treated with LL (Fig. 2). Plants treated with LL maintained a higher level of Φ_{PSII} and a lower level of NPQ than plants treated with high-intensity light throughout the 6-h light treatment. Plants treated with 500 or 1,000 µmol(photon) m^{-2} s^{-1} had Φ_{PSII} and NPQ values that were similar to those for plants treated with LL (data not shown). Photochemical quenching (q_P) values were up to 20% higher in leaves treated with, as compared to leaves treated with LL, 500 or 1,000 µmol(photon) m^{-2} s^{-1} light (data not shown).

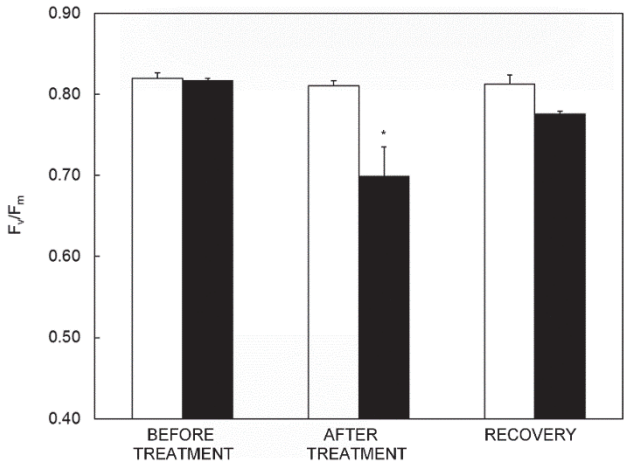

Fig. 1. Response of maximum photochemical efficiency of photosystem II (F_v/F_m) for *Artemisia annua* leaves before, and after, a 6-h exposure to 100 µmol(photon) m^{-2} s^{-1} (*open bars*) or 2000 µmol(photon) m^{-2} s^{-1} (*closed bars*). * – significant difference (p<0.01) between plants exposed to different light treatments. Bars represent means ± SD, n = 6.

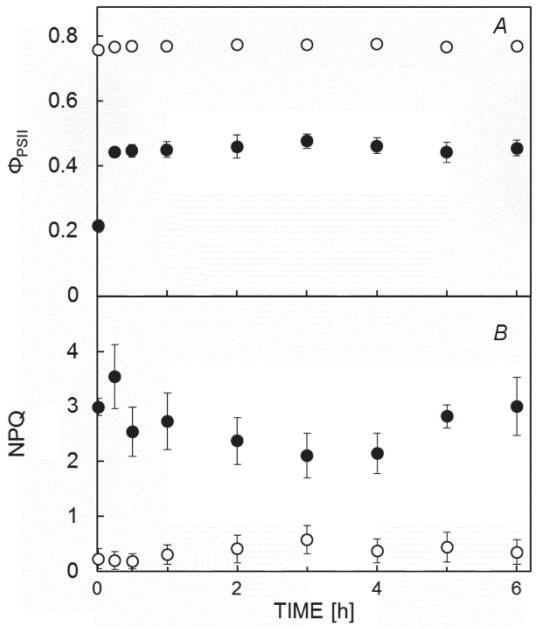

Fig. 2. Response of photochemical efficiency of photosystem II (Φ_{PSII}, *A*) and of nonphotochemical quenching of fluorescence (NPQ, *B*) to time of exposure for *Artemisia annua* leaves exposed to 100 µmol(photon) m^{-2} s^{-1} (○) or 2,000 µmol(photon) m^{-2} s^{-1} (●) for up to 6 h. Each time point represents means of measurements from 6 plants ± SD (some error bars are smaller than symbols). All differences for plants receiving different light exposures are statistically significant (p≤0.001). The first measurement was taken at one minute.

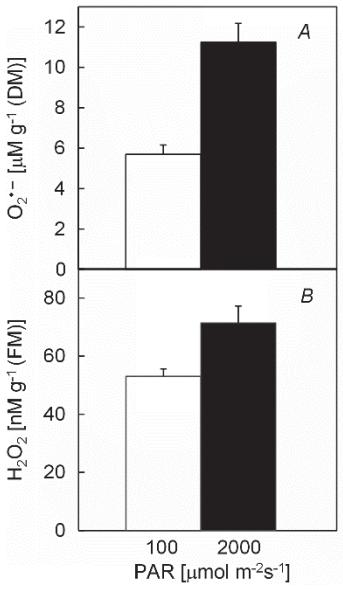

Fig. 3. Superoxide ($O_2^{\bullet -}$), *A*, and hydrogen peroxide (H_2O_2) production, *B*, for *Artemisia annua* leaves exposed to 100 µmol(photon) m^{-2} s^{-1} (*open bars*) or 2,000 µmol(photon) m^{-2} s^{-1} (*closed bars*) for 6 h. Differences for plants receiving different light exposures are statistically significant (p≤0.001). Bars represent means ± SD, n = 8.

Oxidative stress: To investigate the mechanism by which the 6-h irradiance treatment affected artemisinin production, concentrations of the ROS, such as superoxide anion ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2), and singlet oxygen, ($^1O_2$) were assessed. Results of the $O_2^{\bullet -}$ assay indicated a 85% increase in $O_2^{\bullet -}$ for plants treated with 6 h of HL, as opposed to LL (Fig. 3). The H_2O_2 concentration for leaves irradiated with HL increased by 35% after 6 h compared to those irradiated with LL (Fig. 3).

Production of $^1O_2$ in leaves after light exposure was monitored in a bleaching assay. The production of $^1O_2$ in leaf tissue leads to bleaching of a reaction media at 440 nm. The reaction medium was monitored for 2.5 h after completion of the light-treatment. Plants irradiated with LL and HL exhibited a postillumination burst of $^1O_2$ (Fig. 4). The relative size of the burst of $^1O_2$ measured for plants irradiated with HL was two times that from plants irradiated with (Fig. 4).

Artemisinin concentration, as determined by assay with HPLC, was nearly two fold higher for plants irradiated with HL for 6 h, as compared to plants irradiated with LL (Fig. 5) for the same duration. Plants irradiated with lower-intensity irradiation had artemisinin concentration similar to that which was measured prior to the irradiation period (data not shown).

Discussion

Artemisia annua exhibited a small but measurable photo-inhibition resulting from a high-irradiation treatment for 6 h, as indicated by decreased dark-adapted maximal photochemical efficiency of PSII (Kyle 1987). Overnight

recovery was adequate for protein synthesis required to fully repair photosynthetic reaction centers (Ohad *et al.* 1993). Decreased photochemical efficiency of PSII in *A. annua* during the high intensity–light exposure indicates that rapidly reversible energy dissipation reactions operated to increase nonphotochemical quenching of fluorescence (NPQ) during the light treatment (Genty *et al.* 1989, Horton *et al.*1996). *Artemisia annua* is a plant that grows well in a range of light intensities and has increased photosynthetic rates at higher growth light-intensities up to full sun (Wang *et al.* 2008). This light-adaptability, coupled with the plant ability to effectively dissipate excess excitation energy even when grown at light intensities lesser than full sun appears to be adequate to limit photoinhibition during a 6-h high-light intensity treatment.

Good agricultural practices described for *A. annua* note that the plant naturally occurs and is best-grown in high-light-intensity areas such as the edges of forests or in open fields (WHO 2006). Our results indicate that in cases where it is necessary to grow the plant in more shade-like areas due to space limitations or resource constraints, exposure to high light prior to harvest could be used as a practical means for increasing postharvest artemisinin concentration in the leaves of *A. annua*. Since *A. annua* is able to recover from short-term photoinhibition, it may be possible to expose mature plants to repeated high light–intensity treatments with minimal photoinhibitory losses to plant productivity. However, because photoprotection mechanisms such as NPQ reduce photosynthetic capacity of plants, their initiation in the presence of high light may lead to decreased plant yields that serve to offset the benefits of increased artemisinin production.

High irradiance produces ROS in chloroplasts at two prominent sites: oxygen can be reduced at PSI forming superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) and, if photoactivated Chl cannot transfer its excitation energy at PSII, singlet oxygen ($^1O_2$) can be formed from long-lived triplet Chl (Logan 2005, Asada 2006). Although plants have both nonenzymatic ROS-scavenging, such as carotenoids and enzymatic scavenging mechanisms, such as superoxide dismutase (SOD) to catalyze $O_2^{\bullet -}$ conversion to H_2O_2 and further detoxification of H_2O_2 by ascorbate peroxidase (APX), oxidative stress occurs when production of ROS outpaces their dissipation (Asada 1999, Apel and Hirt 2004). Oxidative stress can lead to loss of plant productivity through lipid peroxidation, membrane leakage, and eventually cellular death (Mishra and Choudhuri 1999, Triantaphylidès *et al.* 2008).

In this investigation, exposure of *A. annua* to high intensity light induced measurable production of $O_2^{\bullet -}$, H_2O_2, and $^1O_2$. At the same time, the light treatment increased artemisinin production in the plants. Production of artemisinin in response to chilling stress *via* conversion of its immediate precursors was first suggested by Wallaart *et al.* (1999). A mechanism for abiotic stress–induced artemisinin production was later proposed by Qureshi *et al.* (2005) who suggest that artemisinin precursors are converted into artemisinin through utilization of oxidative species produced in response to environmental stresses. Additional evidence for a catalytic mechanism for artemisinin production from its precursor, dihydroartemisinic acid, using the burst of ROS that occurs under abiotic stresses, has been given for *A. annua* exposed to boron (Aftab 2010b), and cadmium (Li *et al.* 2012) toxicity and through the application of DMSO (Mannan 2010).

In addition to production of artemisinin by conversion of precursors for the product, oxidative stress that is induced upon treatment of *A. annua* plants with salicylic acid or methyl jasmonate (Pu *et al.* 2009, Guo *et al.* 2010), fosmidomycin (Zeng *et al.* 2011) or UV radiation (UV-B or UV-C, Rai *et al.* 2011) has been shown to

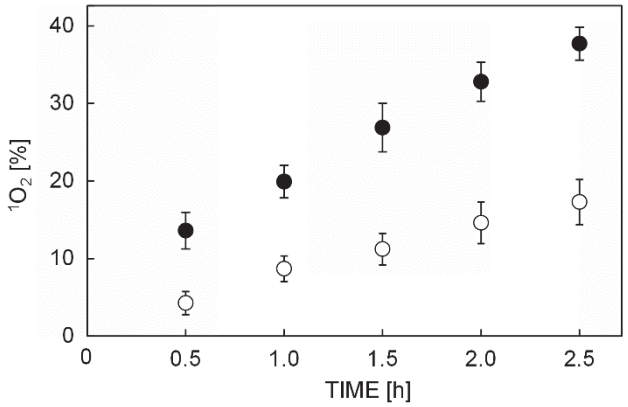

Fig. 4. Singlet oxygen burst from a 2.5 h bleaching assay that correlates with loss of A_{440}. $^1O_2$ emitted by *Artemisia annua* leaves exposed to 100 µmol(photon) m^{-2} s^{-1} (○) or 2,000 µmol(photon) m^{-2} s^{-1} (●) for 6 h prior to bleaching assay. Bars represent means ± SD, $n = 8$. Differences between treatments for each time point are statistically significant ($p<0.001$).

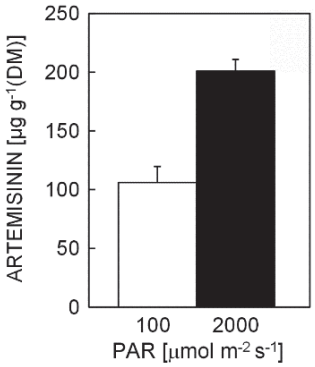

Fig. 5. Artemisinin concentration for *Artemisia annua* leaves exposed to 100 µmol(photon) m^{-2} s^{-1} (*open bars*) or 2,000 µmol(photon) m^{-2} s^{-1} (*closed bars*) for 6 h. Bars represent means of measurements from 6 plants ± SD. Difference is statistically significant at $p \leq 0.01$.

increase artemisinin production through a signaling mechanism that includes upregulation of artemisinin biosynthetic genes. Pu *et al.* (2009) gave evidence that $^1O_2$ production upon exposure of plants to salicylic acid increased artemisinin on a relatively short timescale (measureable differences occurred 8 h after treatment) through both mechanisms: conversion of dihydro-artemisinic acid to artemisinin and upregulation of genes encoding for artemisinin biosynthesis. Given the relatively short time scale of our exposure of *A. annua* to high light, it seems likely that increased production of artemisinin observed in this study was induced by conversion of precursors into artemisinin through a ROS-mediated processes; however, induction of artemisinin *via* a signaling event mediated with or without ROS is also possible within this time range and cannot be ruled out without further research.

On a broader scope of increasing artemisinin production for developing countries, we have provided evidence that suggests artemisinin production can be enhanced when leaves are irradiated with full sunlight for a 6-h period. Continuous exposure of *A. annua* to high-intensity light induced mechanisms for nonphotochemical quenching of excess excitation energy and oxidative stress for plants thereby triggering increased production of artemisinin over a relatively short time scale. Recovery of *A. annua* from high intensity light-induced photoinibition within 24 h indicates that repeated exposure of the plant to high-light intensity may serve to further increase artemisinin content of leaves without loss of plant yields. However, because oxidative stress also decreases plant yields, it may be that the benefits of high intensity light diminish over repeated exposure.

Methods as simple as harvesting *A. annua* from the field on clear days with sunny skies may be useful for increasing artemisinin yield for farmers as well as small-scale growers. Unfortunately, in many growth locations, such as those in which *A. annua* plants are completely shaded, exposure of plants to high intensity light may not be practical without artificial lighting. However, in locations where at least partial or directional sunlight is available for at least some hours of the day, our results indicate that harvest after maximum high intensity–light exposure should serve to increase yields of artemisinin for *A. annua* in the field.

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