The medicinal plant industry is under increasing scrutiny due to wide variance in active ingredient (AI) concentration from values claimed on labels. Reasons for this disparity include environmental and genotypic variation which influence AI concentration. St. John’s wort (Hypericum perforatum) is a popular herbal remedy which also exhibits marked variance in AI concentration among products. This study evaluated concentration changes of three biologically active metabolites of H. perforatum after exposure to ultra-violet light while plants were still vegetative. Treatments were performed with 55 day old plants grown under 400 -µmol m-2 s-1 PAR for 16 hours a day. Three ultra-violet light treatments were evaluated: a single dose, a daily dose, and an increasing daily dose. Concentrations of hyperforin, pseudohypericin and hypericin were monitored for seven days after each treatment. A daily dose and an increasing daily dose did not produce significantly greater increases in secondary metabolites compared to single dose treatments. These results suggest small but significant transient metabolite concentration increases in H. perforatum can be induced by ultra-violet light exposure. Information from this study can be useful in optimizing total biomass and metabolite production in controlled environments.
Effects of UV-B on Secondary Metabolites of St. John’s wort (*Hypericum perforatum* L.) Grown in Controlled Environments

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ABSTRACT

The medicinal plant industry is under increasing scrutiny due to wide variance in active ingredient (AI) concentration from values claimed on labels. Reasons for this disparity include environmental and genotypic variation which influence AI concentration. St. John’s wort (Hypericum perforatum) is a popular herbal remedy which also exhibits marked variance in AI concentration among products. This study evaluated concentration changes of three biologically active metabolites of H. perforatum after exposure to ultra-violet light while plants were still vegetative. Treatments were performed with 55 day old plants grown under 400 µmol m⁻² s⁻¹ PAR for 16 hours a day. Three ultra-violet light treatments were evaluated: a single dose, a daily dose, and an increasing daily dose. Concentrations of hyperforin, pseudohypericin and hypericin were monitored for seven days after each treatment. A daily dose and an increasing daily dose did not produce significantly greater increases in secondary metabolites compared to single dose treatments. These results suggest small but significant transient metabolite concentration increases in H. perforatum can be induced by ultra-violet light exposure. Information from this study can be useful in optimizing total biomass and metabolite production in controlled environments.
INTRODUCTION

St. John’s wort (*Hypericum perforatum*) is currently being used as an alternative therapy to treat many medical maladies such as depression, retroviruses and cancer. Traditional uses dating back to the ancient Greeks include its activity as a reported antidepressant, anti-fungal, anti-inflammatory, and antibacterial. More recently, a key metabolite associated with St. John’s wort, hyperforin, has been reported to possess antitumoral and antiangiogenic activity (1). Most of the plant’s remaining beneficial uses are attributed to another important metabolite, hypericin. A review of the medical uses and efficacy for the metabolite hypericin may be found in Kubin et al., 2005 (2). Pseudohypericin is a secondary metabolite that is often quantified (3-5) and although it does not have an identified pharmacological use, it shares a common precursor with hypericin and, in general, the concentration of the two metabolites may be found to increase in tandem. Hypericin is produced throughout the entire plant and accumulates in dark-colored glands that may be seen without the aid of a microscope (4). Hyperforin is produced in the chloroplasts and stored in the clear glands that surround that tissue. Chemical structures of hypericin and hyperforin are presented in Figure 1.

The medicinal plant industry is currently supplied with plant material produced either through field cultivation or wild crafted. Field cultivated *H. perforatum* has been found to vary widely in hyperforin and hypericin content because of annual environmental variation as well as variation among crop locations. Factors that have been shown to clearly affect hypericin content include drought stress, light intensity, heavy metal contamination of the soil, and nitrogen availability (4-6).

Exposure to ultra-violet (UV) light is a natural elicitor of secondary metabolite responses in higher plants. There are 3 sub-categories of UV light which are separated according to wavelength. UV-A has the longest wavelengths ranging from 400-315 nm and the least amount of energy at 3.1-3.93 eV per photon. UV-B ranges from 315-380 nm (4.43-
12.4 eV per photon) while UV-C is the shortest at 280-100 nm and has between 8.28 and 124 eV per photon. UV-C is generally filtered out by the ozone layer and is not a factor in plant development. UV-A is less harmful to plants due to the lower energy level per photon and is transmitted through glass, meaning that all plants grown in glass greenhouses are exposed to UV-A radiation. UV-C radiation which can be obtained through germicidal lamps will kill plants. Research on plant response to UV-B light is often coupled with the decrease in total biomass production caused by UV-B stress and attempts to predict the effect of a decreasing ozone layer. Reviews on the general plant response to UV light can be found in 7,8. Additionally, supplemental exposure to UV-B light has been shown to increase the concentration of secondary metabolites in maize, basil, peanut, and lettuce (9-12). UV-B has also been associated with anti-feedent properties in many higher plants, leading to enhanced secondary product production in some plants (13).

The goal of this work is to explore the addition of practical quantities of supplemental UV-B light to optimize UV-B exposure time/duration in order to maximize hyperforin, hypericin and pseudohypericin content in plants grown in a controlled environment.

**MATERIALS AND METHODS**

Growing conditions and experimenta treatments. *Hypericum perforatum* L. cv New Stem, (Richter’s Herbs, Goodwood ONT) was rinsed with purified (reverse osmosis; EC = 2 μS cm⁻¹) water to remove germination inhibitors. It was triple seeded into rockwool cubes (1550 plants m⁻² center hole filled with sifted peatlite) and thinned to one plant per cube three weeks after seeding, selecting for crop uniformity. Plants were placed into one of three walk-in growth chambers (Model M1 Environmental Growth Chambers, Chagrin Falls, OH), such that one plant per treatment per day was sampled from each growth chamber. Initially the
plants received 100 µmol m⁻² s⁻¹ for two weeks and after 14 d, the light intensity was increased to 400 µmol m⁻² s⁻¹ by fluorescent lamps (Sylvania, CW, VHO, Danvers, MA) for the duration of the experiment. Rockwool cubes were transplanted 30 days after seeding into 15 cm containers filled with MetroMix 360 (Scott's Horticultural Products, Inc., Marysville, CA). Planting density of St. John’s wort in each growth chamber was 36 plants m⁻². Exposure to UV-B radiation from UV-B-313 bulbs (Q-Panel Co., Cleveland, OH) occurred on day 55. During exposure, tops of the plants were 5 cm from the lamps and the intensity of the UV light was 10 µmol m⁻² s⁻¹ (Apogee UV meter, Logan UT). Single exposure periods for UV-B irradiation were 10, 20, 40, 80 and 160 minutes. Three plants per harvest period were exposed and samples were taken from each treatment at the following time intervals: 12, 24, 48, 96 and 108 hours. The repeated exposure treatment received 10 minutes of UV light each day for 7 days. Three plants were sampled per day and plants were sampled for 7 days starting on the initial day of exposure. An additional experimental group (called the progressive exposure plants) received an increasing exposure to UV-B light each day, starting with 10 minutes and increasing 5 minutes per day for 4 days and then by 15 minutes for the last two days as summarized in Table 1. Three plants were sampled per harvest and plants were exposed to light and harvested for 7 days. Harvests were destructive, and plants were not re-sampled.

<Table 1>

Quantification of metabolites. Quantification of hypericin, pseudohypericin, and hyperforin was performed using a modification of Couceiro et al. 2006 (3). Upon harvest, 10 cm from the growing tips of the main stems of nine plants was pooled into one mixed sample and immediately placed into an aluminum foil packet and dropped into liquid nitrogen. One such sample was obtained for each replicate, for a total of three samples per light condition. The frozen material was ground in the presence of liquid nitrogen into fine powder to which 4 ml of 2% (v/v) dimethylsulfoxide in methanol was added to 1 g fresh weight, weighed after
grinding. The extracted solution was placed in a sonicating bath (8890 Cole Parmer) for 30 minutes and centrifuged at 4000 rpm at 4 degrees C for 15 minutes (5810 Eppendorf). From the supernatant, 2 ml was filtered through a 0.2 μm Acrodisc PTFE syringe filter (Pall Corp, East Hills, NY, USA) and diluted 2-fold with the same solvent. A portion of this extract was placed into an amber vial for the evaluation of hyperforin and another was placed into a clear vial for hypericin and pseudohypericin analysis (Waters Corp, Milford, MA, USA). The above steps were performed under low light conditions at room temperature with the aid of a photography darkroom red light to prevent degradation of hyperforin, which will degrade upon exposure to UV light which exists in fluorescent lamps in the laboratory. The clear vials were then placed 15 cm from a 100 W tungsten lamp for 30 minutes to allow for the full conversion of protopseudohypericin to pseudohypericin and protohypericin to hypericin.

The three metabolites were quantified simultaneously. A 20 μl sample of the extract was injected onto a Waters xTerra C18 column (3.5 μm; 3.9 x 100 mm) with a C18 Waters xTerra guard column (3.9 x 20 mm). The HPLC system utilized was a Waters 2695 Separations module with a 996 photodiode array detector with a detector range of 220 to 750 nm. The mobile phases for this separation were as follows: A: 0.1% triethylammonium acetate (Calbiochem) solution adjusted to pH 3.5 with acetic acid (Fisher Scientific, Pittsburgh, PA, USA) B: Acetonitrile (Fisher) adjusted to pH 3.5 with acetic acid (Fischer). The flow rate was 1 mL/min with a gradient elution beginning with 50:50 (A:B) for 2 minutes increasing linearly to 20:80 in 12 minutes, isocratic at 20:80 for 3 minutes, then linearly increasing to 0:100 in 3 minutes and finally isocratic at 0:100 for 10 minutes after which flow was stopped for a total run time of 32 minutes. All solvents utilized were HPLC grade. Hyperforin was quantified at 270 nm and hypericin and pseudohypericin at 588 nm.

Significant linear calibration curves were generated for hypericin, pseudohypericin and hyperforin (standard retention times of 15, 11 and 17 minutes respectively), and the quantification of these compounds was calculated by comparison to a standard curve. The
RESULTS

Overview

Figure 1 shows the responses of secondary metabolites in St. John’s wort shoot tissues during and after treatment with UV-B light. The data presented has been normalized such that the levels reported represent the increase in chemical concentration relative to control plants harvested the same day. Visible tissue damage to the tips of the leaves was observed 12 hours after UV-B exposure in the 160 minute treatment. For exposures of 40 minutes or greater, visible tissue damage could only be observed after 48 hours. If the plants were not re-exposed to UV-B, the new tissue growth appeared healthy with a greater number of lateral branches.

Single dose experiment

In the single dose experiment, both the dosage effect and the hours after treatment effect were significant (p < 0.01) for all the metabolites tested. Each metabolite showed significantly different concentrations based on the UV-B dose administered, and concentration observed was also dependant on the elapsed time after treatment. There was no interaction between dosage and harvest time, so metabolite concentration levels followed the same pattern for all harvests and dosages. This response is presented in Figure 2, with different letters denoting significantly different metabolite concentrations. Error bars indicate variability between samples. The standard errors observed were greater than expected for reasons that were
unknown, although statistical analysis indicated that the error was not significant. Raw data may be found in Brechner, 2008 (14).

The highest levels of hyperforin were seen in the 40 and 80 minute single dose treatments with a maximum hyperforin concentration 2.5 times greater than the control. Intermediate concentrations were observed in the 10 and 20 minute treatments, with a maximum hyperforin concentration averaging 2 times greater than the control. The lowest concentration was observed in the 160 minute treatment with a maximum of 1.25 times the control values. The pattern of response by the treatments was consistent across all harvests. Time elapsed after treatment was significant and all harvests could be classified into 2 significant rankings for hyperforin. The highest concentrations of hyperforin were seen in the 12, 24 and 48 hour harvests averaging 1.5 times control values for all three harvests, and the lowest concentrations shown at the 96 and 144 hour harvests with the average metabolite response being equal to the control values.

Concerning time of exposure to UV-B, the highest levels of pseudohypericin (Figure 3) were also associated with the 40 and 80 minute treatments. An intermediate level was found with the 20 minute treatment and the lowest levels were seen with the 10 and 160 minute treatments. The harvest performed at 12 hours showed the highest pseudohypericin concentration at 2.5 - 3.5 times control values, with intermediate concentrations observed at 24, 48 and 96 hours, with values equal to the control. The lowest concentration was found at 144h after exposure with metabolite concentrations at half the control value.

Similarly, hypericin (Figure 4) showed the highest increase in concentration compared to the control at a 40 minute exposure time with a maximum at 1.8 times the control value. Intermediate concentrations were observed at 20 and 80 minute exposure times, averaging 1.4 and 1.1 times control values. The lowest concentration was seen with the 10 and 160 minute exposures, averaging 0.3 and 0.4 times the control value. The time after treatment that showed the greatest metabolite concentration was 96 hours, averaging 1.2 times the control value over
the course of the experiment. The lowest concentrations were found at 96 hours after exposure and were equal to the control values.

In general a clear pattern of metabolite formation was observed immediately following the application of UV stress. The metabolite response, from highest to lowest concentration per g shoot tissue was as follows: 40 min, 80 min, 20 min and finally, 160 or 10 min. Although a physiological explanation for this particular response pattern could not be immediately determined, maximal UV-B exposure may result in induction of other critical plant protective systems over time, including the likely production of free radical scavengers required to protect plant photosynthetic apparatus and fragile membrane structures from excessive UV exposure, thereby limiting resources available for hypericin and hyperforin production.

<Figure 2>
<Figure 3>
<Figure 4>

Repeated and Progressive Experiment

UV-B exposure in the repeated and progressive exposure models, did not significantly influence hyperforin concentrations, however the time after treatment did have an impact on metabolite production (Figure 5). Assessment of metabolite concentration on day 4 showed significantly (p < 0.01) higher levels of pseudohypericin than all other days evaluated, with pseudohypericin averaging 1.5 times the control value. Concentrations of pseudohypericin on days 2 and 6 were significantly lower than all the other days averaging 0.6 times the control. Although no clear explanation for this pattern exists, pseudohypericin levels may peak over time and may later degrade over time without repeated UV exposures (Figure 6). In our experiments, it is important to note that there were no significant deviations in temperature, light quality and quantity, and nutrient solution composition during the course of the
experimental period. Additionally, the plants were not exposed to insect or disease pressure at any point during this experiment. For pseudohypericin (Figure 6) the light quality treatment did not significantly impact concentrations, however time of harvest did have a significant impact. Highest concentrations were observed at day 4 harvest where the values averaged 1.3 times the control, intermediate concentrations were observed at days 1,3,5,6,7 with average values equal to the control, and lowest concentrations were seen at day 2. For hypericin (Figure 7), the UVB exposure was significant (p = 0.0001) with the progressive treatment providing higher hypericin concentrations (averaging 0.1 times higher) for all the harvests than the repeated treatment. The time of harvest after treatment was not significant.

**DISCUSSION**

In general, the maximum response in metabolite production in St. John’s wort tissue was demonstrated within 24 hours after the first UV-B exposure in all treatments evaluated. The induction of secondary metabolite production by higher plant tissues is widely recognized, in response to UV treatment and other plant stressors. Depending on the plant, the specific metabolite and the stressor, variation in metabolite production over time is frequently observed. Furthermore, within the plant itself there is a variation in metabolite concentration over time. It is hypothesized that these metabolites are often unstable and are typically degraded and recycled in the plant. In other studies evaluating UV induced responses in secondary product formation by higher plants, similar trends have been observed. In table grapes, short to intermediate exposure to UV light has resulted in maximal production of
resveratrol, whereas longer or shorter treatments resulted in potential degradation of metabolites over time or potential damage due to the inhibitory effects of the UV treatment itself (15). Peanut plants that were exposed to UV-B light showed an increase in the secondary metabolite resveratrol that was an order of magnitude greater 12 hours after exposure, compared to 3 hours after exposure (11).

Given the expense of maintaining the plants under controlled conditions for additional time, and limited increases observed in metabolites with delayed harvest, it is recommended that the UV-B light challenge be administered within 24 hours prior to harvest for maximal metabolite recovery. One exception to this in our studies was observed with the metabolite hypericin, where the greatest enhancement in harvested St. John’s wort tissues was determined to be 4 days after UV-B challenge. However, the final hypericin concentration, while much greater than the control plants, was not significantly greater than the values observed in plants at the time of flowering. Physiologically, most flowers are open on the plant, and not yet senescing, up to 10 times greater hypericin contents can be observed in harvested tissue. Hypericin content appears to be maximal at time of flowering and then gradually decreases with flower senescence (data not presented).

The maximum response elicited in this experiment was shown in the single dose experiment and included a 3.7% increase in hypericin concentration compared to control plants. The repeated and progressive treatments of UV light exposure over time did not result in significantly greater increases in metabolite concentration in comparison to the single dose treatment, and involved more labor to complete. Treatment type was only significant in the induction of increased hypericin concentration. To our knowledge, this is the first reported study of impacts of repeated UV-B exposure upon metabolite formation and retention in St. John’s wort tissues.

For the purposes of increasing secondary metabolite production, we observed that a single dose of 40 minutes of UV-B light followed by harvest (within 12 hours after exposure) was optimal. This resulted in the shortest amount of exposure time and the fastest time to
harvest. However, it must be cautioned that when comparing the natural levels of metabolites produced when the plants are at the peak of flowering before setting seed (on average 60 days after seeding), the increases observed in this study (5% or less) due to UV-B light exposure do not merit harvesting the plants before full flowering is achieved (Brechner, unpublished data).

Future work should include the exploration of metabolite response specifically during the response period from 0-24 hours after exposure to determine if 12 hour exposure indeed produces the optimal response in metabolite production. Additionally, exposing plants in full bloom to UV-B before harvest would be worthwhile to determine if an increase in metabolite production beyond the range of the normal high flowering values can be achieved. Finally, surrounding the plants on all sides with UV irradiation rather than overhead exposure alone would also be worthwhile to evaluate in an attempt to enhance overall metabolite production in the shortest production period.

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REFERENCES


**FIGURE CAPTIONS**
Figure 1. Chemical structures of hypericin (left) and hyperforin (right).

Figure 2. Normalized hyperforin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

Figure 3. Normalized pseudohypericin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

Figure 4. Normalized hypericin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

Figure 5. Normalized hyperforin concentration over time for repeated and progressive dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.
Figure 6. Normalized pseudohypericin concentration over time for repeated and progressive
dose of supplemental UV-B experiment. Average values represent five plants. Bars represent
+- standard error. Different letters at each time represent significantly different values. Lower
case letters represent the overall effect caused by the harvest day and upper case letters
represent significantly different treatments.

Figure 7. Hypericin concentration over time for repeated and progressive dose of supplemental
UV-B experiment. Average values represent five plants. Bars represent +/- standard error.
Different letters at each time represent significantly different values. Lower case letters
represent the overall effect caused by the harvest day and upper case letters represent
significantly different treatments.