SOME EFFECTS OF LIGHT QUANTITY AND QUALITY ON SECONDARY METABOLITES HYPERFORIN, PSEUDOHYPERICIN AND HYPERICIN, IN *HYPERICUM PERFORATUM*

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by

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SOME EFFECTS OF LIGHT QUANTITY AND QUALITY ON SECONDARY METABOLITES HYPERFORIN, PSEUDOHYPERICIN AND HYPERICIN, IN *HYPERICUM PERFORATUM*

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Hypericum perforatum or St. John's wort is a plant grown commercially for use as a medicinal plant. The consistency of secondary metabolites that act as active ingredients in *H. perforatum* preparations is a constant problem and is attributed partially to environmental conditions experienced by the plants during growth. Controlling the light provided to plants has been an effective way to manipulate plant growth in other crops. The optimal lighting conditions and time to harvest *H. perforatum* grown in controlled environments was the primary goal. The effects of light intensity, quantity and quality on biomass and secondary metabolites hyperforin, pseudohypericin and hypericin over time were investigated in four experiments. An additional experiment demonstrated that *H. perforatum* will flower under the long-day conditions (16 hours of light) used in all experiments. Light intensities from 90 to 340 $\mu mol\ m^{-2}\ s^{-1}$ were investigated while daily light integral was held constant. Effects of daily light integral were demonstrated by holding the light intensity constant and varying the light integral from 8.6 to 20 $mol\ m^{-2}\ d^{-1}$. The response of metabolite production to the presence or absence of UV-A and UV-B was also explored. Finally, the usefulness of stressing the plants with supplemental UV-B light just prior to harvest was determined. Results showed a very small or no significant increase in the secondary metabolites quantified in response to increasing light intensity, light integral or the addition of UV-A or UV-B light. Biomass production was shown to increase

with exposure to increased light intensity and light integral. It was demonstrated that all of the metabolites increased their concentrations as plants transitioned from a vegetative to reproductive state. For growth in controlled environments, increased light integral did increase metabolite production indirectly as biomass increases led to a more rapid time to flowering. Since metabolite concentrations were shown to rise so dramatically when plants were flowering, the best protocol for maximizing metabolite production per square meter of growing space is to furnish plants with as much total light as possible which would hasten the time to flowering, then harvest plant material at the full bloom stage.

BIOGRAPHICAL SKETCH

Melissa Lynn Brechner was born Melissa Lynn Kizis in central New Jersey in 1977. Her primary and secondary education was completed in Allentown New Jersey, graduating from Allentown High School in 1995. She entered Rutgers University and received Bachelor of Science degrees in Bioresource Engineering from the College of Engineering and Horticultural Engineering from the agricultural school, Cook College, in 2001. In August of 2001 she married Adam Brechner and moved to Ithaca to obtain her Master of Science degree, which she received in 2003. Her research project involved aphids and the induction of the winged form of the insect in response to differing photoperiods. In the spring of 2004, she began her PhD work and simultaneously started her tenure as a teaching assistant for Biology 105/106, the autotutorial biology course. For the 2005-2006 school year, she was awarded a National Science Foundation Cornell Science Inquiry Partnership fellowship which allowed her to teach the process of scientific inquiry at the middle school and high school levels. Following that, she returned to the Biology program as the lead teaching assistant, and mentored undergraduates for the remainder of her PhD career. Her next project will include the analysis of transgenic protein from tobacco as a Postdoctoral Research Associate with the Controlled Environment Agriculture research group at Cornell University.

To my husband, Adam.

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I would like to thank my advisor, Lou Albright for helping me to find a project that matched my research interests and for helping me to determine a problem that could be investigated in a reasonable amount of time. He also supported my teaching interests even though they may have extended the time necessary to finish this degree. Finally, he provided the financial support necessary to complete my research and thoughtfully and patiently read numerous drafts of papers and proposals over the years. My chemistry analysis was completed in Leslie Weston's laboratory and she guided me in the refinement of lab protocols and provided much encouragement during times when research results provided more questions than conclusions. My remaining committee members, Beth Ahner and Roger Spanswick, provided rapid answers to any questions I presented them with. The Controlled Environment Agriculture research group including Lou Albright, Bob Langhans, David de Villiers and Tim Shelford helped with the refinement of experimental design, ideas for further experimentation, and an immense amount of knowledge about previous experience with all aspects of hydroponic experimentation during weekly research meetings. David de Villiers assisted me in the setup of many experiments and demonstrated the intricacies of working with the computer control systems. Matt Balestrino helped with some of the later experiments and laboratory quantification, and without whose help I would not have been able to finish this task in the time allotted. Finally, I would like to thank my husband, Adam Brechner for being my unpaid assistant for many experiments and donating many Friday nights to data collection after putting in a long week at his own job and for providing emotional support during all the times in which it was required.

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LIST OF ABBREVIATIONS

ABC	American Botanical Council
CCES	Closed Controlled Environment System
CEA	Controlled Environment Agriculture
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
DSHEA	Dietary Supplement Health and Education Act
DW	Dry Weight
FDA	Food and Drug Association
FW	Fresh Weight
gFW	Grams Fresh Weight
gDW	Grams Dry Weight
GMP	Good Manufacturing Practices
HPLC	High Pressure Liquid Chromatography
HPLC/MS	High Pressure Liquid Chromatography/ Mass Spectroscopy
MAO	Monoamine oxidase
$mol\ m^{-2}\ d^{-1}$	Moles per meter squared per day
MS	Mass Spectroscopy
NCCAM	National Center for Complementary and Alternative Medicine
NFT	Nutrition Film Technique
NIH	National Institute of Health
NMR	Nuclear Magnetic Resonance
SSRI	Selective Seratonin Reuptake Inhibitor
TLC	Thin Layer Chromatography
$\mu mol\ m^{-2}\ s^{-1}$	Micro-moles per square meter per second
US	United States
USP	United States Pharmacopeia
UV-Spectroscopy	Ultra-Violet Spectroscopy
WHO	World Health Organization

CHAPTER 1

Introduction and Literature Review

1.1 MEDICINAL PLANT INDUSTRY

It is generally acknowledged that the last two decades have seen a tremendous increase in the use of medicinal and aromatic plants in developed countries. New markets have opened with the advent of new fields of retail consumerism including nutraceuticals, cosmeceuticals, phytotherapy, aromatherapy and functional foods (Husnu Can Baser, 2005). Valiant attempts are made each year at quantifying the total sales of each medicinal plant-based product, but it is difficult to quantify sales from large vendors such as Wal-Mart, Costco and online merchants. Nevertheless, numbers are generated, often with a long list of merchants that were not included in the sales estimates. One popular source cites that in 2004, 'Supplement Sales' enjoyed $60 billion globally and $20 billion (that includes 29,900 products) in the US with an average growth rate of 4% per year since 2000 (IRI, 2005). Products included under this framework include herbals, vitamins, minerals, sports nutrition supplements and diet complements (IRI, 2005). Globally, sales growth rates hover around 9% (Husnu Can Baser, 2005). The 1990-1997 Unconventional Medicine National Survey Statistics agrees that it is difficult to really understand these trends because there is no publicly accessible group that puts out nationwide or worldwide information on a regular basis (Druss and Rosenheck, 1999). No further national surveys have been published in the last decade, and accurate usage and sales information continues to be difficult to obtain. The Nutrition Business Journal publishes an annual report for the US and seems to be one of the most complete sources of compiled sales information. Their most recent report shows sales figures for 2005 where the total sales for all herbals were $4,410 million with a 2.1% increase in sales from 2004. Information Resources Inc. reports annual sales and percent change for each of the top 20 best

selling herbal dietary supplements annually (See Table 1.1 for summary of top 10 best selling herbals). However, it does not include sales from large vendors such as Wal-Mart, Sam's Club, and convenience stores. It is estimated that IRI's figures might double if it included such data since it has been determined that Wal-Mart is the largest retail seller of dietary supplements in the United States (Blumenthal et al., 2006).

Table 1.1. Sales of top herbal dietary supplements in the food, drug and mass market channel in the US in 2005 from Information Resources Inc. (Blumenthal et al., 2006.).

Common Name	Latin Name	$ 2005 Sales	% change from 2004
1. Garlic	*Allium sativum*	26,244,200	-3.28
2. Echinacea	*Echinacea spp.*	21,114,160	-11.21
3. Saw palmetto	*Serenoa repens*	19,252,980	-5.42
4. Ginko	*Ginko biloba*	16,553,030	-14.54
5. Cranberry	*Vaccinium macrocarpon*	15,839,160	16.97
6. Soy	*Glycine max*	14,497,100	-17.12
7. Ginseng	*Panax ginseng*	11,444,550	-6.19
8. Black cohosh	*Actaea racemosa*	9,736,738	-19.05
9. St. John's wort	*Hypericum perforatum*	9,035,399	-1.34
10. Milk thistle	*Silybum marianum*	8,312,867	6.77

Regulation

There is no worldwide regulation of supplements although the World Health Organization (WHO) has published a series of monographs on common plants used as supplements that detail the identification of cultivars thought to be useful as medicine. In 2003, WHO published a document entitled "Guidelines on Good Agricultural and Collection Practices" of herbs. In an appendix to this document, the WHO recommends that all monographs use names and control standards set forth by the monographs in the Japanese Pharmacopoeia (WHO, 2003). The WHO has also authored Good Manufacturing Practices (GMP). It is the hope of the WHO and every

country where there is a significant market for herbal medicines that these protocols will be followed and a uniform and safe product will be available to consumers.

Individual countries produce documents entitled 'Pharmacopeia' periodically. These pharmacopeia contain monographs on individual plants that are similar to the WHO monographs and detail agricultural production suggestions as well as extraction, quantification and processing protocols. More famous pharmacopeia include the European Pharmacopeia, Japanese Pharmacopeia, Korean Pharmacopeia, American Herbal Pharmacopeia, and the United States Pharmacopeia (USP). Some of these Pharmacopeia's serve as a basis for quality standards and governmental regulation as the USP does in the United States for all prescription and over the counter medicines.

Asia

Korea - Not only does Korea have the regular quality control issues that are common to all countries, but the current cultural practice of mixing herbs and boiling them together in order to benefit from proposed synergistic effects adds an additional level of complexity to the regulation of herbal medicine. In 1991, the Korean government imposed strict regulations on herbal medicine preparations and Korean traditional medicine by domestic pharmaceutical corporations and mandated that Korean Good Manufacturing Practices be followed.

Australia

All human medicine must be placed on the Register of Therapeutic Goods in compliance with a 1989 law that groups all medicines under either a listed or registered category. Listed drugs are substances regarded by the Therapeutic Goods Administration to be of low public health concern and comply with the Therapeutic Goods Advertising Code. There are about 4500 plant-based products in the listed drug

category. The registered category contains only 5 plant-based products, usually many plants in combination, and these are products that are restricted by the federal standards for uniform scheduling of drugs and poisons or those whose efficacy claims are more substantial. For more on the drug categories and herbal regulations, see Drew and Myers, (1997).

Canada

The health protection branch is an expert advisory committee on herbs and botanical preparations, created to study the labeling of herbal products in 1984. In 1986 a report was published recommending a new class of remedies called "Folklore Mediares".

Europe

The European Directorate for the Quality of Medicines requires that medicinal products obtain pre-marketing approval showing quality, safety and efficacy before the product may be sold on the market. A more widely accepted criterion for the assessment of herbal medicinal products is being sought and many monographs have been proposed, but currently the only standards are found in the European Pharmacopoeia. Specifically in Europe, Germany formed a group called Commission E, which is a special scientific committee of Bundesgesundheitsant (Federal Department of Health) that created a German monograph in 1984 (called Germany's Commission E Monographs) that was translated to English and published by the American Botanical Council in the fall of 1996. These monographs are some of the first of their kind published, and as a result, other monographs are modeled after them.

USA

There was no governmental regulation prior to the Dietary Supplement Health and Education Act (DSHEA) of October 1994. This act mandated that the Food and Drug Association (FDA) does not oversee the introduction of specific supplements (as it does in the costly and lengthy process of introduction of pharmaceutical products), but they require supplement manufacturer to notify the FDA of new products, produce labeling information that is clear and not misleading, and show adequate evidence of safety and efficacy for all products and ingredients.

DSHEA provided a definition of dietary supplements, a new framework for addressing safety of supplements, and required that guidelines for third party literature be provided at point of sale, appropriate use of statements of nutritional support, ingredient and nutrition information labeling standards and granted authority to the FDA to establish GMP regulations.

Federally funded National Institute of Health (NIH), National Center for Complementary and Alternative Medicine (NCCAM), and Office of dietary Supplements facilitate and conduct research exploring the role of dietary supplements in health and disease. The general belief is that the United States has the least amount of regulation of the more developed countries although this is difficult to quantify.

1.1.1 Problems with herbal medicine

Recurring problems exist within the production and use of herbal medicine worldwide that can lead to less than optimal results for the consumer. Mistakes may occur at every step of the process from the selection of the seed to the dosage and timing of ingestion of the finished product. First, the plant cultivar that is used may be incorrect either entirely or a less biologically active cultivar may be substituted. The growing conditions may not be conducive to maximum metabolite production as some

types of stress may increase production while other types of stress will decrease the same metabolite. Different parts of any given field may experience different growing conditions including soil type, water availability and heavy metal contamination. Plants may be exposed to bacteria, viruses and/or fungi, all of which may induce a modification in metabolite concentration (Murch et al., 2000). The time of harvest may not coincide with maximum metabolite production, and all plants in a given field may not be at the same growth stage leading to a situation where a single time of harvest that would optimize the chemical concentration in all of the plants at the same time is impossible. Plant material may not be stored correctly and many metabolites are broken down before the plants may be processed. For many products there is a lack of standardized preparation for each batch of plant material and the resulting amount of active ingredient in the final product may vary greatly both within and between companies (for more on this topic see '1.2.3 Studies of Material in Marketplace). Finally, there may be contamination of the final product with other plant material or biological impurities. Bombardelli and Riva, (2005) discuss the necessity for greater amounts of standardization and the need for the adoption of Good Manufacturing Practices (GMP's) for the entire industry worldwide.

The preceding pitfalls detail sources of variability in the quality of the product as it sits on retail shelves. An entirely different set of problems can occur once the consumer is in possession of the product. One of the largest problems is the consumption of the wrong dosage or herbal-pharmaceutical drug interactions (and this assumes that the amount of active ingredient in the dose of the herbal medicine corresponds with the quantity that appears on the label) which is often due to self-medication (Drew and Myers, 1997). See Appendix C for western doctors' views on herbal medicine study and use.

1.2 HYPERICUM PERFORATUM OR ST. JOHN'S WORT

1.2.1 Market Information

H. perforatum is sold as dry bulk herb, capsules of ground plant material, tinctures (generally an alcohol based product) and tablets. It is grown for profit in North and South America, Europe, Australia and China (German monograph Expanded Commission E translated by ABC to English, 1998). As mentioned previously, in early 2005, retail sales in the US of St. John's wort were at least nine million dollars. This estimate does not include sales figures from Wal-mart or some of the warehouse clubs which may represent a significant portion of total sales.

Worldwide Distribution

Common St. John's wort is native to Western Europe, Western Asia and Northern Africa. It is naturalized in Asia, South Africa, North and South America and Australia (USDA, 2008). Controlled cultivation of the herb has been increased and is now common in Europe, North and South America, Australia and China (German monograph Expanded Commission E translated by ABC to English, 1998).

St. John's wort was introduced in to the United States in the 17th century (Kirakosyan et al., 2004), and is considered a noxious weed in California, Colorado, Montana, Nevada, Oregon, Washington, Wyoming, Manitoba and Quebec (USDA, 2008). In human studies on the effectiveness of *H. perforatum* on the human immunodeficiency virus (HIV) where very large doses were used, 16 of the 30 patients experienced cutaneous phototoxic effects (sunburn) that was so painful that they discontinued the treatment (Kubin et al., 2005). Consumption of the weed can

cause hypersensitivity to the sun and thus severe sunburn in sheep and cattle and sometimes cause death and birth defects in these animals.

1.2.2 Botanical Information

Hypericum is a short-lived perennial herb that has a life span of about six years. There are about 200 species in the *Hypericum* genus (Osinska and Weglarz, 2000), 59 of which are in the United States (Sirvent, 2001).

Domain-Eukarya

Kingdom – Plantae

Subkingdom – Tracheobionta (Vascular plants)

Superdivision – Spermatophyta (Seed Plants)

Division – Magnoliophyta (Flowering Plants)

Class – Magnoliopsida (Dicotyledons)

Subclass – Dilleniidae

Order – Theales

Family – Clusiaceae – Mangosteen family

Genus – Hypericum (St. John’s wort)

Species – *Hypericum perforatum* L. (Common St. John’s wort)

This shrubby herb may range from two to five feet tall depending on cultivar and growing conditions. Flowers develop in clusters from May to September. In an evaluation of 11 different species of Hypericum it was determined that the growth habit, biomass production, time to and duration of flowering, and concentration of hypericin varied significantly by species (Osinska and Weglarz, 2001).

For a detailed general appearance information see the WHO monograph on Herba Hyperici, (2003).

1.2.3 Medicinal portions of plant

Secondary Metabolites

There are many active constituents found in *H. perforatum* that are believed to have medicinal properties and over twenty secondary metabolites have been isolated. Specific metabolites that are thought to be of medicinal value include: pseudohypericin, hypericin, hyperforin, adhyperforin, chlorogenic acid, rutin, hyperoside, isoquercitin, quercitin, xanthones, flavonoids and tannins. It is generally accepted that some combination of the above list of metabolites is responsible for the positive health effects mentioned previously and that whole plant extracts produce greater health benefits than individual extracts.

Two large groups of secondary metabolites with medicinal properties include the naphthodianthrones and phloroglucinols. Specific naphthodianthrones that are often quantified are: hypericin (for chemical structure see Figure 1.1), protohypericin, pseudohypericin and protopseudohypericin. The proto forms of naphthodianthrones are unstable under light, while both types of phlorogoucinols are unstable in solution. Pseudohypericin and hypericin together are often quantified separately and reported together as total hypericins. The two molecules differ at one carbon atom where hypericin has an extra H and pseudohypericin has a hydroxy group. Protohypericin and protopseudohypericin are precursors to pseudohypericin and hypericin. With exposure to light in vitro, the proto forms are converted into hypericin and pseudohypericin within two hours (Sirvent and Gibson, 2000). It is unknown if this conversion happens in vivo.

Light and dark colored glands are found in the leaves of *H. perforatum*. The dark glands are where hypericins accumulate (Figure 1.2), and may also be found in the flowers and stems (Fields et al., 1990). In the dried herb, hypericin is generally found to be about 0.3% weight/weight. For a detailed description of the chemistry of hypericin including biosynthesis and artificial synthesis information, see Vollmer and Rosenson, (2004) and Kubin et al., (2005). See Appendix A for a possible pathway for the biosynthesis of hypericin. Hypericin has been created synthetically (Vollmer and Rosenson, 2004).

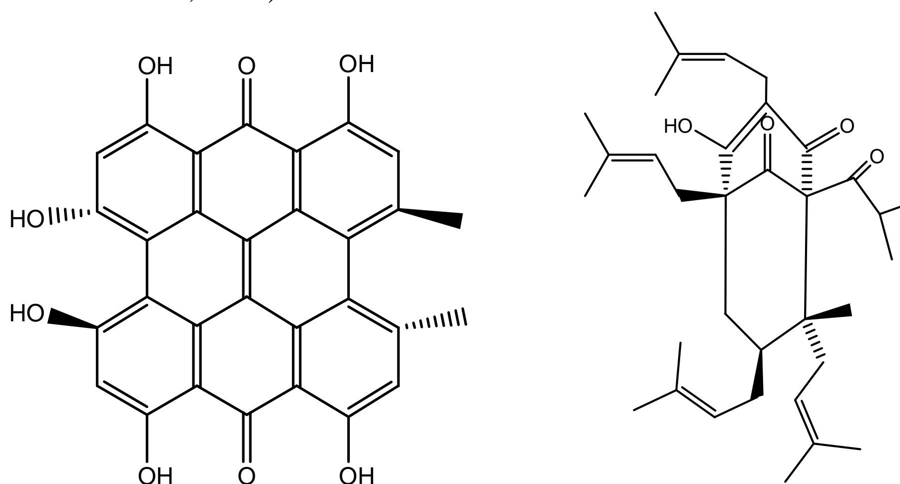

Figure 1.1. Hypericin (Left) and Hyperforin (Right).

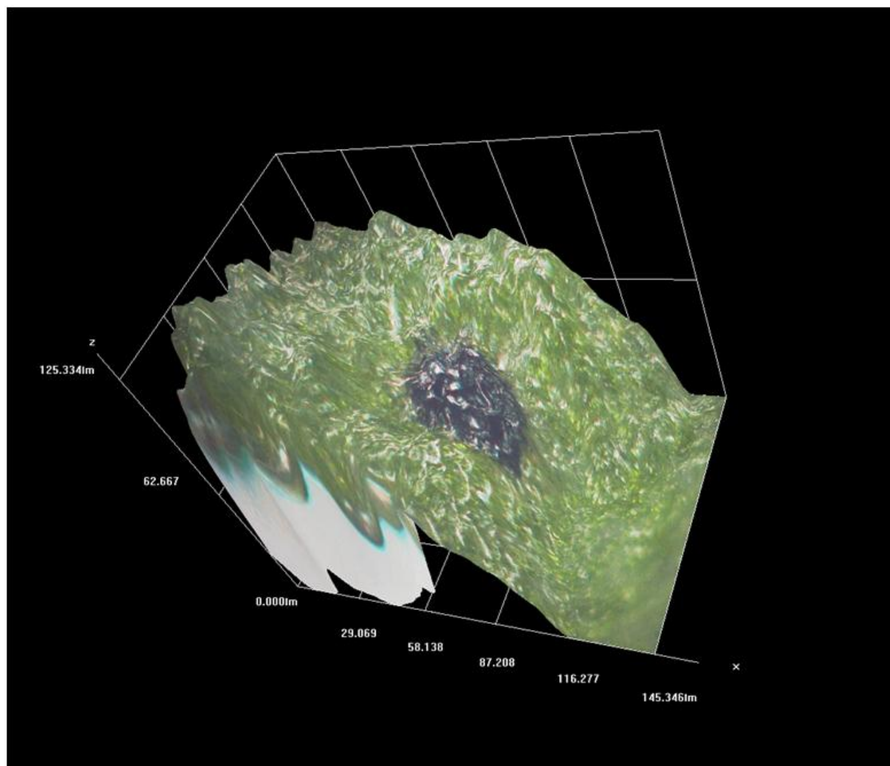

Figure 1.2. Photomicrograph of black gland where hypericin is stored. 520x magnification. Taken on 5/16/07.

Hyperforin (for chemical structure see Figure 1.1) is found at 2-4% w/w in the dried tissue with a greater amount found in the flowers than in stems and leaves (Gray et al., 2003; Poutaraud and Girardin, 2004; Southwell and Burke, 2001). A similar molecule called adhyperforin is sometimes quantified. Often both metabolites are summed and reported as hyperforins. The location of hyperforin synthesis has recently been identified as inside the chloroplasts surrounding the clear glands in the leaves, flowers and stems. Hyperforin is secreted into the clear glands and stored there. An analysis of non-secretory tissue demonstrated that hyperforin is only found in the clear glands (Soelberg et al., 2007). The exact pathway for biosynthesis of hyperforin has not been elucidated. However, Adam et al. (2002), used labeled glucose and NMR spectroscopy to determine that biosynthesis is likely to involve five

isoprenoid moieties derived from the non-mevalonate or deoxyxylulose phosphate pathway. It is generally accepted that hyperforin is made from one molecule of acyl phloroglucinol and five isoprenoid moieties. The isoprenoids that are the precursors for hyperforin are thought to be made via the non-mevalonate pathway (Eisenreich et al., 2004). See Appendix B for a possible hyperforin biosynthesis pathway. An analog of hyperforin called O-(carboxylmethyl)-hyperforin or Aristoforin can be produced synthetically and is stable and soluble in aqueous solutions (Medina et al., 2006). In an in-vitro study, it was found that hyperforin was only present in plantlets with and without roots, and not in suspension cells, undifferentiated callus and callus with vegetative buds suggesting that cell differentiation must occur before hyperforin is produced and hinting at the location of its production (Pasqua et al. 2003). A more detailed description of the chemistry of this molecule may be found in Vollmer and Rosenson (2004).

For both hypercins and hyperforins, the exact biochemical synthesis pathway with detailed information including exact pathway location, pathway intermediates, enzyme names and enzyme kinetics has not been elucidated. As a result, it is not possible to begin to create transgenic plants that have a rate-limiting enzyme amplified so that increased metabolite production might be obtained. This is not a problem that is limited to *H. perforatum*. Many key details of natural product pathways have yet to be elucidated; however, the field of metabolic engineering is not anticipating the manipulation of natural products in the near future (Dixon, 2005).

Over the counter products produced from whole plants, plant parts, or extracts of *H. perforatum* are standardized most often to hypericin, as this was the metabolite that was in the literature first as the important medicinal component. Currently, most products remain standardized to hypericin, though some are standardized to any or some combination of hypericin, pseudohypericin or hyperforin (Chatterjee et al., 1998;

Butterweck et al., 2003). The most common quantification method used by industry and described in Deutscher Arzneimittelcodex (1991) for the quantification of hypericin, still involves spectroscopy as hypericin turns red when exposed to solvent. This laboratory method, however, is known to be less accurate than other available quantification methods, and thus has been attributed to some of the variability in the commercial product (Ruckert et al, 2006).

1.2.4 Location of metabolites

The location of the precursors of hypericin, pseudohypericin and hyperforin are not yet known (Pasqua et al., 2003; Vollmer and Rosenson, 2004). Hypericin may be found in the black glands visible to the naked eye found on the stems, leaves, flower petals and flower pistils. Pseudohypericin may also be found in stems, leaves and flowers, though its storage organ remains unknown. It is well-established that all three metabolites may be found in the greatest concentration in the flowers, followed by the leaves and stems (Couceiro et al., 2006; Sirvent et al., 2002; Zobayed et al., 2005). Figures demonstrating the average total amounts of hyperforin and hypericin in each plant part as affected by temperature (shoot which includes leaves and stems, flower, and bud) may be seen in Figures 1.4 and 1.6. Relative percents of hyperforin and hypericin in each plant part as affected by temperature may be observed in Figures 1.3 and 1.5. An important discovery in 2002 by Murch et al. is that the metabolite levels (μg/mgFW) are significantly different when flowers are analyzed starting at a green bud stage versus when the flowers were fully open. A four time increase was observed for all metabolite concentrations when reproductive parts were analyzed in the green bud stage compared to the yellow bud stage. Maximum levels of all three metabolites were found just before the flowers opened when the buds were yellow but

not fully swollen. This finding should be recalled when determining when to harvest the crop, however it is not typically considered in commercial production situations.

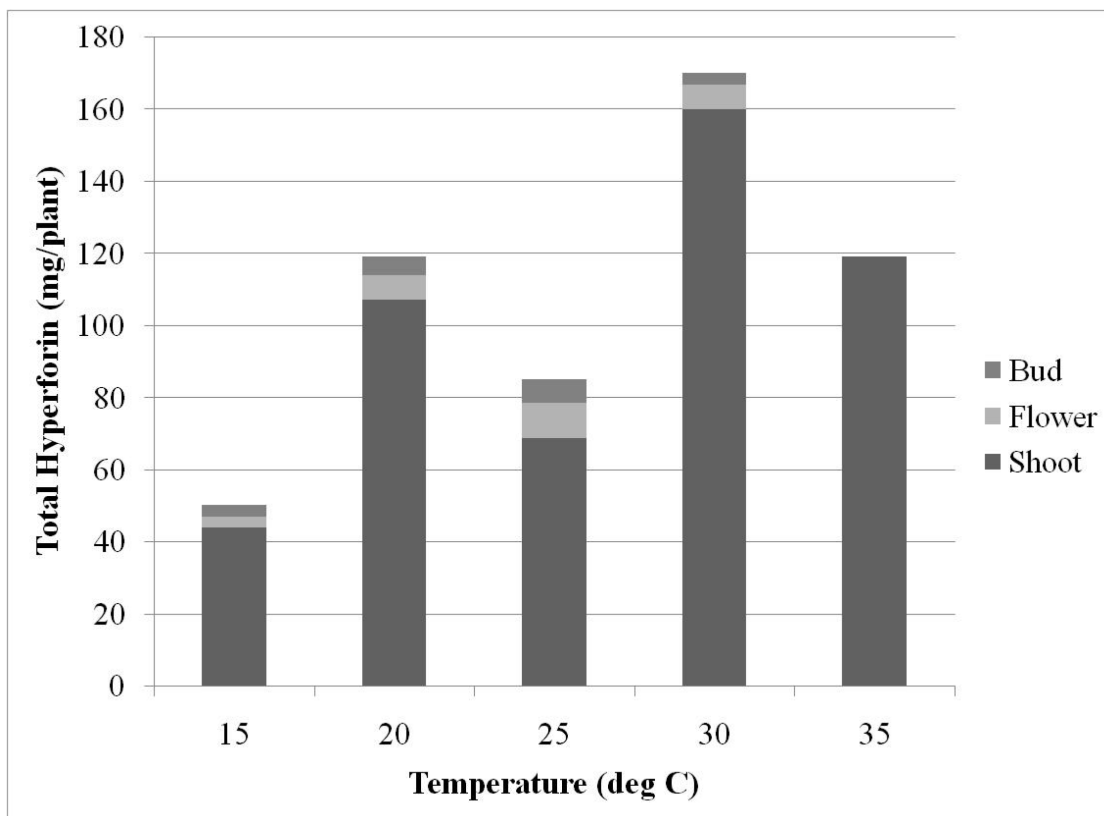

Figure 1.3. Estimated hyperforin amount found in whole plant found in each plant part as affected by temperature adapted from Zobayed et al., 2005. Shoot includes leaves and stems.

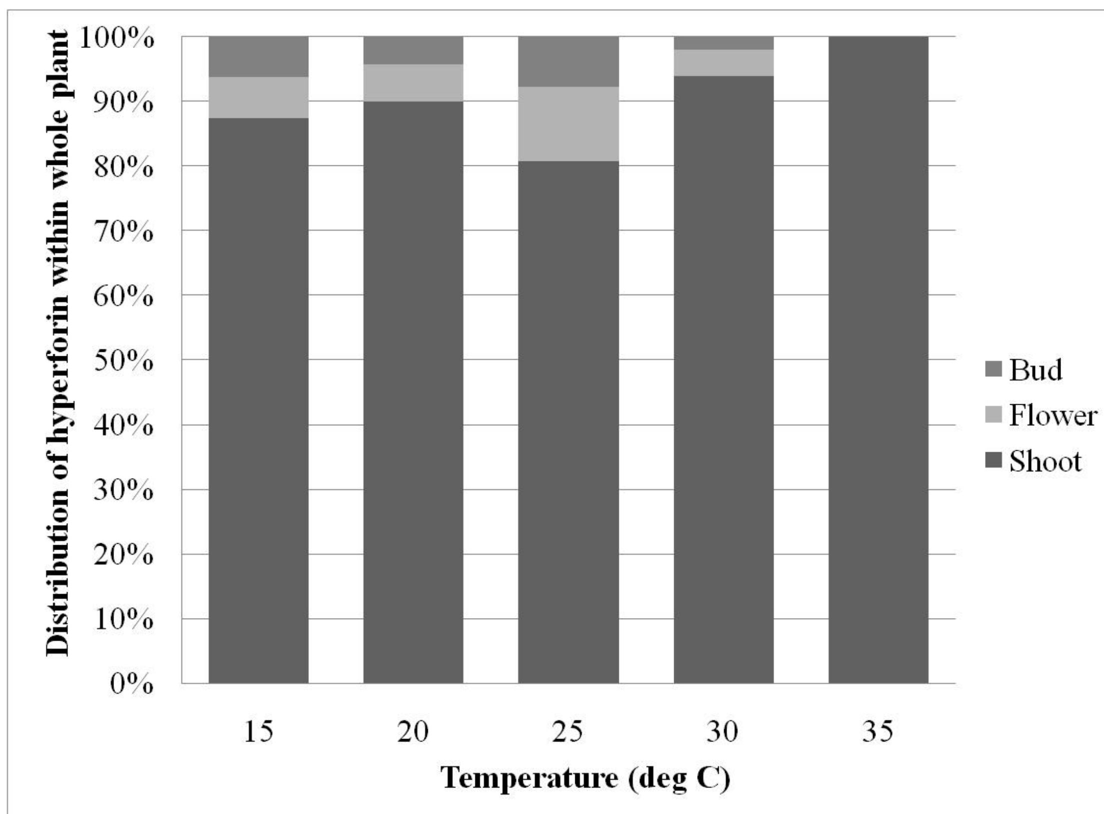

Figure 1.4. Distribution of hyperforin concentration per plant part in whole plant as affected by temperature adapted from Zobayed et al., 2005. Shoot includes leaves and stems.

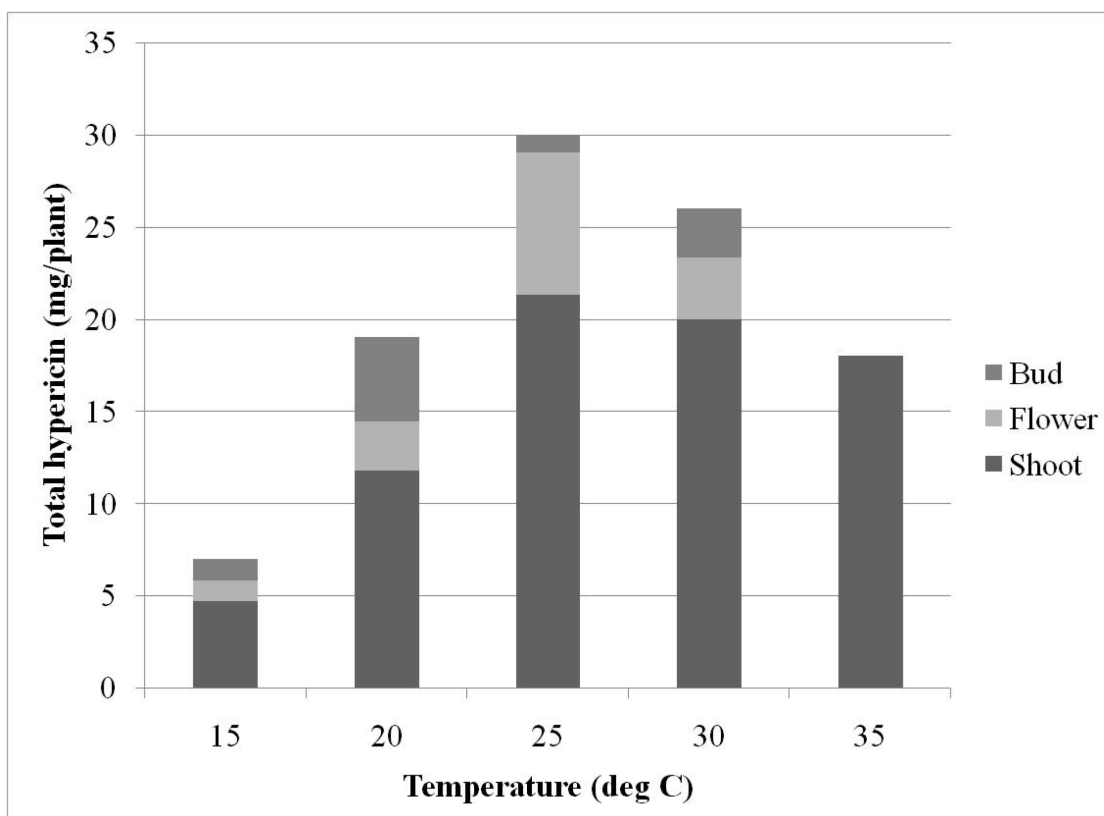

Figure 1.5. Estimated hyperficin amount found in whole plant found in each plant part as affected by temperature adapted from Zobayed et al., 2005. Shoot includes leaves and stems.

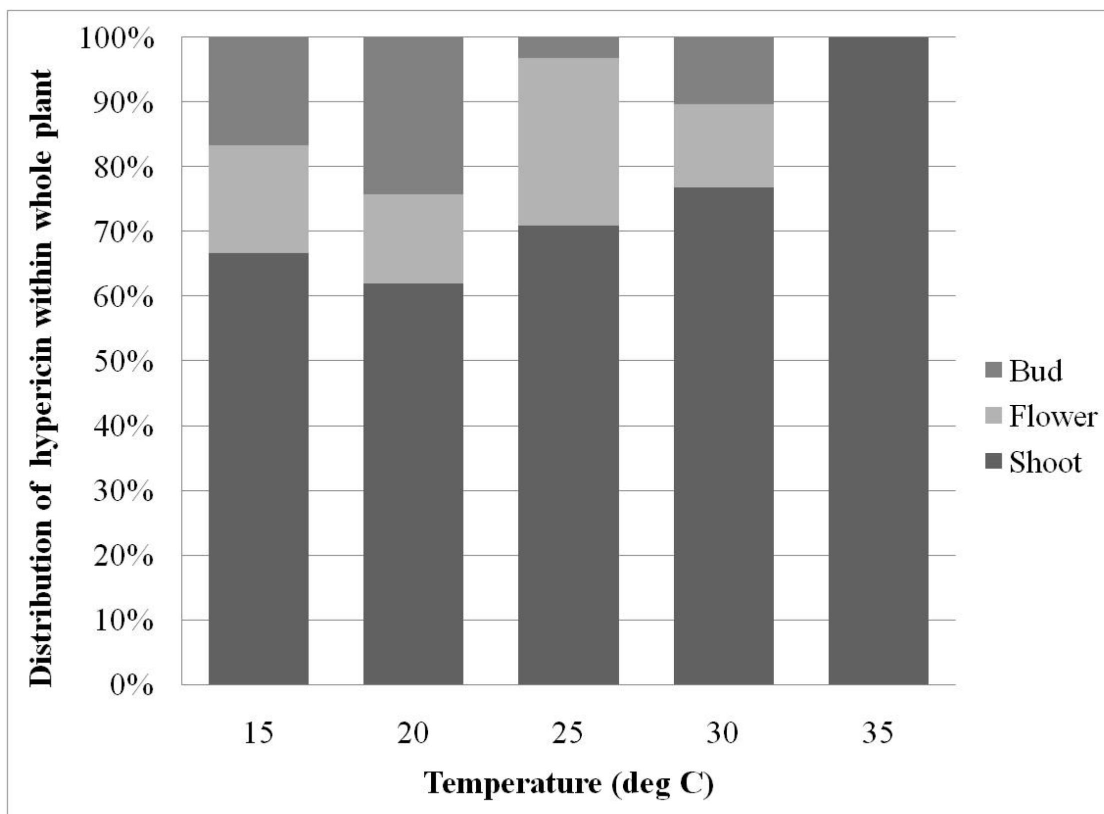

Figure 1.6. Distribution of hypericin concentration per plant part in whole plant as affected by temperature adapted from Zobayed et al., 2005. Shoot includes leaves and stems.

1.2.5 Experimentally-based health claims of *H. perforatum*

Traditional uses dating back to the Ancient Greeks include antidepressant, antifungal, anti-inflammatory, and antibacterial applications. For a review of the medical uses of hypericin including antidepressant, anti-viral, and antiproliferative uses see Kubin et al., (2005). For a review of the many of the beneficial effects thought to be mediated by the metabolite hyperforin including antidepressant, anti-inflammatory, antibacterial, antitumoral, antiangiogenic effects, see Medina et al., (2006).

Mode of action of H. perforatum on humans for depression

Initially, it was thought (Suzuki et al., 1984) that hypericin was the bioactive ingredient in *H. perforatum* that contributed to the alleviation of mild and moderate symptoms of depression through the inhibition of monoamine oxidase (MAO), an enzyme that facilitates the breakdown of many important neurotransmitters including serotonin. Now it is thought that hyperforin is the compound responsible for the alleviation of depression symptoms (Chatterjee et al., 1998; Butterweck et al., 2003; Mennini and Gobbi, 2004; Medina et al., 2006). It was postulated that hyperforin acted as a selective serotonin reuptake inhibitor (SSRI) which is the mode of action of the popular synthetic drug Zoloft. Recent studies have determined that while hyperforin may be a serotonin reuptake inhibitor, it is not a selective inhibitor. The exact mechanism of hyperforin is still being debated, but recent work with human platelets and rat pheochromocytoma cells shows that hyperforin activates nonselective cation channels and that it does not directly inhibit neurotransmitter transporters, but rather interferes with intracellular concentration. Furthermore, it is generally acknowledged that it is a likely (but unproven) synergism of many different secondary metabolites that makes *H. perforatum* an effective treatment, and that the isolation of individual components may not show the same activity as a whole plant extract (Chatterjee et al., 1998; Butterweck, 2003). For a review of the antidepressant mode of action of hyperforin see Medina et al, (2006).

Clinical trials using H. perforatum extracts on humans

A detailed review on the numerous clinical applications of components of *H. perforatum* on humans is beyond the scope of this report. A summary of the major findings of medical trials may be found in the previously mentioned review articles of the main metabolites hypericin and hyperforin by Kubin et al., (2005) and Medina et al., (2006). Additionally, an important study detailing the bioavailability of hypericin, pseudohypericin, hyperforin and other metabolites by single or multiple oral dosing may be found in Schultz et al., 2005. It is well established that although the individual constituents that give *Hypericum* spp. it's antidepressant activity have been identified, whole plant extracts are more effective than any of the isolated metabolites (Butterweck, 2003; Muller, 2005; Noldner, 2005). Nolder (2005) said that the "extract is more than the sum of single components". This suggests that it is important for standardized extracts to be produced and that the concentration of all components be strictly controlled by the manufacturer. The consistency in metabolite concentrations of the raw plant material supplied to the manufacturer becomes very important with such a task.

1.2.6 Studies of variability of material in marketplace

Several studies have analyzed commercially available products and reported on deviations between actual content of the metabolite and labeled content. While their quantitative and statistical analysis methods differed, the conclusion was always that the concentration of metabolites in the products tested was generally not what was labeled on the package.

In Germany, eleven *H. perforatum* products available in pharmacies were evaluated with respect to total hypericins and hyperforin for batch to batch (different bottles) reproducibility. Hyperforin showed great variability in the content per dose

ranging from less than 0.5 to 26 mg/dose. There was no mention of hyperforin concentration on the product label. The authors suggest that some of the products be labeled "hyperforin-free" because some brands contained such a small dose of hyperforin that it would not be therapeutically useful. The average measured hypericin content per dose ranged from 0.6 to 1.7 mg/dose. According to the label strength on the products, hypericin concentration should have been 250-600 mg/dose. An interesting aspect of this study was that the authors' disclose the names the products are sold under instead of reporting the results as commercially available but keeping the manufacturer anonymous (Wurglics et al., 2001).

Wang et al. (2004) looked at five commercially available *H. perforatum* tablets purchased from a store in California and analyzed them for hypericin and pseudohypericin. They found that the amount of hypericin varied between products from 130 to 198 μg/tablets and pseudohypericin varied from 257 to 465 ug per tablet and that there was no correlation between the two chemical concentrations for any given product. One product was exceptionally low for both chemicals. All but one of the tablets should have had 900 μg per tablet which is 0.3% by weight. The product that was supposed to have 340 μg/tablet demonstrated 388 μg/tablet or 114% of the labeled amount. The extraction efficiency was over 90% and the authors attribute low amounts of hypericin found in the products to poor product quality, degradation or polymerization. Although the authors used a reverse-phased high pressure liquid chromatography method with fluorescence detection which is the most common apparatus for quantification of secondary materials in St. John's wort, the solvent and buffer used as well as the wavelengths used for detection were not standard. While slight evidence of some statistical analysis was evident in the form of standard deviations, significance tests and analysis of variance were absent. Commercially available medicinals may vary greatly in the amount of active ingredient that is in each

dose as has been suggested in the popular press (Good Housekeeping Institute, 1998), but this study is not the best example of such a statement.

In another study attempting to draw conclusions about the consistency of labeling and actual quantities in commercially available botanical dietary supplements, the top five best selling supplements were investigated (Krochmal et al., 2004). Six bottles of each of two lots from nine nationally available manufacturers were purchased and analyzed for the marker compound standard to each supplement. The herbs investigated were: saw palmetto, kava kava, echinacea, ginseng and *H. perforatum*. Overall, not much marker compound variability was found within a given brand. However, there was found to be a large difference in marker compound (hypericin) concentration among brands. For *H. perforatum*, the products were found to contain 88-139% of the amount claimed on the label. Compared to another herbal product tested, *Piper methysticum* (Kava Kava) where the percent of the amount found on the label varied between 42 and 110%, *H. perforatum* was much less variable overall. If one considers that *H. perforatum* was shown to be the herb that contained active ingredients that were closest to what was on the label despite (perhaps unfairly) a reputation as one of the most inconsistent products on the market, it may be noted that the consistency of the industry as a whole may be worse than previously suspected. The authors note that the analysis methods used were identical to those used commonly by commercial producers and cite the quantification method for *H. perforatum* as high pressure liquid chromatography (HPLC) (Krochmal et al., 2004). Investigation of different manufacturers at any local drugstore will demonstrate that HPLC is not the most commonly used method for metabolite standardization. An evaluation of product labels of locally available *H. perforatum* supplements shows the method used by most companies to quantify the metabolite concentration during the manufacturing process is UV spectroscopy. For more information on these

quantification methods, see section 1.4.4 Chromatography and other quantification methods.

1.2.7 Commercial Production

Until the renewed interest in herbal medicine in the 1990's, much of the *H. perforatum* that was sold in the world was wild-collected. Most of the monographs mentioned above caution against the use of such material for both environmental and quality-control reasons. While *H. perforatum* is considered an invasive species in many areas where it grows uncultivated, regulatory suggestions are encouraging drug companies not to use any wild-collected herbs to reduce the negative impact on those plant species that are endangered. Furthermore, they uniformly acknowledge that the misidentification of cultivars leads to an inferior product with little or no active ingredient. Currently, the majority of the *H. perforatum* that is processed and sold as a medicinal plant is field-grown in temperate regions. Though this crop is a perennial species, a single planting is often maintained for only three years due to a vulnerability to fungal diseases (Poutaraud and Giradin, 2005).

Following a drought brought on by El Nino conditions in 1998 that negatively impacted the plant material supplied by growers in South America, there was a shortage of *H. perforatum* that provided an opportunity for new suppliers of plant material. This prompted an influx of money available for research into the improved agricultural practices for this plant as well as much encouragement by agricultural extension offices to increase the availability of plant biomass. The following is an example of a fairly detailed paraphrased summary of growing procedures from the crop development branch of the Canadian government, most recently updated April 5, 2006 to reflect the results of agricultural research conducted in Guelph at the agricultural experiment station (www.agr.gov.sk.ca, accessed 3/14/08):

There are four improved commercially available strains that are recommended based on their physical and chemical properties: Hypericum perforatum L. New Stem, H. perforatum L. Helos, H. perforatum L. Elixir J and H. perforatum L. Topas, For commercial production, the top half of the plant should be removed to obtain flowering parts while the top seventy percent of the plant may be removed to obtain the aerial portion of the plant. This harvesting should occur when fifty percent of the flowers are fully open and before the seed formation. Plant material should be allowed to dry out of the sun in order to preserve color and quality. Artificial heat alone or in combination with fans may be used during the drying process. Once dried, the plant material may be baled and transferred to a warehouse to await further processing. Industry wants leaves and flowers but does not always separate out stems. Producers typically harvest the top 50-70% of aerial portion of the plant.

Extension specialists in the United States also authored bulletins on the cultivation of *H. perforatum*, for an example see the bulletin from Kansas State MF-2629 written by Janke, (2004), which is quite similar to the Canadian document with the addition of more extensive market potential analysis and results of a multi-year field analysis conducted in Kansas.

1.3 PLANT GROWTH AND METABOLITE STUDIES[1]

1.3.1 Genotypic and Phenotypic variation[2]

Studies have reported variation in hypercin levels for wild-captured *H. perforatum* from the following areas: Nova Scotia (Jensen et al., 1995), America (Sirvent et al., 2002), America vs. Armenia (Kirakosyan et al., 2003)[3], Poland (Osinska and Weglarz, 2000), Australia- broad vs. narrow leaf (Southwell et al., 1991), Switzerland (Buter et al., 1998)[4]. The results of these studies suggest a significant effect of biotope on metabolite production. The study by Buter et al. was the only study that attempted to find a connection between genetic and environmental effects on hypericin. A detailed list of additional studies examining the metabolite variation in various accessions of *H. perforatum* (not wild-type) may be found in Poutaraud and Girardin (2005), where the difficulty of comparing such studies due to lack of a universal control cultivar is noted.

A three year study of 39 wild collected *Hypericum* accessions (30 *H. perforatum* and 9 *H. angustifolium*) were grown in a field to assess genotypic and phenotypic variation simultaneously, and it was found that hyperforin concentrations varied from 0.65 to 5.7% depending on the accession and plant part investigated.

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[1] Because of the effects of different extraction techniques as described in section 1.4 (extraction technique can result in a variance as large as 50% depending on the method used see Poutaraud et al., 2001), the vast differences in quantification protocols makes studies very difficult to compare. Unless otherwise noted, the quantification procedure used in these studies is High Pressure Liquid Chromatography which removes some of the variability in extraction efficiency, but differences in the remaining variables associated with quantification makes comparison among studies very difficult.

[2] Some of these studies are done by sampling wild-type plant material from different locations to look indirectly at genotype and phenotype (as affected by environmental conditions), while others look at the effect of only genotype by producing plants in a single location.

[3] Genetic material was wild-collected and plants were grown in tissue culture to determine chemical concentration.

[4] It must be noted that many of these studies used quantification methods that are less accurate than those currently used. The abnormally high metabolite levels reported in some of them (hyperforin concentrations of 15% of dry weight when it is normally 4%, or hypericin levels of 0.23% instead of the more commonly accepted 0.03%) may be a result of the quantification method used.

Similarly, hypericin content varied from 0.7 to 3% (Poutaraud and Girardin, 2004). The biomass and metabolite results of this study were confounded by the fact that many of the plants succumbed to the fungal disease anthracnose at various times. Authors of all studies on genotypic/phenotypic variation agree that concentrations of hypericin and hyperforin will vary according to environmental conditions during plant growth as well as the portion of the plant harvested. The Armenian study cited that the difference between the highest and lowest producing site for hypericin was 950% and for pseudohypericin was 833% (Kirakosyan et al., 2004). The authors attribute some of this variability to the altitude, average temperature and amount of sun each site received per year. While these values are dramatic, all of the studies citing metabolite concentration and harvest location show similarly high variation.

Couceiro et al. (2006) looked at variation of metabolites in response to germplasm variation. This growth chamber conducted study is detailed below (See Time to Harvest under 1.3.3 Growth Chamber Research).

1.3.2 Field Research

For most field studies, attempts to control conditions (water, nutrient availability etc.) were not made to the extent that might be expected. Occasionally plants were watered and/or fertilized. It is difficult to compare results from year to year because of various unexpected happenings such as disease outbreak, drought and excess precipitation. This created problems in evaluating multi-year studies and added to the difficulty associated with the comparison of different studies.

In field grown plants, the following variables have been considered: drought, enhanced rainfall, seasonal variation, and geographic location (see'1.3.1 Genotypic and Phenotypic variation' section above). See Table 1.2 at the conclusion of this

section for a summary of growing conditions and metabolite concentrations for studies conducted in the field.

Drought stress and Harvest time

In Missouri during the summer of 1998 Gray et al. grew *H. perforatum* L., Clusiaceae from seed in 11.5 liter pots with drip irrigation and investigated the effect of drought stress for two growth stages that were approximately two weeks apart, first the initial period of flowering and then during seed development. Ten phytochemicals were quantified, among them hyperforin, pseudohypericin and hypericin. While the authors quantified both flower and leaf metabolite concentrations, the numbers that follow are for the flowers only, and it must be noted that differences that are detailed below between the well-watered condition and the water-stressed condition were not significant[5]. However, differences in metabolite concentration were statistically significant between the stage in which the plants were in full flower and seed development. The concentration of metabolites in the leaves showed the same trends with the exception of hypericin, which decreased during seed production (down 9%)[6]. Compared to the control plants which were adequately watered throughout the drydown periods, hyperforin levels decreased during both initial flowering period (down 4%) and during seed development (down 12%) if the plant was drought stressed. Pseudohypericin concentrations decreased for drought stressed plants in full flower (down 2%), but increased (up 37%) if the drought was imposed when the plants were in the seed development stage. Hypericin levels increased in drought stressed plants during both flowering (up 8%) and seed production (up 10%). It is

[5] A detailed summary of the metabolite increases and decreases is given here to establish the amount of variation possible when these conditions are imposed upon plants. Since most changes are less than 15% and are not significant, similar low percentage changes in concentration should also be looked upon as potentially not significant.
[6] Relative to control values.

hypothesized that the reason for an increase in hyperforin during the setting of the seeds is due to the antimicrobial action that has been attributed to hyperforin (Gray et al., 2003). In addition to its quantification of the effects of a short water stress period just prior to harvest, a major contribution of this paper is the demonstration that the timing of harvest with respect to the reproductive stage is important. For most metabolites, the concentration changed significantly in the two weeks between full flowering and seed production. An analysis of the author's data shows that the harvest date for the optimization of hyperforin would be different than that for pseudohypericin or hypericin. The exact age at harvest was not disclosed, although the planting date of the seeds started in a greenhouse for transplanting was cited as 1997, and the drydown periods that constituted the experimental treatments were started on June 6 making the plants at least 6 months old. Compared to plants that are grown directly in the field where new biomass may be seen emerging from the soil in May with flowering occurring in July, these plants were fairly old. There were omissions of details that would allow this data to be analyzed more completely. Additional pieces of information that would have been valuable were: where the plants were grown (inside or outside), average diurnal temperatures, light intensity, photoperiod, and temperature during the periods of drought stress.

Enhanced Rainfall

In northern England, the effects of enhanced rainfall and drought on reproductive biomass and herbivory of an established cultivated crop were evaluated. The effects of withholding and supplementing precipitation increased the time to germination and decreased the seed produced by 15%. Since this is an ongoing study and non-destructive analysis methods were used when possible, whole plant biomass was not assessed.

Seasonal Variation

In New South Wales Australia where it is possible to harvest two crops of *H. perforatum* per year, Southwell and Bourke (2001) found a 50-fold difference in hypericin concentration between summer and winter grown crops.

1.3.3 Growth Chamber Research[7]

Controlled environment growth rooms or growth chambers are excellent tools for the elucidation of the effects of a single variable on the growth and development of plants because of an ability to have control over light intensity, light duration, photoperiod, light quality, temperature and sometimes carbon dioxide and/or humidity. The following variables have been investigated in growth chambers: light intensity, temperature, carbon dioxide concentration, nutrition and water status[8]. See Table 1.3 at the conclusion of this section for a summary of growth conditions and metabolite concentrations for research conducted in growth chambers.

Light

In 2001, Briskin and Gawienowski evaluated hypericin levels as light intensity was varied between 100-400 µmol/m²/s. Hypericin levels showed a 3-fold increase with increasing light intensity. This study showed a linear relationship between leaf gland number and level of leaf hypericins (See 1.4.4 Hypericin Concentration and Leaf Gland Number). This contradicts an earlier study by Briskin et al. in 2000 that showed no correlation between leaf gland number and hypericin level. The

[7] Studies detailed in the following papers were all completed in the same facility with CO_2 supplemtation and identical quantification methods: Couciero et al., 2006; Zobayed et al., 2005; Zobayed et al., 2007

[8] Most studies did report air temperature, light intensity and photoperiod and watering frequency. Root zone temperature, however, was not reported for any study.

Table 1.2. Summary of growing conditions and metabolite data for experiments conducted in the field.

			Plant age from seeding		Light				
Authors	Year	Variable	Treatment applied (days)	Harvest (days)	Source	Intensity (umol/m^2/s)	Photoperiod (hours)	Planting density (plants/m^2)	Cultivar
Southwell and Bourke [1]	1991	Broad v. narrow leaf	n/a	n/a	sunlight	unknown	unknown	unknown	wildtype
Osinska and Weglarz	2000	Species	n/a	n/a	sunlight	unknown	unknown	32	various
Sirvent et al.	2002	State	n/a	n/a	sunlight	unknown	unknown	n/a	wildtype
Poutaraud and Girardin	2004	Accession	n/a	n/a	sunlight	unknown	unknown	4.75	Topaz as control
Gray et al. [2]	2003	Drought stress	6	at least 6 months	sunlight	unknown	unknown	unknown	Clusiaceae
Buter et al. [3]	1998	Accession and environmental variation	n/a	at least 6 months	sunlight	400-550 MJ/m2	unknown	unknown	unknown
Mosaleeyanon et al.	2005	Control plants	n/a	72	sunlight	1000-2000	13.25-14.75	12	unknown

		Metabolite concentrations			
Authors	Year	Hyperforin % DW	Pseudohypericin % DW	Hypericin % DW	Dry weight at harvest (grams)
Southwell and Bourke [1]	1991	not measured	Reported as 'hypericins'	0.02-0.47	unknown
Osinska and Weglarz	2000	not measured	not measured	0.035-.0.183	75
Sirvent et al.	2002	not measured	0.0019-0.85	0.0003-0.12	unknown
Poutaraud and Girardin	2004	0.65-3.2	Reported as 'hypericins'	0.7-3	15-39
Gray et al. [2]	2003	1.2-2.9	0.05-0.18	0.04-0.125	53-64
Buter et al. [3]	1998	not measured	40	0.25-0.5	unknown
Mosaleeyanon et al.	2005	0.67	0.07	0.03	0.375

[1] Only narrow leaf metabolite concentrations are summarized

[2] Weights and metabolites are complicated and differed by treatment and harvest. Averages give a general idea of the range of values observed.

[3] Concentration in flowers only

quantification technique done in the Briskin studies was thin layer chromatography, which is not as quantitatively consistent as high pressure liquid chromatography, the technique used by most researchers in subsequent studies.

The next major controlled environment study investigating the effect of light intensity on metabolite production was by Mosaleeyanon et al. (2005). Different combinations of light intensity and carbon dioxide concentrations were used with the light intensity varying from 100-600 $\mu mol/m^2/s$ and CO_2 concentration ranging from 500-1500 ppm. Metabolite concentrations were compared to concentrations in control plants grown in a field condition at the same time. Pseudohypericin and hypericin were 41 and 30 times greater than the field-grown control plants. The chamber grown plants with the combination of highest light and highest CO_2 produced the plants with the greatest concentration of secondary metabolites and the largest total biomass. Interestingly, hyperforin concentrations were the highest (45 times greater than control values) under the medium light and high CO_2 condition not under high light and high CO_2 as might be expected. When the plant was analyzed for biomass by plant part (leaves, stems, roots, or total biomass), the treatments were statistically different. Leaf biomass was the same for all high light treatments and the medium light treatment with the highest CO_2 concentration, but total biomass was highest when plants received high light and high CO_2.

Temperature and Time to Harvest

The effects of temperature, harvesting time and germplasm were examined by Couciero et al. in their study published in 2006. Plants that were approximately halfway to flowering were placed in growth chambers that were either 25 or 30C and grown for 30 more days with a final harvest at 72 days at which time plants were flowering. Metabolites were quantified approximately 10 days apart and plants were

repeatedly sampled such that a single germplasm could be evaluated over time. Though the authors failed to take enough samples at each harvest to evaluate the variability within a single germplasm, variance among plants is highlighted. Variation from germline to germline was demonstrated to be as high as 55% for hyperforin, 200% for pseudohypericin, 100% for hypericin and 100% for both fresh weight and dry weight at final harvest. It is important to note that the carbon dioxide concentration was elevated to 1000 ppm for this study. The major contribution of this study, besides further confirmation that there is great variability between different germplasms, was that a peak in metabolite concentrations was noted on day 52 where metabolite concentrations doubled with respect to their initial and final values, a phenomenon that has not been reported previously or subsequently. The authors also noted that the ratio of metabolites (hyperforin to hypericins and pseudohypericin to hypericin) was significantly affected by temperature, observations that demonstrate another variable that must be considered when attempting to determine a protocol to optimize the production of specific metabolites.

In 2005, Zobayed et al. looked at temperature stress under a CO_2 enriched environment over a 20 C range from 15-35 C under a relatively low light intensity, 200 µmol m^{-2} s^{-1}. Metabolite concentration is reported for each plant part including vegetative (stems and leaves), fully open flowers and buds. Different temperature optimums were found for all metabolites; hypericin was optimized at 25 C and hyperforin at 30 C. An important finding was that plants did not flower at 35 C, suggesting that high temperature exposure at a critical period would suppress flowering. This condition is somewhat limited to a specialized CEA production facility that would prevent radiation to the sky at night that would allow the leaf temperature to be lower than 35 C. The variation in metabolite concentration between fully open flowers and buds confirmed the observations by Murch et al., 2002.

Carbon dioxide concentration

Another study published by Zobayed and Saxena in 2004 focused on plants that were started in tissue culture and transplanted to rockwool and grown either in a specialized growth chamber called a Closed Controlled Environment System (CCES) that was unique because it could supply plants with CO_2, or a greenhouse. The CCES setup was given regular or elevated carbon dioxide levels. The same chemicals were evaluated as in the previous study. The important conclusion of that study was that metabolite concentration may be increased by CO_2 supplementation to a level that is at least twice that of ambient conditions. This specialized form of growth chamber was custom made and would not be economical to use for commercial production and has not been used in subsequent studies.

Nutrition

In 2000, Briskin et al. looked at the effects of reducing the supply of nitrogen to *H. perforatum* grown in a growth chamber. They found that decreasing the level of nitrogen to the plants resulted in a two to three-fold increase in the amount of total hypericins (pseudohypericin and hypericin) that the plant contained. The treatment conditions were 1/20, 1/100 and 1/300 of a standard solution and were applied for 56 days. While total hypericins (pseudohypericin plus hypericin) on a μg/gFW basis was not significantly higher with any concentration lower than the 1/20 condition, the lower levels of nitrogen induced a steady and significant decline in the shoot fresh weight, plant height and number of branches per plant. It may be concluded that decreasing the nitrogen levels to the plant by 1/20 compared to the standard solution has just as much of a positive effect on increasing hypericin while limiting the negative effect on shoot fresh weight, total height and branching. In this study, the authors also looked at the effects of a short (28 d) low nitrogen stress (zero nitrogen)

on the plants. They found that there was an increase in the hypericin levels in the upper leaves of the plants. The level of hypericins in the lower leaves remained constant over the course of the experiment. Standard signs of nitrogen stress were found by the end of the low nitrogen treatment that were consistent with the longer low-nitrogen study showing decreased fresh weight, decreased branching, decreased plant height and lower leaf chlorosis. The hypericin levels were determined by thin layer chromatography which is a method that is not as accurate in a quantitative sense as HPLC and thus re-evaluation of the plant material with such a method could show greater variation in metabolite concentration.

In the same 2000 study, Brisken et al. also looked at nitrogen supplementation at levels either 1 or 3 times the amount in the nutrient solution and found that both conditions showed a decrease in hypericin levels to 30 percent of the amount in the control treatment that had the standard composition of nitrogen.

Water availability

The effect of a longer water stress condition than the earlier field experiment (12 days instead of 6 days) was investigated by Zobayed et al., (2007). Plants were sampled at day 1, 6 and 12 of the experiment and a significant decrease was observed after 12 days of water stress in hypericin (85% decrease) and pseudohypericin (81% decrease) while an increase was observed in hyperforin (45%). Water potential measurements for 12 day old plants were: -0.12 MPa for control plants and -3.12 MPa for stressed plants. It is important to note that there was no significant difference in metabolite concentration after 6 days of drought stress implying that plants can undergo drought conditions for some time before metabolite concentration is affected. This contradicts the earlier field study by Gray et al. (2003), where hyperforin and pseudohypericin decreased and hypericin increased in response to water stress.

Studies have shown that increasing salt concentration and hence the electrical conductivity measurements in hydroponically grown plants can cause water stress symptoms and increase the concentration in plant produced secondary metabolites such as lycopene in tomato plant production (Wu et al., 2004).

Photoperiod

To date, no published work may be found in the extant literature on photoperiod, although one publication (Mosaleeyanon et al., 2005) alluded to examinations underway at Chiba University in Japan.

1.3.4 Tissue Culture Research

Tissue culture is a growing system that has been extensively used with *H. perforatum* for the following reasons:

1. The resulting material may be certified virus and bio-contaminant free.

2. It is easy to manipulate the medium that the plants are grown in to facilitate the investigation of specific metabolic pathways.

3. It was once thought that a higher concentration of secondary metabolites could be produced on a per weight basis in such a system, but analysis of metabolite concentrations on a percent dry weight basis shows this supposition to be incorrect (See Tables 1.3 and 1.4).

The optimization of some aspects of a tissue culture protocol including media composition, plantlet age, and type of in-vitro system have been performed. However, the prohibitively high cost for such a production method has halted the further investigation into this system.

Table 1.3. Summary of growing conditions and metabolite data for experiments conducted in growth chambers.

			Plant age from seeding			Light						
Authors	**Year**	**Variable**	**Treatment applied (days)**	**Harvest (days)**	**Source**	**Intensity ($umol/m^2/s$)**	**Photoperiod (hours)**	**Reproductive stage**	**Planting density ($plants/m^2$)**	**Temperature (deg C)**	**Cultivar**	**Carbon Dioxide (ppm)**
Briskin et al. [1]	2000	Nitrogen concentration	21	77	unknown	345	18	unknown	unknown	25	Topas	unknown
Briskin and Gawienowski	2001	Light intensity and nitrogen concentration	21	77	unknown	100, 200, 300, 400	18	unknown	unknown	25	Topas	unknown
Zobayed and Saxena [2]	2004	CCES system	n/a	45	unknown	200	16	vegetative	178	23/19	New Stem	950-1050
Mosaleeyanonn et al. [3]	2005	Light intensity, Carbon dioxide concentration	30	75	fluorescent	100, 300, 600	16	flowering	178	28/26	unknown	500-1500
Zobayed et al. [1,4]	2005	Temperature stress	70	85	fluorescent	250	16	flowering	unknown	15-35	New Stem	1000
Couceiro et al. [1,5]	2006	Harvesting time, temperature, germplasm	42	72	flourescent	300	16	flowering	unknown	25 or 30	New Stem	1500
Zobayed et al. [6]	2007	Water stress	60	72	unknown	250	16	vegetative	unknown	27	New Stem	1000

		Metabolite concentrations			
Authors	**Year**	**Hyperforin % DW**	**Pseudohypericin % DW**	**Hypericin % DW**	**Dry weight at harvest (grams)**
Briskin et al. 1	2000	not measured	Reported as 'hypericins'	0.1-0.4	unknown
Briskin and Gawienowski	2001	not measured	Reported as 'hypericins'	0.05 - 0.24	unknown
Zobayed and Saxena 2	2004	3.00	0.09	0.015	0.3
Mosaleeyanonn et al. 3	2005	2.67	0.075	0.033	1.6-3.8
Zobayed et al. 1,4	2005	0.875	0.31	0.123	5-6
Couceiro et al. 1,5	2006	2-4	0.05-0.125	0.025-0.085	unknown
Zobayed et al. 6	2007	3-6	0.05-0.26	0.002-0.14	unknown

[1] Assumed that FW/DW ratio was 5

[2] Assumed that the units on the graph were labelled incorrectly and should have read 'ug/gDM'

[3] Values reported are for 300 $umol/m^2/s$ PPF and 500 ppm CO_2

[4] Metabolite values are for plants at 25 C

[5] Assumed units on graph should have read ug/gFW

[6] Metabolite values are for control plants - stressed/recovered plants

Another category of experiments performed in-vitro include the evaluation of metabolite concentration due to biotic challenges from insects, fungi, bacteria, viruses and abiotic challenges chemical inductors such as salicylic acid and methyl jasomate (Sirvent and Gibson., 2002). While most of these treatments did significantly increase metabolite concentration and this fact should be recalled when evaluating plants exposed to such elicitors, only a review of those studies that may be applied to CEA production will be addressed here, however, a more extensive review of in-vitro work may be found in Kirakoysan et al., (2004). See Table 1.4 at the conclusion of this section for a summary of growing conditions and metabolite concentrations for studies conducted in the tissue culture.

Effects of Supplemental Carbon

In 2004, Zobayed et al., compared chemical constituents among six different types of tissue culture systems with elevated levels of carbon both in the media (15-60 g/l) and as gaseous CO_2 at a concentration of 1600 ppm. Hyperforin concentration was increased by 50% when supplied with 45 g/l sucrose and supplemented with CO_2. A 25% decrease in hyperforin concentration was found with an increase in sucrose in the absence of supplemental CO_2, suggesting that the extra carbon used for metabolite production was fixed from the air, and not transported from the roots. There was no increase in hypericin and pseudohypericin in response to supplemental carbon supplied as either sucrose or CO_2.

Mechanical Injury

A study was conducted investigating the effects of either mechanical or biotically induced injury. Plantlet leaves were cut with scissors in an attempt to elicit chemical changes similar to those that develop when a plant is being attacked by

insects. Beetles that were either generalist or specialized to *H. perforatum* were encouraged to feed on plants and all three treatments were analyzed for hypericin. It was determined that mechanical injury and feeding by generalist beetles did not have a significant impact on chemical concentration, though specialist beetle feeding increased hypericin concentration (Sirvent, 2001). The findings are important in the consideration of hypericin concentration for any study where plants are repeatedly sampled.

Nickel contamination

Another important tissue culture study attempted to mimic the potential effects of nickel contamination in the soil. In 2003, Murch et al., found a reduced production of hypericin (21 fold) and pseudohypericin (15 fold) in response to an increase in nickel concentration from 0 to 50 mM. Hyperforin production was disabled entirely upon exposure to increased nickel concentration.

1.3.5 Greenhouse Research

Almost all plants produced for greenhouse experiments were started from a seed, then put into tissue culture and finally transferred to a greenhouse. With the exception of the study by Murch et al. (2002) that demonstrated a successful transition of in-vitro produced plantlets to greenhouse production, plants grown in greenhouses were used as a control for other studies that were conducted in a growth chamber or in the field. (See Tables 1.3 and 1.5) Plants were grown in pots with soil mix, sand:peat, or rockwool systems. No work on photoperiod or light integral has been reported. There has been no attempt to optimize either a growing system or nutrient solution composition.

Table 1.4. Summary of growing conditions and metabolite and biomass data for plants produced in tissue culture.

Authors	Year	Variable	Plant size at start	Harvest	Source	Light Intensity (umol/m²/s)	Photoperiod (hours)	Reproductive stage	Planting density (plants/m²)	Cultivar	Temperature (deg C)	Carbon Dioxide (ppm)
Sirvent and Gibson	2002	Biotic and chemical challenge	apical meristem	7	unknown	234	16	vegetative	unknown	New Stem	25	unknown
Kirakosyan et al.	2003	Country	n/a	n/a	fluorescent	40	unknown	unknown	unknown	accessions	unknown	ambient
Murch et al. [1]	2003	Nickel concentration	seeds	36	fluorescent	35	16	vegetative	unknown	New Stem	24	unknown
Pasqua et al.	2003	Stage of growth	unknown	varied	unknown	70	16	vegetative	unknown	Topas	26	unknown
Zobayed et al. [1]	2003	Elevated carbon supply	stems with 4-5 nodes	25	unknown	unknown	unknown	vegetative	unknown	New Stem	24	1500-1800 or ambient
Zobayed et al.	2004	6 types of in-vitro systems	2-3 nodes, 4-6 leaves	25 days	unknown	35	16	vegetative	25 plants/ L	New Stem	23	ambient
Zobayed and Saxena [2]	2004	Plantlets in magenta boxes	unknown	45	unknown	60	16	vegetative	66 plants/L	New Stem	23	ambient

Authors	Year	Metabolite concentrations: Hyperforin % DW	Pseudohypericin % DW	Hypericin % DW	Dry weight at harvest (grams)
Sirvent and Gibson	2002	0.035	0.050	0.007	0.001-0.005
Kirakosyan et al.	2003	15 Armenia 7 US	0.05-0.3	0.025-0.2	n/a
Murch et al. [1]	2003	0.1	0.2	0.01	n/a
Pasqua et al.	2003	2.22-7.41	0.02-0.2	0.007-0.015	n/a
Zobayed et al. [1]	2003	0.2-0.48	0.015-0.05	0.0004-0.003	5-14
Zobayed et al.	2004	0.1-0.8	0.025-0.125	0.002-0.0007	0.025-0.065
Zobayed and Saxena [2]	2004	0.300	0.0490	0.00200	0.02

A NFT system was developed for the production of tissue culture grown plantlets by Murch et al., 2002. Hypericin, pseudohypericin and hyperforin from the greenhouse grown plants were found to be produced at concentrations similar to or higher than (10% increase) values reported for field-grown plants.

Zobayed et al, 2004 grew plants in greenhouse as a control to evaluate tissue culture grown plants. The authors found three or four significant different amounts of metabolites and biomass. Compared to in-vitro grown plantlets, greenhouse grown plantlets produced the highest total biomass and the highest metabolite production for hypericin and hyperforin.

Other medicinal plant species have been grown hydroponically in a greenhouse (Dorias et al., 2000, Pedneault et al., 2002,). Four species of medicinal plants (*Taraxacum officinale, Achillea millefolium, Tanacetum parthenium, Inula helenium)* underwent such a trial and the levels of some active ingredients were found to be higher (up to 6 times) in hydroponically grown plants that were raised in a greenhouse than those raised in the field (Pedneault et al., 2002). However, some compounds were found to be at a higher concentration in the field grown plants. Since plants were not grown outside hydroponically, it must be acknowledged that it is unknown if the difference in metabolite production was an artifact of being grown hydroponically, or if it was due to environmental parameter differences found between the two growing environments (Pedneault et al., 2002).

In-vitro plantlets to greenhouse production

A protocol for producing plantlets in-vitro and then transplanting to greenhouse for production in a hydroponic nutrient film technique (NFT) system may be found in Murch et al., (2002). Furthermore, the optimum duration of supplemental lighting and relative humidity reduction was determined as part of a procedure for

acclimatization to aid the transition from fragile tissue culture plantlets to those suitable for greenhouse production (Couciero et al., 2006).

1.3.6 Cell culture

Attempts have been made at the elucidation of the pathways that produce the secondary metabolites of interest using cell suspension cultures combined with mass spectroscopy (MS) or nuclear magnetic resonance (NMR) technology (Adam et al., 2002). Phenolic compound induction by fungal spores has also been investigated to examine the importance of xanthones as a component of *H. perforatum's* defense mechanism against biotic stress (Conceicao et al., 2005).

1.4 QUANTIFICATION METHODS

The above experiments on environmental parameter and cultivar and pathogen effects attempt to quantify some metabolite(s). The development of a rapid and reliable method to accomplish this task has proven to be complicated, resulting in a plethora of different protocols that will be summarized here.

The general procedure for isolating secondary products from St. John's wort includes the following steps: harvesting the biomass, drying or freezing immediately upon harvest, breaking the material into smaller pieces, extracting the metabolites with a solvent, filtering the supernatant and, finally, using Thin Layer Chromatography (TLC) or High Pressure Liquid Chromatography (HPLC) or Ultra Violet Spectroscopy (UV-spec) to quantify and, sometimes further separate the metabolite.

Table 1.5. Summary of growing conditions and metabolite and biomass data for plants produced in tissue culture.

Authors	Year	Variable	Plant size at start	Harvest	Source	Light Intensity (umol/m^2/s)	Photoperiod (hours)	Reproductive stage	Planting density (plants/m^2)	Cultivar	Temperature (deg C)	Carbon Dioxide (ppm)
Sirvent and Gibson	2002	Biotic and chemical challenge	apical meristem	7	unknown	234	16	vegetative	unknown	New Stem	25	unknown
Kirakosyan et al.	2003	Country	n/a	n/a	fluorescent	40	unknown	unknown	unknown	accessions	unknown	ambient
Murch et al. [1]	2003	Nickel concentration	seeds	36	fluorescent	35	16	vegetative	unknown	New Stem	24	unknown
Pasqua et al.	2003	Stage of growth	unknown	varied	unknown	70	16	vegetative	unknown	Topas	26	unknown
Zobayed et al. [1]	2003	Elevated carbon supply	stems with 4-5 nodes	25	unknown	unknown	unknown	vegetative	unknown	New Stem	24	1500-1800 or ambient
Zobayed et al.	2004	6 types of in-vitro systems	2-3 nodes, 4-6 leaves	25 days	unknown	35	16	vegetative	25 plants/ L	New Stem	23	ambient
Zobayed and Saxena [2]	2004	Plantlets in magenta boxes	unknown	45	unknown	60	16	vegetative	66 plants/L	New Stem	23	ambient

Authors	Year	Metabolite concentrations			
		Hyperforin % DW	Pseudohypericin % DW	Hypericin % DW	Dry weight at harvest (grams)
Sirvent and Gibson	2002	0.035	0.050	0.007	0.001-0.005
Kirakosyan et al.	2003	15 Armenia 7 US	0.05-0.3	0.025-0.2	n/a
Murch et al. [1]	2003	0.1	0.2	0.01	n/a
Pasqua et al.	2003	2.22-7.41	0.02-0.2	0.007-0.015	n/a
Zobayed et al. [1]	2003	0.2-0.48	0.015-0.05	0.0004-0.003	5-14
Zobayed et al.	2004	0.1-0.8	0.025-0.125	0.002-0.0007	0.025-0.065
Zobayed and Saxena [2]	2004	0.300	0.0490	0.00200	0.02

[1] Assumed FW/DW ratio was 5

[2] Assumed that the units on the graph were labelled incorrectly and should have read 'ug/gDM'

1.4.1 Freezing or drying

As mentioned above, most of the plant material produced for commercial purposes is dried either directly in the field, or in drying ovens. Sirvent (2001), investigated the optimal drying temperature and time to preserve the maximum amount of hypericin. Studies on the breakdown of metabolites due to long-term storage could not be found. It was observed that hyperforin concentration was reduced by as much as 20% after a two hour exposure to sunlight, suggesting that plant material should be dried out of the sunlight (Poutaraud et al., 2001a). With very few exceptions, plant material that is going to be quantified for the evaluation of secondary metabolites is frozen in liquid nitrogen and stored in a freezer at -80C until extraction and quantification procedures may be completed.

1.4.2 Extraction chemicals

The most popular extraction chemicals are methanol, ethanol, acetone, pressurized liquid, and sometimes dimethyl sulfoxide (DMSO). Pressurized water extraction has been also been evaluated (Mannila and Wai, 2003), but has not been adopted as a common technique. The industry uses ethanol primarily and sometimes methanol to extract metabolites, but the most popular solvent used in laboratory analysis is methanol (Liu et al., 2005). It has been demonstrated that the combination of solvent composition and extraction method can change the amount of metabolites quantified by as much as 50% for hypericins and 17% for hyperforin (Poutaraud et al., 2001). Numerous studies have evaluated these solvents both with and without direct (inside the sample) or indirect sonication (samples inside a sonicating bath). Perhaps the current best technique to enhance metabolite extraction is direct sonication

(Smelcerovic, 2006), but this requires long treatments (30 minutes per sample) of in-sample sonication and is not conducive to processing a large number of samples.

1.4.3 Chromatography and other quantification methods

The following lab techniques have been used for the quantification of the metabolites of interest (including but not limited to pseudohypericin, hypericin and hyperforin): Thin-Layer Chromotography (TLC), High Pressure Liquid Chromotography (HPLC) both normal and reverse phased, High Pressure Liquid Chromotography coupled with Mass Spectroscopy (HPLC/MS), Nuclear Magnetic Resonance spectroscopy (NMR) and ultra violet spectroscopy (UV-spec). Non-aqueous capillary electrophoresis has also been used as a quantification technique (Jensen and Hansen, 2002), but is not a common method and will not be summarized here. Currently, HPLC is the method that combines the ability to process a large number of samples in a reasonable amount of time; however, if available, the preferred method is HPLC/MS.

Thin Layer Chromatography

This was one of the first methods used to quantify extracts from St. John's wort, and the metabolite of interest at the time was generally hypericin. A less precise method that requires more preparation steps before yielding results, this method is no longer widely used and has been replaced by HPLC or HPLC/MS.

High Pressure Liquid Chromatography

Many groups have published protocols for the quantification of *H. perforatum* metabolites using HPLC and most often reverse-phased high pressure liquid chromotagraphy (de los Reyes et al., 2001; Poutaraud et al., 2001b; Sirvent and

Gibson, 2000). Both isocratic and gradient systems have been developed and are used today. The choice between which system is used seems to depend on whether the researcher is more interested in rapid run times, or sharp peaks. Detector type is generally a photodiode array, but sometimes fluorescence is used. It has been shown that electrochemical style detection is more accurate than traditional detection methods (Ruckert et al., 2006), however, most researchers do not have access to this detector type.

Liquid Chromatography-Mass Spectrometry

This technique is used for both quantitative analysis and structure revelation. It is the preferred analysis tool for the rapid identification of transformation products and may also be used for quantification (Liu et al., 2005). Nevertheless, it is an expensive piece of equipment that requires additional laboratory time and expense, and therefore it is not widely used for the large-scale quantification of plant biomass samples.

Nuclear Magnetic Resonance Spectroscopy

This technique has been used in the study of metabolite structure and also for the positive identification of metabolites (Wolfender et al., 2003). The techniques used to prepare a sample for analysis in this technique is labor intensive and necessitates a large amount of biomass (Liu et al., 2005). For this reason, it is not an appropriate method for analyzing numerous samples quickly.

Ultra-violet Spectroscopy

Ultra-violet spectroscopy is the method that is currently used by the majority of the medicinal plant industry to quantify hypericin because hypericin will turn red

when placed in solvent and then exposed to light. As mentioned above, it is fairly imprecise compared to methods such as HPLC, and may be the cause of much of the variability of the product that is on the market.

Hypericin Concentration and Leaf Gland Number

A relationship reported in the low nitrogen study in (2000) by Brisken et al. reported the correlation between the number of dark glands on the leaves and the amount of hypericin extracted and found no difference between the number of glands on leaves known to have higher hypericin concentration and those with lower hypericin concentration. This would suggest that the plant puts more hypericin into glands that are already formed and that counting the number of glands on a portion of the plant is not an accurate quantification method. The same research group observed a significant positive relationship between gland number and hypericin content the following year when observing the effect of light intensity, but did not observe the same trend when looking at the effect of nitrogen concentration and abandoned that method. Zobayed and Saxena, (2004) also quantified dark glands/leaf and found a positive correlation between the number of glands and the amount of hypericin. This potential method of metabolite quantification has been abandoned due to the availability of more precise quantification methods.

1.4.4 pH

In 2006, Fourneron and Nait-Si looked at the effect of eluent pH on the quantification of hyperforin. The two mobile phase eluents used were acetonitrile and methanol. Both chemicals were investigated with pH was adjusted to 2.5 and 7.5. Additionally, pH was investigated at 3.5 and 5.5 for methanol. They found that there

is a dramatic difference in hyperforin elution time when the pH is below 3 for both mobile phases, and attributed that result to a conversion of the hyperforin from an enol form above pH 3 and to a diketone form below that. An important finding for anyone that wishes to monitor hyperforin was made when the authors observed that higher absorption levels could be observed with acetonitrile than with methanol at all pH values.

1.4.5 Hyperforin instability

It is important to remember when attempting to quantify hyperforin that the compound is light sensitive and will break down if exposed to light after solvent has been applied. This also has implications for the use of *H. perforatum* in functional foods and powdered material in solvent-extracted capsules. Degradation of hyperforin has also been demonstrated in acidic beverages (Ang et al., 2004). Specific degradation products have been identified, and may be quantified separately if desired (Vugdelija et al., 2003). Furthermore, the lack of hyperforin in some over the counter capsules may be attributed to the processing method which includes extraction of the dried and powdered material in solvent and then the drying of the extracted solution to produce a powder that is placed into capsules and sold. In 2001a, Poutaraud et al. found that exposure to sunlight for five minutes could result in a loss of 96% of hyperforins once solvent was added to the plant material. Other studies have confirmed this loss and determined that complete conversion of hyperforin occurs after exposure to light for 12 hours at pH 7 (Liu et al., 2005). For this reason, hyperforin must be quantified in amber vials and extraction must be performed in the dark.

1.4.6 Conversion of protohypericins

As mentioned above, protohypericin and protopseudohypericin is converted to hypericin and pseudohypericin upon exposure to light. As a result, most quantification protocols require the extracted material be placed in clear glass vials and exposed to light for at least 30 minutes to allow for this conversion to take place in order to more accurately measure the total amount of hypericin and pseudohypericin produced.

1.5 OBJECTIVES

The focus of this dissertation is how light impacts some of the most economically valuable metabolites in *Hypericum perforatum*. The objectives of these studies were to evaluate the effect of light quality, quantity and daylength from seedling to flowering stages on the production of hyperforin, pseudohypericin and hypericin by *H. perforatum*. A parallel objective was to determine the time to harvest that best optimizes the production of all three metabolites on a $\mu g/m^2$ growing space basis.

1.6 EVOLUTION OF EXPERIMENTS

As stated in the previous paragraph, it was the original intention of this dissertation to determine the optimum time to harvest the plant material such that production of all metabolites was maximized on a $\mu g/m^2$ growing space basis. However, the first experiment conducted which investigated the effects of photosynthetic photon flux density (PPFD) or light intensity that is reported in Chapter 2, illuminated a curious phenomena that involved the absence of production of a significant amount of the metabolite hypericin, which required the original cascade of experiments to be modified in order to determine some probable causes for this

observation. Evaluation of ultra-violet light effects on metabolite production that may be found in chapters 3 and 4 provided some indirect answers to the question that asked, ‘Under what conditions are significant amounts of hypericin produced?’. A summary of a possible explanation to this question may be found in Chapter 6, the conclusion chapter.

1.7 DISSERTATION ORGANIZATION

This document begins with an introduction to the herbal medicine industry and literature review, followed by a chapter dedicated to each of the experiments designed to evaluate the objectives stated above including an investigation of the effects of light intensity (Chapter 2), light integral and exposure to ultra-violet light (Chapter 3), the effect of supplemental UV light (Chapter 4), and a demonstration of the effect of a long or short photoperiod on flowering and internode number. Finally, a conclusion chapter (Chapter 6) summarizes the major findings from each paper and future work is suggested. Appendices provide further details about the experiments including manipulated quantitative data.

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CHAPTER 2

Impact of a variable light intensity at a constant light integral: effects on biomass and production of secondary metabolites by *Hypericum perforatum*

2.1 Abstract

Hypericum perforatum, or St. John's wort, is currently used medicinally to treat neurological disorders, while research continues to seek practical methods to harness the plant's proven potential as an anti-cancer and anti-retroviral drug source. More than other medicinal plant preparations, bioactive components of *H. perforatum* are often found to vary by a factor of two compared to concentrations reported on labels for the prepared drug. This is a serious problem for medical researchers, physicians and consumers. Variability is attributable to environmental fluctuations to which the plants were exposed during development and growth. Growing *H. perforatum* in controlled environments, such as greenhouses or growth chambers, can remove wide variations of common variables such as temperature, insect and disease pressures, and water status. Furthermore, plants may be exposed deliberately to stressors known to elicit increases in secondary metabolite concentrations. High light intensity (also known as photosynthetic photon flux density) has been shown to increase metabolite production, for example. Daily light integral control has been shown to produce predictable, consistent biomass gain in other crops but has not been related yet to secondary metabolite production in *H. perforatum*. This project focused on production of three important secondary metabolites, hypericin, pseudohypericin and hyperforin, from plants grown in floating hydroponic systems in controlled environments at light intensities of 90, 160 and 340 µmol m^{-2} s^{-1} (at 25C, 16 hour photoperiod achieved with the use of incandescent lights) which received a constant daily light integral of approximately 5 mol m^{-2}. Plant growth data were collected and

metabolite concentrations were (determined by use of RPHPLC), from seedling stage to day 104. Hypericin and pseudohypericin concentrations did not vary significantly over time. Hyperforin concentration increased with increasing plant maturity and the 90 µmol/m^{-2}/s treatment was consistently higher than the other two light intensities. An exponential model for biomass estimation per square meter of growing space is presented, valid between the intensities of 90 and 340 µmol m^{-2} s^{-1}, a photosynthetic period of 16 hours, 25C constant air temperature and ambient CO_2 concentration.

2.2 Introduction

Consumer interest in alternative medicines in the United States has increased during the last two decades. The increasing popularity of a 'back to nature' sentiment as detrimental side effects from prescription drugs continue to make headlines has contributed to this renewed attention. In 2004, 'Supplement Sales' as defined by the United States Food and Drug Association reached $60 billion globally and $20 billion (includes 29,900 products) in the US with an average growth rate of 4% per year since 2000. Products included under this framework include herbals, vitamins, minerals, sports nutrition supplements and diet complements (IRI, 2005). Studies of commercially available products highlight the large variability in the chemical composition of various herbal supplements; some variation may be attributed to differences in the quantity and quality of secondary metabolites obtained from raw plant material (Wurglics et al., 2001; Krochmal et al., 2004). In 2005, *Hypericum perforatum*, or St. John's wort was one of the top ten best-selling herbs in the US market with more than nine million dollars worth of retail sales (Blumenthal, 2005). Current chemical standardization of *H. perforatum* – containing products is based on the secondary metabolite hypericin, and tablets must contain a minimum of 0.3% of hypericin according to World Health Organization guidelines. Production of

secondary metabolites such as hyperforin and hypericin is highly variable in *H. perforatum* (Wurglics et al., 2001; Krochmal et al., 2004). Some of this inconsistency may be removed through an ability to optimize production parameters for maximal expression of these compounds. However, an improved knowledge of plant response to environmental variables is necessary for optimization of production in controlled environments. Environmental variables that have been shown to have a significant effect on hypericin and hyperforin levels include light intensity (Briskin et al., 2001; Mosaleeyanon et al., 2005), temperature (Couceiro et al., 2006; Zobayed et al., 2005), carbon dioxide concentration (Mosaleeyanon et al., 2005), nutrient availability (Briskin et al., 2001) and water availability (Zobayed et al., 2007).

The daily light integral, also occasionally termed the "light sum", is defined as the number of photons received during one day, per unit area of plant growing area, where the photons are characterized by wavelengths within the region of the light spectrum effective for photosynthesis (400 to 700 nm). Daily light integral has been shown to be a key factor in producing consistent and predictable foliar biomass in plant production systems (Both et al., 1997; Albright et al., 2000). Current studies which address the impact of light intensity (also known as photosynthetic photon flux density or PPFD) on secondary product production may inadvertently vary light integrals. No information regarding the impact of light integral during vegetative growth stages on secondary metabolite production and expression was found in the extant research literature. A review of current literature shows that in growth chamber studies where light integral was held constant some other variable was also altered; therefore the effect of light integral cannot be separated from other confounding parameters. Thus, the objective of the present study was to evaluate the effect of varying light intensities while maintaining a consistent light integral to determine whether the previously observed increase in secondary metabolite production in *H.*

perforatum with increasing light intensity was in fact, associated with an increased daily light integral.

2.3 Materials and Methods

Plant production system

A single 2.5 by 3.6 m with ceiling height of 2.1m growth chamber (Environmental Growth Chambers, 1965) was used for seedling production. Three identical growth chambers were used once seedlings were placed into experimental conditions. *Hypericum perforatum* L. CV New Stem, (Richter's Herbs, Goodwood ON) was triple seeded into rockwool cubes (center hole filled with sifted peatlite) and thinned to one plant per cube three weeks after seeding, selecting for crop uniformity. Rockwool cubes (1.25 cm) were transplanted 30 days after seeding into 25 mm thick blue polystyrene floats at a plant density of 206 plants m^{-2}. Silver reflective barriers were placed around groups of nine plants to reduce edge effects (Appendix F). Each growth chamber contained three 265 L ponds and represented one lighting condition. The nutrient solution utilized for these experiments was half strength Sonneveld solution prepared with reverse osmosis water adjusted to pH between 5 and 7 with potassium hydroxide and nitric acid (Sonneveld and Straver, 1994). Electrical conductivity was maintained at 1200 $\mu S\ cm^{-1}$ +/- 100 µS. Air was supplied to the nutrient solution with aquarium air pumps and air stones. Carbon dioxide and relative humidity were not controlled, but were determined to be approximately 400 ppm CO_2 and 50-55% relative humidity for the duration of the experiment. Air temperature was 25 C +/- 0.5 C. Temperature of the nutrient solution was 24 C +/- 1 C.

Light quantity

The photosynthetic period varied by light integral treatment and the lighting source was fluorescent lamps (Sylvania, CW, VHO, Danvers, MA, USA). Light treatments were 90, 160 and 340 µmol m^{-2} s^{-1}at plant level for a total of 5.18, 4.61 and 4.9 mol m^{-2} d^{-1}. The photoperiod was fixed at 16 hours and provided by incandescent lamps. See Table 2.1 for a summary of lighting schedules.

Table 2.1. Hours of photosynthetic lighting by fluorescent lamps and photoperiod extension lighting by incandescent lamps.

Treatment	Hours of Fluorescent Lighting	Hours of Incandescent Lighting
90	16	0
160	8	8
340	4	12

Biomass data

Plants were harvested once per week beginning when plants were 1 cm tall (which was at 21 days). Harvests continued until 100+ days after seeding at which point it was determined that flowering was not going to occur in the near future, and the experiment was terminated. Each week, nine plants per pond were individually harvested at the soil line and plant fresh weight was recorded.

Hypericin, pseudohypericin and hyperforin quantification

Quantification of hypericin, pseudohypericin, and hyperforin was performed using a modification of Couceiro et al., 2006. Upon harvest, 10 cm from the growing tip of the main stem 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 pond, for a total of three samples per light condition. The frozen material was ground with 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 the breakdown of hyperforin. The clear vials were 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.

All 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 with a detector range of 220 to 750 nm. The mobile phases for this separation were as follows: A: 0.1% triethylammonium acetate (Calbiochem) 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 were 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 (retention times of 15, 11 and 17 minutes respectively), and the quantification of these compounds was calculated by comparison to this curve. The limit of detection for hypericin was 20 ug/gFW. Standards were purchased from Alexis Biochemicals (San Diego, California, USA).

2.4 Results and Discussion

Figure 2.1. Hypericum perforatum grown for 78 days (104 days after sowing) as affected by light intensity with all plants receiving 4.6-5.2 mol PAR/day. From left to right: low light intensity 90 µmol m^{-2} s^{-1}, medium light intensity 160 µmol m^{-2} s^{-1}, high light intensity 340 µmol m^{-2} s^{-1}. Entire bar = 10 cm.

Statistical Analysis

Four multi-level models (one for biomass and three for metabolite concentration) with light and time as fixed independent variables and pond as a random effect were developed for statistical analysis (JMP 6.0.3, SAS Institute).

Biomass accumulation

At the conclusion of the experiment, no harvested plants showed visible signs of imminent flowering, 110 d after seeding. A dramatic difference in plant size and morphology may be observed in Figure 2.1 and was dependent upon light treatment. Significant differences occurred in biomass accumulation, with biomass increasing more rapidly under lower light intensities and longer photosynthetic periods. Intuitively, the assumption could be made that increased light intensity would provide a larger opportunity for photosynthesis and biomass accrual than lower intensities. The discrepancy between that assumption and the results of this experiment may have been due to the differing photosynthetic periods which were associated with the light intensity treatments. Due to the fact that we wished to maintain uniform PAR conditions in each treatment, 340 µmol m^{-2} s^{-1} treatment was given only four hours of photosynthetic light per day and twelve hours of photoperiod control light, which created an artificially long episode in which the plants were respiring. Consequently, carbon pools may then have been too depleted to allow increased growth at increased light intensity.

An exponential equation of the form: FW = A exp (0.0649 (DAY)), with A= -0.0013 (INTENSITY) + 0.5551 and with INTENSITY limited to a range of 90 – 340 µmol m^{-2} s^{-1},was developed to predict fresh weight accumulation for light intensities between 90 and 340 µmol m^{-2} s^{-1}. The fitted growth curves are shown in Figure 2.2 and the fit of the model may be observed in Figure 2.3.

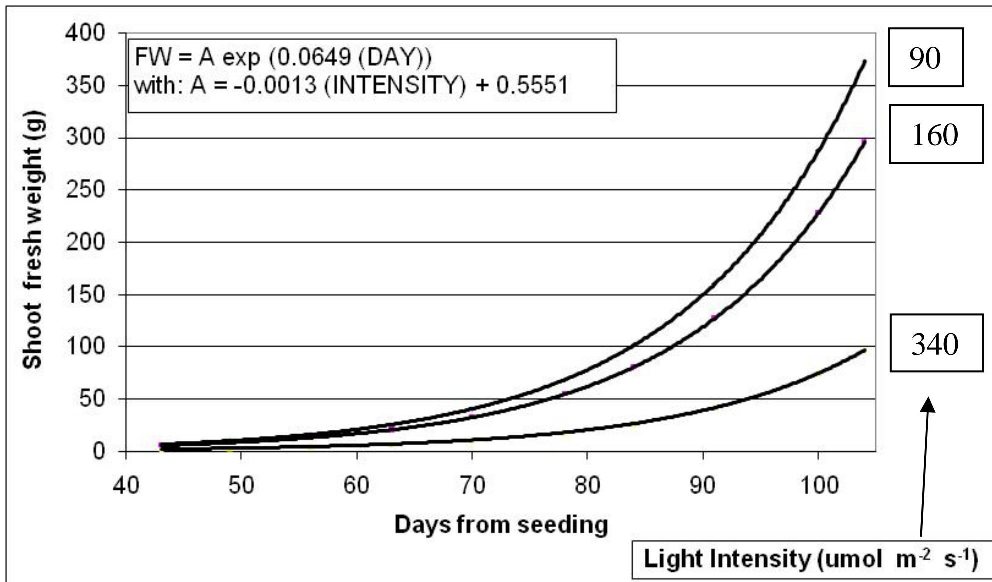

Figure 2.2. Curves based on the biomass model for light intensities investigated.

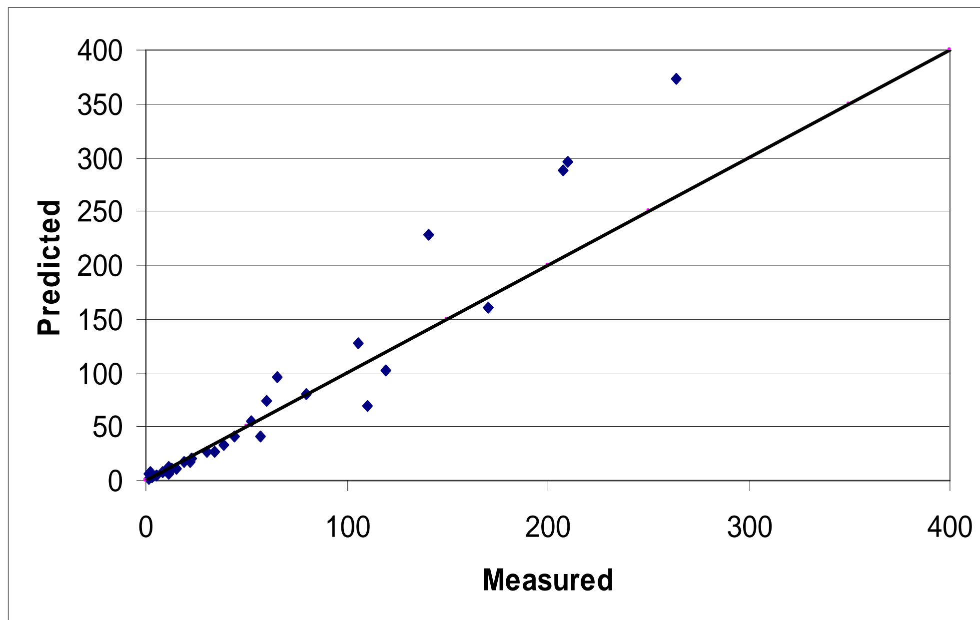

Figure 2.3. Measured vs. predicted values for the biomass model. The 1:1 line represents a perfect fit between the predicted and measured values.

Secondary metabolite production

No significant trend was observed in hypericin concentration with respect to increasing light intensity (Table 2.2). The hypericin levels in plants harvested in these experiments were so low that they may be considered insignificant. Table 2.3 shows the change in pseudohypericin concentration over time. While there were statistically significant differences in chemical concentrations among light intensity treatments for each harvest, there was no overall trend showing a steady increase or decrease of pseudohypericin over time. Hyperforin was the only metabolite that demonstrated a significant response with increasing light integral. The 90 µmol $m^{-2}s^{-1}$ treatment consistently yielded the highest hyperforin concentration, though a statistically significant difference in chemical concentration between harvests did not occur until 104 days from seeding (Table 2.4).

Our findings regarding secondary metabolite production do not agree with the previous work with *H. perforatum* reported by Briskin et al., 2001, where it was found that increasing light intensity over the range of 100-400 µmol m^{-2} s^{-1} resulted in increased concentrations of hypericin and pseudohypericin. Our results also did not concur with the results obtained by Mosaleeyanon et al., 2005, where increased light intensity between 100 and 600 µmol m^{-2} s^{-1} PAR was evaluated along with increasing carbon dioxide concentration and a resulting increase was observed in secondary metabolite production. Because total light integral was not controlled for in those experiments, and the photosynthetic period was held constant for all treatments, the results presented here suggest the increased light integral (a fourfold increase between the low and high intensity treatments) may have accounted for the increased metabolite production. However, in our investigation, we saw no significant impact of light intensity on secondary metabolites other than the hyperforin in this species.

Table 2.2. Hypericin concentration over time. Means represent 18 or 27 plants sampled and pooled into two or three samples for chemical analysis. ND denotes below the detection limit. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

	Chemical concentration from each harvest (µg/gFW)			
	Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [A]	ND ± 0	10 ± 20	ND ± 0	20 ± 40
160 umol m^{-2} s^{-1} [B]	20 ± 30	ND ± 0	ND ± 0	60 ± 10
340 umol m^{-2} s^{-1} [C]	ND ± 0	ND ± 0	ND ± 0	60 ± 10

Table 2.3. Pseudohypericin concentration over time. Means represent 18 or 27 plants sampled and pooled into two or three samples for chemical analysis. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

	Chemical concentration from each harvest (µg/gFW)			
	Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [B]	491 ± 284	462 ± 115	829 ± 156	645 ± 151
160 umol m^{-2} s^{-1} [A]	863 ± 363	746 ± 36	658 ± 241	1300 ± 72
340 umol m^{-2} s^{-1} [AB]	713 ± 266	969 ± 214	323 ± 225	1240 ± 160

Table 2.4. Hyperforin concentration over time. Means represent 18 or 27 plants sampled and pooled into two or three samples for chemical analysis. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

	Chemical concentration from each harvest (µg/gFW)			
	Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [A]	1014 ± 119	991 ± 186	1015 ± 125	1289 ± 207
160 umol m^{-2} s^{-1} [A]	649 ± 11	562 ± 129	868 ± 92	1360 ± 257
340 umol m^{-2} s^{-1} [A]	574 ± 262	225 ± 317	455 ± 115	816 ± 34

2.5 Conclusions

Biomass accumulation in *H. perforatum* increased significantly with decreasing light intensity. An exponential equation of the form: FW = A exp (0.0649 (DAY)) provided a reasonable estimate of fresh weight accumulation per square meter when A= -0.0013 (INTENSITY) + 0.5551 and INTENSITY is limited to a range of 90 – 340 µmol m^{-2} s^{-1}. This equation may be combined with the chemical concentration data in Figures 2.4 – 2.6 and used to estimate potential metabolite production per square meter within the light intensity and elapsed time parameters of the model. Since all three metabolites failed to show significant changes in concentration over time, a more general model could not be created to optimize harvest date. It may be assumed from the perusal of biomass information in Figure 2.2 and chemical concentration data from Tables 2.2 – 2.4 that any operation that may be performed to increase biomass will subsequently increase the yield of metabolite in the harvested biomass, and no benefit may be found by harvesting the crop before it flowers.

Hyperforin showed a weakly increasing concentration over time with the low light intensity, with 90 µmol m^{-2} s^{-1}, consistently producing the highest levels of metabolites, followed by 160 and 340 µmol m^{-2} s^{-1}. Compared to previous published data for field and controlled environment produced plants, very little hypericin could be detected in our growth chamber produced plants, and there was no difference in concentration between various light treatments imposed in these experiments. At the time of the final harvest, the plants used in this experiment were not flowering nor were any signs of imminent flowering present. It should be noted that most previously published data was collected when the plants were flowering, and a link between that reproductive stage and hypericin concentration may exist. The lack of hypericin may perhaps be explained by a lack of ultra-violet light in our growth chambers due to

selective UV filtration of the barrier between the light compartment of the growth chamber and the plants that functions to provide an opportunity for independent temperature control for the optimal operation of the fluorescent lamps. Results of further experimentation to address the impact of ultraviolet light upon plant quality and secondary product composition may be seen in Chapter 4.

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CHAPTER 3

Evaluation of light integral and light quality on biomass and some secondary metabolites of *H. perforatum*

3.1 Abstract

Hypericum perforatum, a medicinal plant used to treat depression that also has anti-retroviral and anti-cancer properties, was grown at 380 μmol m^{-2} s^{-1} light intensity and light integrals of 8, 13 and 20 mol m^{-2} d^{-1} to evaluate the effect of light integral on biomass production and secondary metabolite production from seedling to flowering stages at a constant light intensity. Additionally, the same parameters were evaluated when plants were grown exposed to UV-A, UV-A and UV-B, or not exposed to UV light. Plants were grown in a growth chamber at 25 C for the evaluation of light integral. Plants used for the evaluation of the effect of UV exposure were produced in a growth chamber (no UV exposure), a glass greenhouse (UV-A only), or in the field (UV-A and UV-B). Secondary metabolites hyperforin, pseudohypericin and hypericin were quantified from 21 to 110 days after seeding by HPLC analysis. A significant increase in biomass was demonstrated with increasing light integral. An exponential model for biomass estimation is presented on a grams per square meter basis, valid between the daily light integrals of 8.6 and 20 mol m^{-2} d^{-1}, a photosynthetic period of 16 hours, 25 C constant air temperature and ambient CO_2 concentration. Secondary metabolite production was not affected by increasing light intensity over time until the final harvest, when the 20 mol m^{-2} d^{-1} treatment was flowering, suggesting that flowering is associated with the production of the secondary metabolites investigated, and is thus more important than the total amount of light received. Interestingly, the largest biomass and highest secondary metabolite concentrations were observed in

plants that were not exposed to UV light, suggesting that the absence of UV stress allows the conversion of more primary metabolites into biomass and secondary metabolites.

3.2 Introduction

Evaluation of commercial herbal preparations has demonstrated a large variance in active ingredients with the metabolites in *H. perforatum* varying as much as fifty percent from what was reported on the label (Wurglics et al., 2001; Wang et al., 2004). Part of this quality control problem may be attributed to a lack of consistency in the plant material that is used to create the product. It has been shown that metabolite production can vary widely by a factor of 10 due to drought stress (Gray et al., 2003), geographic location (Jensen et al., 1995), temperature (Couciero et al., 2006), nitrogen concentration (Briskin et al., 2000; Briskin and Gawienowski, 2001), heavy metal exposure (Murch et al., 2003), chemical challenge (Sirvent and Gibson, 2002), and insect pressure (Sirvent, 2000).

The parameters of light intensity, quantity and quality have been shown in other crops to be very important for uniformity and consistency in plant production (Albright et al., 2000; Both et al., 1997). Experiments designed to demonstrate an increase in metabolite production with increasing light intensity (Briskin et al., 2001; Mosaleeyanon et al., 2005) inadvertently simultaneously varied light integral. In 2007, Brechner et al. demonstrated that an increase in light intensity was not related to increases in metabolite production if the integral was held constant. Daily light integral describes the total quantity of daily energy received that is in the range useful for photosynthesis, generally defined as 400-700 nm. No research relating daily light integral to metabolite concentration could be found in the extant literature.

Controlled Environment Agriculture (CEA) is an ideal system in which to attempt to produce medicinal plants of higher quality and consistency because of the ability to manipulate environmental parameters such as light quality and quantity, temperature, humidity, carbon dioxide concentration and plant nutrition (Both, 1995). Thus, the objectives of this study were to evaluate the effect of light integral and light quality on the biomass production and metabolite concentration over time. The first objective was accomplished by varying light intensities while maintaining a consistent light integral to determine whether the previously observed increase in secondary metabolite production in *H. perforatum* with increasing light intensity was, in fact, an association with an increased daily light integral. Finally, a subset of light quality (involving UV primarily) was analyzed by growing plants in a growth chamber under fluorescent light, in a glass greenhouse, or outside under ambient light. An ultimate goal is to optimize growing conditions and growth stage at harvest to maximize metabolite production per area of growing space.

3.3 Methods and materials

Seedling production

A single 2.5 by 3.6 m with ceiling height of 2.1m growth chamber (Environmental Growth Chambers, 1965) was used for seedling production. Three identical growth chambers were used once seedlings were placed into experimental conditions. *Hypericum perforatum* L CV New Stem, (Richter's Herbs, Goodwood ON) was triple seeded into 1.25 cm rockwool cubes (center hole filled with sifted peatlite) and thinned to one plant per cube three weeks after seeding, selecting for crop uniformity.

Watering practices

The nutrient solution utilized for the light quantity experiments was half strength Soneveld solution (Sonneveld and Straver, 1994) prepared with reverse osmosis water (EC = 2 $\mu S\ cm^{-1}$) adjusted to pH between 5 and 7 with potassium hydroxide and nitric acid. Electrical conductivity was maintained at 1200 $\mu S\ cm^{-1}$ +/- 100 $\mu S\ cm^{-1}$. Air was supplied to the nutrient solution with aquarium air pumps and air stones. Carbon dioxide and relative humidity were not controlled, but were approximately 400 ppm CO_2 and 50-55% relative humidity for the duration of the experiment. Air temperature was 25 C +/- 0.5 C. Temperature of the nutrient solution was 24 C +/- 1 C.

Plants in the light quality experiment received enough water (Reverse osmosis EC = 2 $\mu S\ m^{-1}$) for the excess to drain from the bottom of the pot, or diluted fertilizer once a week with Peters 21-5-20 (Scott's Horticultural Products, Inc., Marysville, CA) diluted to package recommendations for weekly fertilization.

Experimental plant production

For the light integral experiment, rockwool cubes were transplanted 30 days after seeding into 25 mm thick blue polystyrene floats at a plant density of 206 plants m^{-2}. Silver reflective barriers were placed around groups of nine plants to reduce edge effects (see photo, Appendix F).

The photosynthetic period varied by light integral treatment and the lighting source was fluorescent lamps (Sylvania, CW, VHO). The photoperiod was fixed at 16

hours and provided by incandescent lamps. The three light integrals were 8.6, 13 and 20 mol m^{-2} d^{-1}. The instantaneous light levels averaged 380 μmol m^{-2} s^{-1} PAR.

For the light quality experiment, seedlings were produced as above, and then Rockwool cubes were transplanted 30 days after seeding into 15.25 cm containers filled with MetroMix 360 (Scott's Horticultural Products, Inc., Marysville, CA) and surrounded by metal foil to reduce increases in root zone temperature such that root zone temperatures were not higher than air temperatures. The planting density of the plants in both the greenhouse for the UV-A light only condition and field locations for the UV-A and UV-B condition was 36 plants m^{-2}.

The light quality experiment involved plants grown in growth chambers under fluorescent lamps (Sylvania, CW, VHO) with acetate covers providing an environment free of UV, in a greenhouse with ambient light and shade sprayed on the glass for a condition with UV-A light only, and outside with ambient light providing both UV-A and UV-B. Plants were outside starting June (22) 2007 until September (9) 2007. The value of the average daily light integral for plants grown with exposure to both UV-A and UV-B was approximately 40 mol m^{-2} d^{-1} and the daily integral may be seen in Figure 3.1.

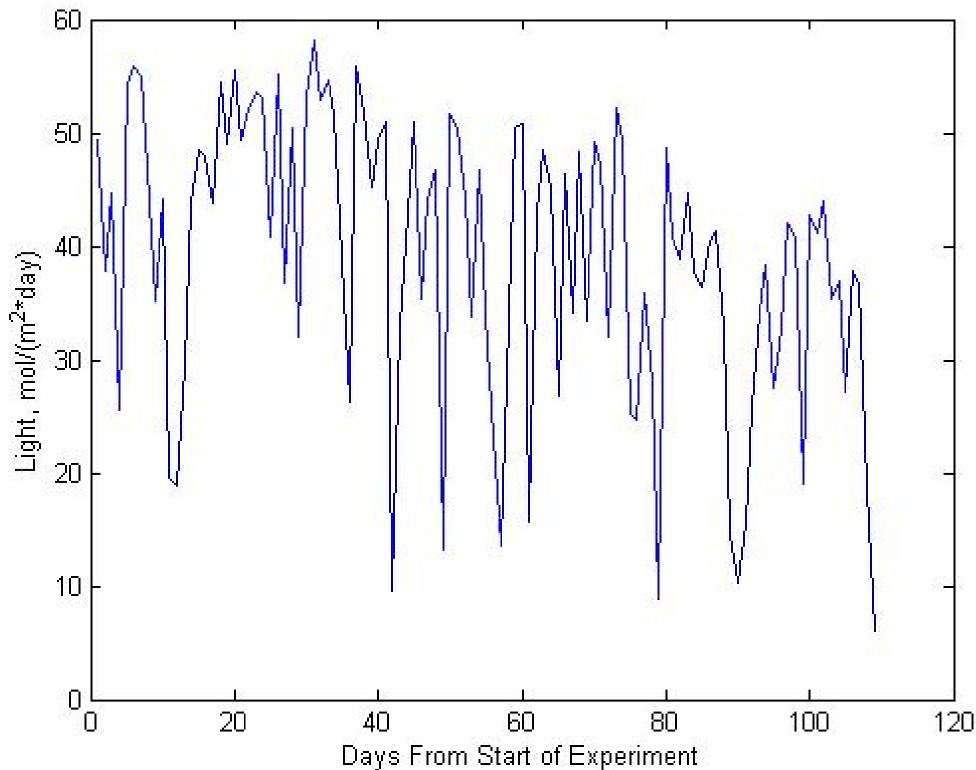

Figure 3.1. Daily light integral from when plants were moved outside to begin exposure to ultra-violet light for plants grown with exposure to both UV-A and UV-B.

Biomass data

For the integral experiment, plants were harvested once per week beginning when plants were 1 cm tall (which was at 21 days from seeding). Nine plants per pond were individually harvested at the soil line and plant fresh weights were recorded. Harvests continued until the plants were 77 days old for the integral experiment. These were times when it was determined that the plants were not going to flower and when the plants could no longer be effectively separated. For the light quality experiment, five plants were harvested every two weeks starting when plants were 21 days old and ending when two of the three treatments reached the flowering

stage where most of the buds were fully open. At each harvest, individual plants were sampled 1 cm from the soil line and both fresh and dry weights obtained.

Hypericin, pseudohypericin and hyperforin quantification

Quantification of hypericin, pseudohypericin, and hyperforin was performed using a modification of Couceiro et al., 2006. See Chapter 2 for details.

3.4 Results and Discussion

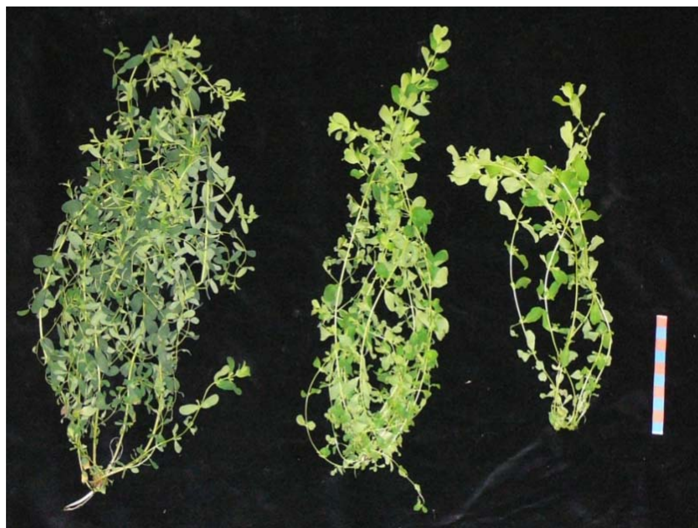

Figure 3.2. Hypericum perforatum grown for 89 days (110 days after seeding) in a deep water hydroponic system in a growth chamber as affected by light integral, with all plants receiving 380 µmol m^{-2} s^{-1}. From left to right 20 mol m^{-2} d^{-1}, 13 mol m^{-2} d^{-1} and 8.6 mol m^{-2} d^{-1}. Entire bar = 10 cm.

Statistical analysis

Four multi-level models (one for biomass and three for metabolite concentration) with light treatment and time as fixed independent variables were developed for statistical analysis. For each experiment, each metabolite was analyzed

for three statistically significant differences with the Tukey-Kramer honest significant difference test at $p < 0.05$ where treatment significance, harvest and treatment x harvest interactions were evaluated. Biomass was also statistically analyzed in the same way.

Biomass

The physiological difference in growth habits may be observed in Figure 3.2. An exponential equation was developed for the light integral range investigated. A polynomial of the form: FW = A exp (0.1162 (DAYS FROM GREEN)), with A= 0.0432 (INTEGRAL) and INTEGRAL refers to the daily light integral and is limited to a range of 8.6 – 20 mol m^{-2} d^{-1}. (Figure 3.3). The accuracy of the model may be assessed by observing the graphs showing measured compared to predicted values next to each biomass model graph (Figure 3.4). There were significant increases in biomass with increasing light integral. There were also significant increases in biomass for each harvest. The 20 mol d^{-1} treatment had flower buds 92 days after seeding with an average weight of 15 gFW, while the other two light integrals showed no signs of flowering at the conclusion of the experiment. This suggests that larger biomass may be linked to the induction of flowering.

Biomass production for the light quality experiment may be seen in Figure 3.5. Plants grown without exposure to UV light showed statistically consistently higher biomass than the other treatments. The treatments exposed to UV light showed no difference in biomass from each other, but were significantly lower in biomass compared to the plants grown without UV exposure. Large divergences in biomass accumulation began after approximately 1.5 months of growth (84 days from seeding) under the treatment regimes. At the conclusion of the experiment, plants grown without UV exhibited a 2-fold increase (250 gFW) in biomass compared to the other

treatments (average 100gFW). Biomass production increased significantly with each successive harvest for all treatments. Flower buds were observed 60 days after seeding in the treatment that was not exposed to UV, when plant weight averaged 29 gFW. The vast difference in days to flowering between the light integral and light quality experiments is most likely related to the fact that the plant densities were different between the two experiments. The light integral density was 206 plants m^{-2} while the density in light quality experiments was 16 plants m^{-2}. The difference in plant densities and, thus, in light received per plant, may account for the difference in biomass production and total biomass production is likely to be linked to the induction of flowering.

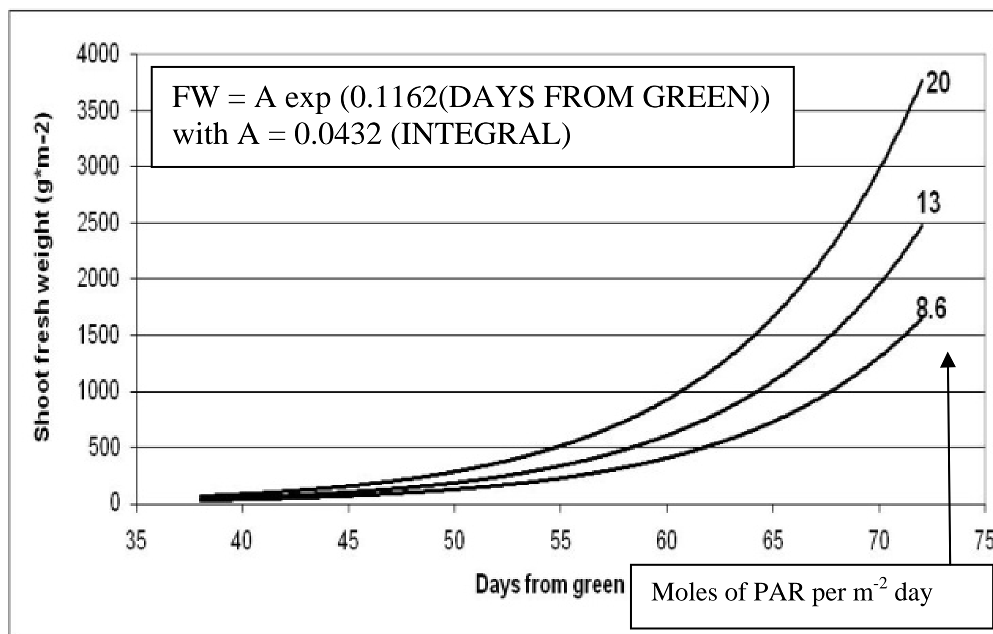

Figure 3.3. Biomass model for plants grown under varying daily light integrals from 8.6 to 20 mol d^{-1} for 61 days.

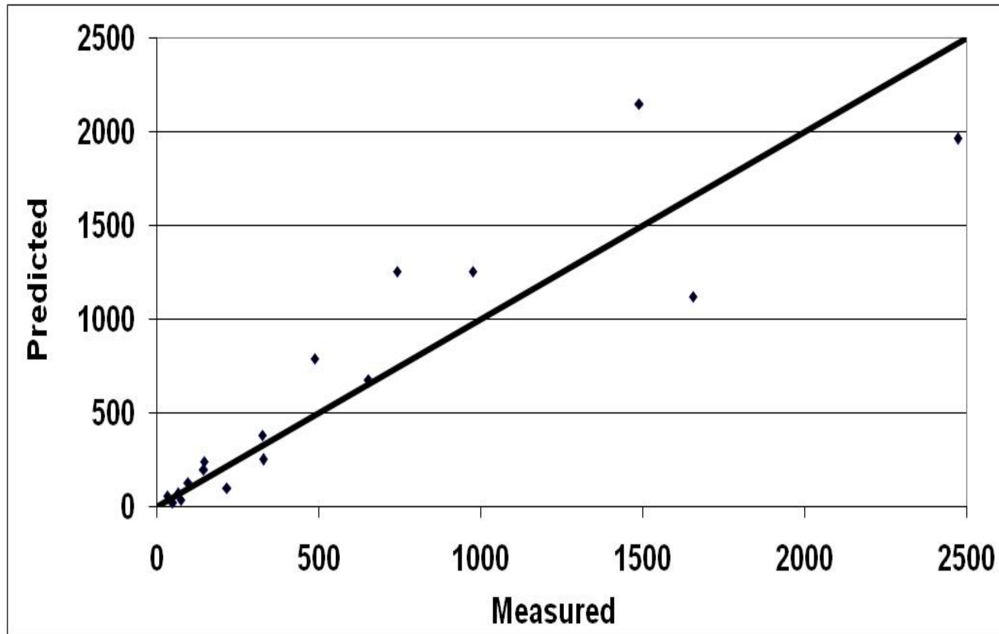

Figure 3.4. Actual vs. predicted values for the biomass model. The straight line represents a perfect fit between the predicted and measured values.

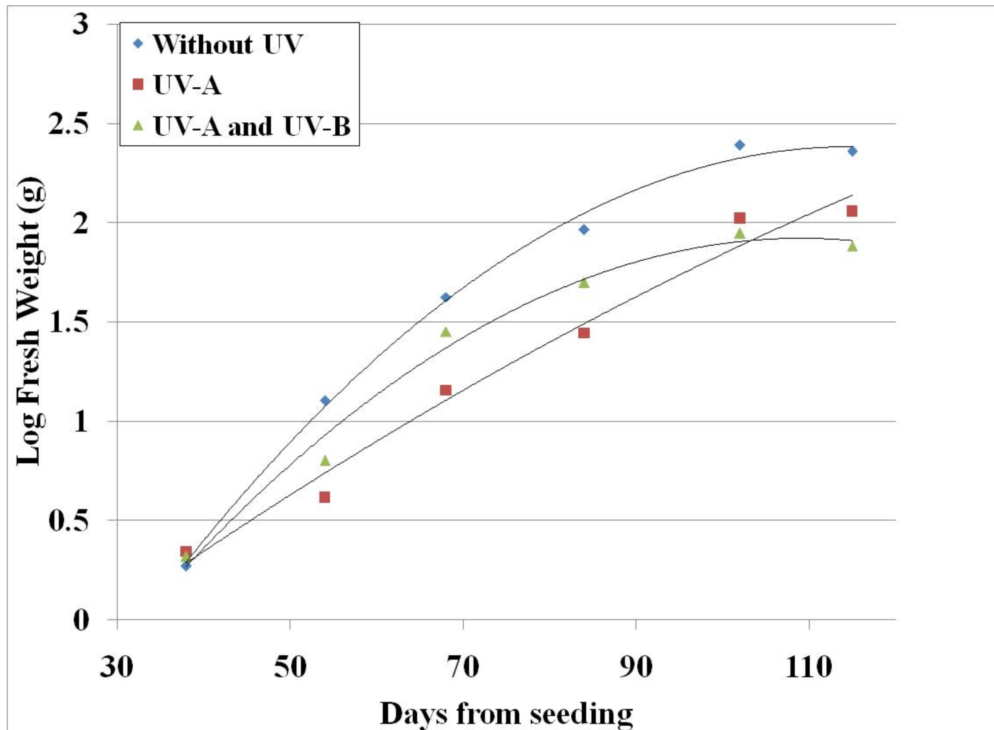

Figure 3.5. Log transformed average biomass fitted with second order polynomial lines for plants grown with various UV exposures.

Hyperforin

The hyperforin concentration for the light integral experiment averaged 1700 µg/gFW and demonstrated a slightly higher concentration with increasing light integral, but this was not a statistically significant difference. There was no difference in hypericin concentration among harvests (Figure 3.6).

Plants grown with no UV exposure showed significantly higher hyperforin values than those exposed to UV-A only, or UV-A and UV-B together, the latter two not being statistically different. The highest hyperforin concentration was in the final harvest of plants not exposed to UV and was 12,000 µg/gFW, interestingly, the final value was nearly three-fold greater than the initial concentration of 4,000 µg/gFW. The lowest hyperforin concentration was in plants exposed to UV-A with an average of 600 µg/gFW. At the conclusion of the experiment, plants produced outdoors where exposure to UV-A and UV-B is greater showed concentrations of hyperforin approaching concentrations achieved by the early harvests of plants grown with no UV exposure (4,600 µg/gFW), and may be viewed in Figure 3.7.

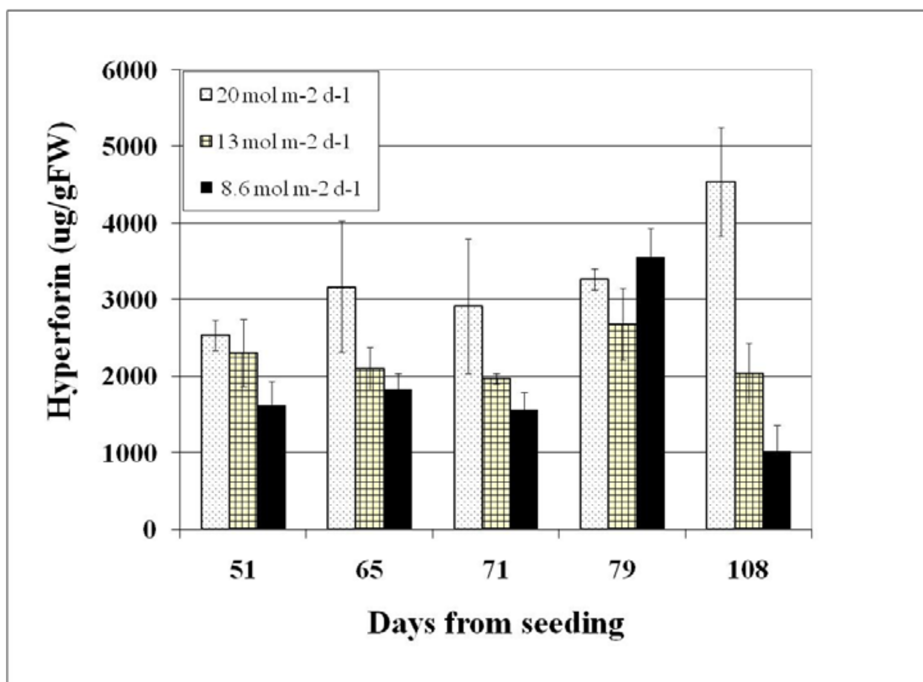

Figure 3.6. Hyperforin concentration over time light integrals 8.6, 13 and 20 mol m^{-2} d^{-1}. Means represent 27 plants sampled and pooled into three samples for chemical analysis. Bars represent +/- standard error.

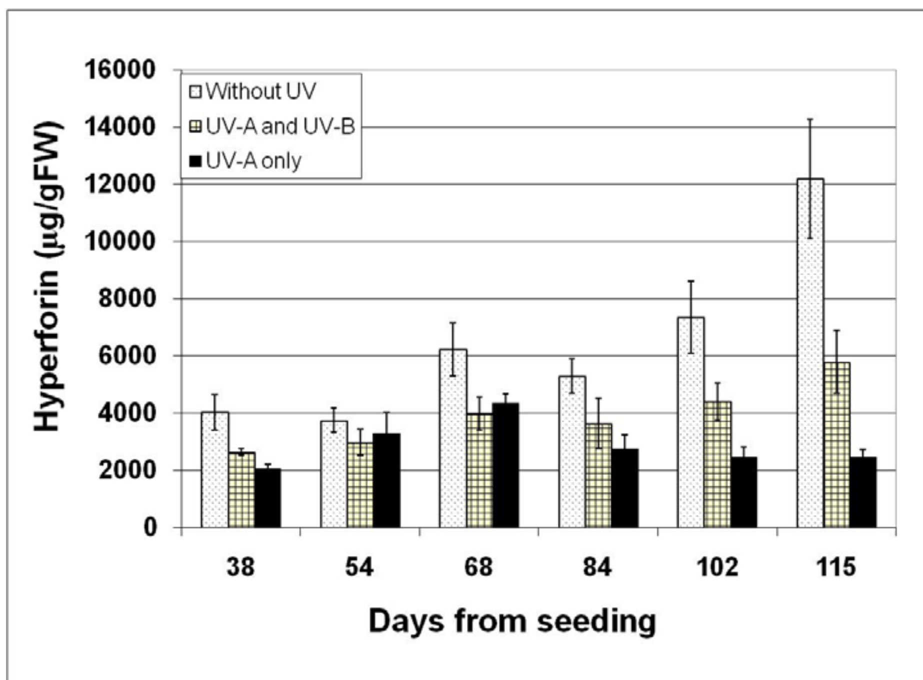

Figure 3.7. Hyperforin concentration over time for plants exposed to UV-A, UV-A and UV-B and No UV. Means represent five plants. Bars represent +/- standard error.

Pseudohypercin

For the light quantity experiment, the only significantly different harvest and treatment was the highest integral, 20 mol m^{-2} d^{-1} at the final harvest, the only one with flower buds, with a value of 2,800 µg/gFW (Figure 3.8). The average concentration for all other treatments and harvests was 1,000 µg/gFW.

Pseudohypericin concentration for the UV exposure experiment (Figure 3.9) followed a similar trend as hyperforin for the same experiment with the plants grown without UV, demonstrating 2 to 3-fold higher concentrations in comparison to plants grown with UV-A and UV-B or those with UV-A only. Plant growth in the absence of UV demonstrated the statistically highest concentration of pseudohypericin at the final harvest, 3,200 µg/gFW, although the final three harvests were similar. The difference in pseudohypericin production for plants grown without UV exposure might be due to the more stable environment experienced by these plants. Lack of insect pressure and reduced transpiration due to reduced wind stress might have resulted in plants grown without allotting greater resources towards biomass production and reduced resources into secondary product production including lignin for support and anthocyanins for protection against UV damage. This increase in biomass may also have contributed to the earlier flowering observed in this treatment. Flowering is known to be linked to an increase in pseudohypericin production.

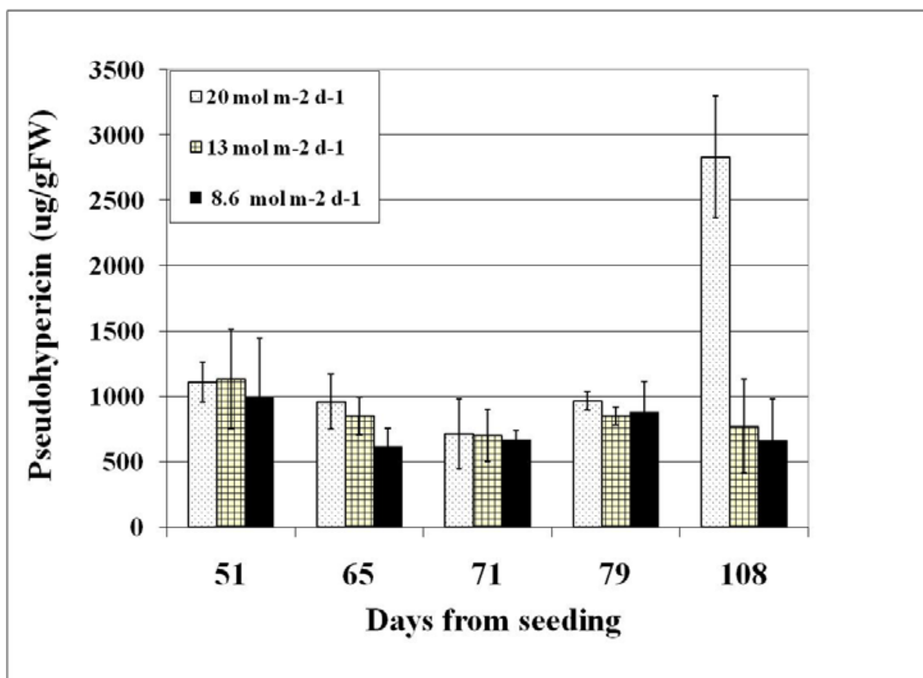

Figure 3.8. Pseudohypericin concentration over time for light integrals 8.6, 13 and 20 mol m^{-2} d^{-1}. Means represent 27 plants sampled and pooled into three samples for chemical analysis. Bars represent +/- standard error.

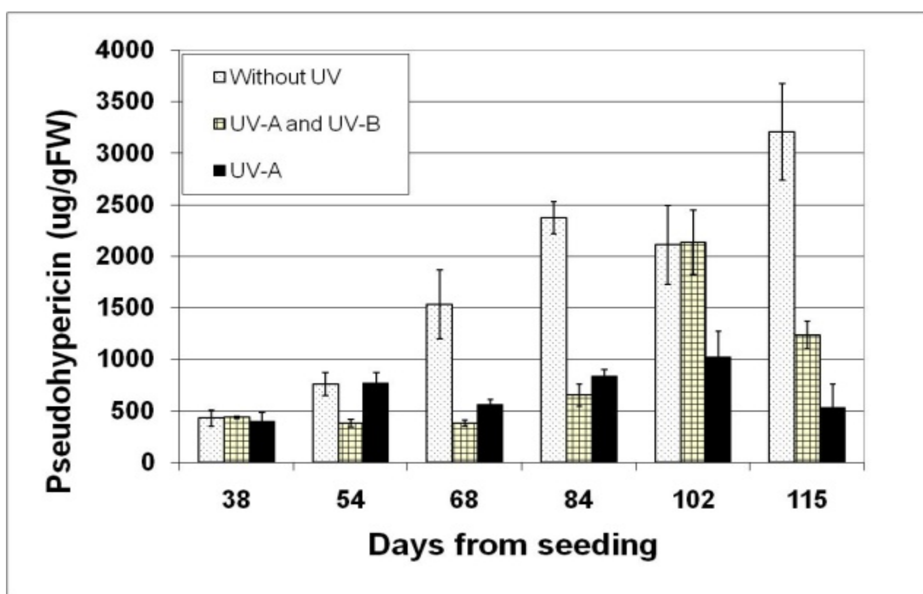

Figure 3.9. Pseudohypericin concentration over time for plants exposed to UV-A, UV-A and UV-B and No UV. Means represent five plants. Bars represent +/- standard error.

Hypericin

There was a small amount of hypericin at each harvest, but there was not a significant difference observed between harvests with the exception of the final harvest where the 20 mol $m^{-2} d^{-1}$ condition produced the highest concentration of hypericin overall with 100 µg/gFW (Figure 3.10). Flowering occurred only in the 20 mol $m^{-2} d^{-1}$ treatment and was observed only at the last harvest which occurred at 108 days after seeding. Since hypericin is associated with *H. perforatum* when plants are in bloom, and equivalent or larger concentrations were not observed before this time, it may be concluded that the possibility exists that the plant does not up regulate hypericin production until this stage of the plant's life cycle.

As with the other two metabolites, the hypericin concentration was always higher in plants not exposed to UV light than other treatments. Likewise, an increasing amount of hypericin was observed in all treatments during the final harvest, but the concentration was the again the highest for plants grown without UV exposure with an average concentration of 200 µg/gFW. Hypericin concentration in the final two harvests for the plants exposed to both UV-A and UV-B was statistically similar to the concentration of the plants not exposed to UV in harvest 4. The highest level of hypericin seen in plants exposed to UV-A only was observed in the second to last harvest at 70 µg/gFW, and was similar to the third harvest of growth chamber grown plants (Figure 3.11).

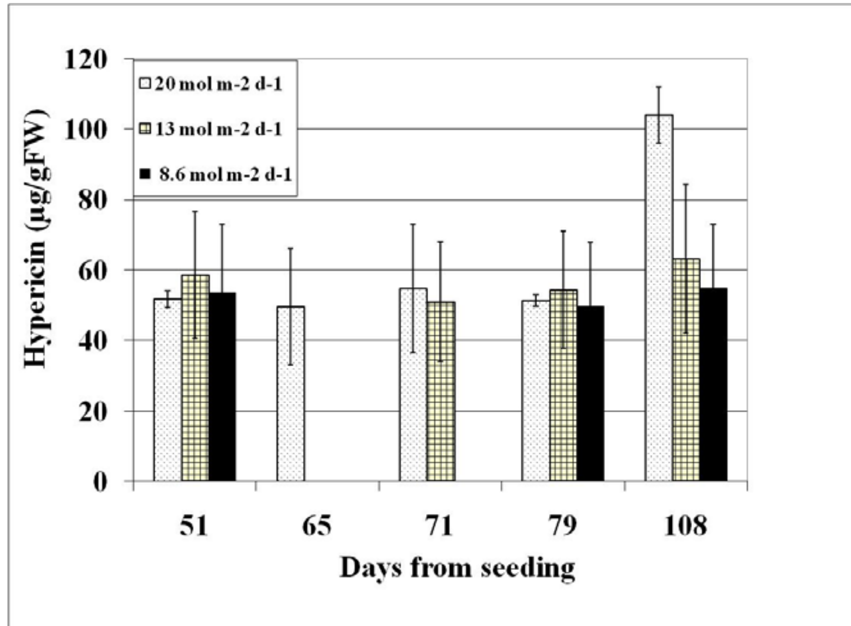

Figure 3.10. Hypericin concentration for light integrals 8.6, 13 and 20 mol m^{-2} d^{-1}. Means represent 27 plants sampled and pooled into three samples for chemical analysis. Bars represent +/- standard error.

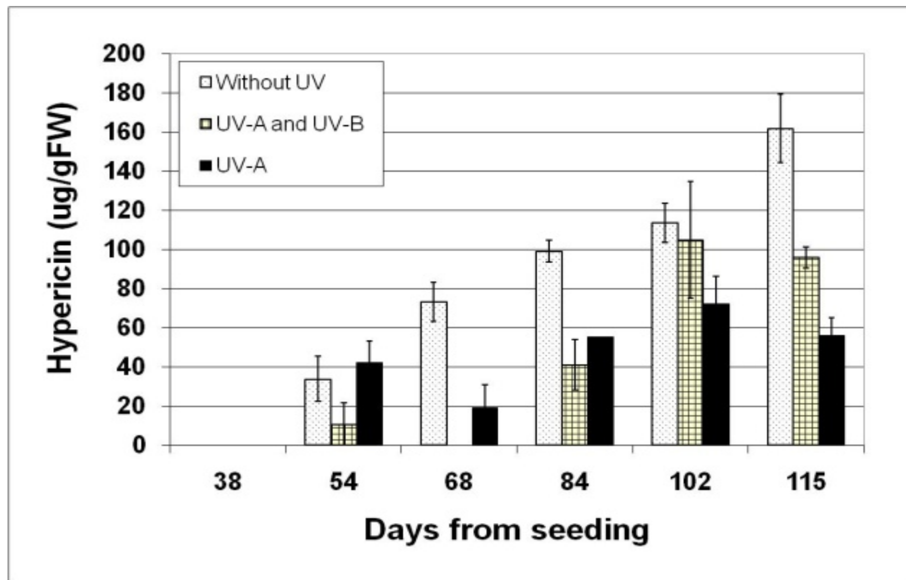

Figure 3.11. Hypericin concentration over time for plants exposed to UV-A, UV-A and UV-B and No UV. Means represent five plants. Bars represent +/- standard error.

3.5 Conclusion

Daily light integral resulted greatly increased biomass and biomass appears clearly linked to reproductive stage. *H. perforatum* does not produce significant amounts of secondary metabolites (especially hypericin) until it reaches the flowering stage, with increased biomass production leading up to this point. If biomass increases are associated with enhanced flowering, our recommendation based on the results of this study would be to consider the use of the largest integral that is practical and allow the plants to be in full flower, that is, between the points at which a minimum of ½ the buds fully open and not more than ½ the buds are in the seed pod stage. An exponential equation of the form: FW = A exp (0.1162 (DAYS FROM GREEN)) provided a reasonable estimate of fresh weight accumulation per square meter when A= 0.0432 (INTEGRAL) and INTEGRAL refers to the daily light integral and is limited to a range of 8.6 – 20 mol d^{-1}. This equation may be combined with the chemical concentration data in Figures 3.6, 3.8, and 3.10 and used to estimate potential metabolite production per square meter within the light intensity and elapsed time parameters of the model. Plants exposed to UV-A only showed a delayed biomass production, possibly due to very small daily light integrals due to heavy shade sprayed on glass. It also demonstrated lower metabolite production than the other treatments, further supporting the supposition that metabolite production may be linked to biomass production.

Daily light integral clearly does not affect metabolite production alone. Secondary metabolite production was generally not affected by increasing light integral. Harvests with significantly increased metabolite production coincided with the observation of flower buds, supporting the theory that secondary product production is increased when flowering is initiated. Since the 20 mol m^{-2} d^{-1} treatment

was the only treatment to flower and this occurred just prior to the final harvest, and it was the only treatment to demonstrate a significant difference with respect to harvest date, the supposition might be made that a greater light integral increases biomass faster and hastens flowering which increases metabolite production. Thus, the observed increase in metabolite production observed by Briskin et al. (2000), Briskin and Gawienoski (2001) and Mosaleeyanon et al. (2005) with increasing light intensity was not due to increases in light integral only as much as increased light integral hastens flowering.

An increased light integral alone is not responsible for increases in biomass, as plants grown with both UV-A and UV-B received on average over twice the amount of daily light (average 40 mol m^{-2} d^{-1}) than those grown without UV (20 mol m^{-2} d^{-1}). Lack of insect pressure and reduced transpiration due to reduced wind stress might have resulted in reduced overall stress, such that the plants grown without UV were able to put more energy into biomass production and less into components such as lignin for support and anthocyanins for protection against UV damage. This increase in biomass may have contributed to the earlier flowering observed in this condition and flowering is linked to increase metabolite production.

Metabolite production is not dependent on UV light. Since metabolite production was lower in plants exposed to both UV-A and UV-B, the presence of UV light might induce removal of some carbon resources from primary biomass production to make defensive compounds to shield plants from the damage caused by the high-energy wavelengths. Pseudohypericin and hypericin share a precursor molecule. The greater conversion of this precursor to pseudohypericin instead of hypericin in the absence of UV light may suggest that UV light plays a role in the induction of the precursor molecule to differentiate into hypericin.

Future work would investigate both larger total light integrals and higher light intensities in an attempt to determine optimum conditions before plants are saturated and suffer detrimental consequences.

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CHAPTER 4

Effects of UV-B on some secondary metabolites of *Hypericum perforatum* or St. John's wort grown in controlled environments

4.1 Abstract

The medicinal plant industry is under scrutiny because of studies finding active ingredient concentrations in products do not agree with values claimed on labels. Metabolite concentrations in herbal preparations can differ by a factor of two compared to labeled concentrations. Reasons include plants not being harvested at physiological stages conducive to producing the desired metabolites. *Hypericum perforatum*, St. John's wort, a popular herbal remedy, has this problem. This study evaluated concentration changes of three metabolites of *H. perforatum* after exposure to ultra-violet light while plants were still in a vegetative state. Treatments were performed with fifty-five day old plants grown under 400 µmol m^{-2} s^{-1} PAR for sixteen hours a day. Three ultra-violet light treatments were evaluated: a single dose, a daily dose, and an increasing daily dose. Metabolite concentrations (hyperforin, pseudohypericin and hypericin) were monitored for seven days after each treatment. A temporary three-fold hyperforin increase was observed in the single-dose experiment while hypericin production increased from zero before treatment to a concentration comparable to the beginning stages of flowering (metabolite concentrations were highest in untreated plants when they flowered). A daily dose and an increasing daily dose did not produce significant greater increases in secondary metabolites compared to single dose treatments. These results suggest 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 product harvest in continuous production in controlled environments.

4.2 Introduction

Hypericum perforatum or St. John's wort is being used currently as alternative therapy to treat many medical maladies such as depression, retroviruses and cancer. Traditional uses dating back to the Ancient Greeks include: antidepressant, anti-fungal, anti-inflammatory, antibacterial. For a review of the many beneficial effects thought to be mediated by the metabolite hyperforin, including antidepressant, anti-inflammatory, antibacterial, antitumoral, antiangiogenic effects, see Medina et al., 2006. Most of the plant's remaining beneficial uses are attributed to another metabolite called hypericin. A review of the medical uses and efficacy for the metabolite hypericin may be found in Kubin et al., 2005. Pseudohypericin is a secondary metabolite that is often quantified (eg. Courceiro et al. 2006, Gray et al. 2003, Murch et al., 2003) and although it does not have an identified pharmaceutical use, it shares a common precursor with hypericin and, in general, the concentration of the two metabolites may be found to increase in tandem.

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 contents because of environmental variability from year-to-year as well as among crop locations. Examples of variables shown to affect hypericin content include: drought stress, light intensity, heavy metal contamination of the soil, and nitrogen availability (Gray et al, 2003; Murch et al., 2003; Briskin et al., 2001).

UV light is a natural elicitor of secondary metabolite responses. Supplemental exposure to UVB light has been shown to increase the concentration of secondary metabolites in maize, basil and peanut (Gao et al., 2004; Johnson et al., 1999; Chung et al., 2001). UV-B has been shown to be the stimulus for anti-feedent properties (Cassi-Lit, 2005).

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 for plants grown in a controlled environment.

4.3 Materials and Methods

Growing conditions and experimental treatments

Hypericum perforatum L CV New Stem, (Richter's Herbs, Goodwood ON) was rinsed with reverse osmosis water) EC = 2 µS cm^{-1} to remove germination inhibitors, triple seeded into rockwool cubes (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 (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 the light intensity was increased to 400 µmol m^{-2} s^{-1} supplied by fluorescent lamps (Sylvania, CW, VHO) for the duration of the experiment. Rockwool cubes were transplanted 30 days after seeding into 15.25 cm containers filled with MetroMix 360 (Scott's Horticultural Products, Inc., Marysville, CA). The planting density of the plants in the growth chamber was 36

plants m^{-2}. Exposure to UV-B radiation from UVB-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, Utah). Single exposure periods were 10, 20, 40, 80 and 160 minutes. 3 plants per harvest were exposed. Samples were taken from each dosing condition on the following time intervals: 12, 24, 48, 96 and 108 hours. 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 seven days starting on the initial day of exposure. 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 4.1. 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 4.1. UV-B exposure periods of *H.perforatum* during the one week experiment. Treatments included a single dose, a daily repeated dose, or a progressively increasing dose.

Day/	Minutes of UV-B exposure per day for each treatment		
Treatment	Single	Repeated	Progressive
1	10,20,40,80,160	10	10
2	none	10	15
3	none	10	20
4	none	10	25
5	none	10	30
6	none	10	45
7	none	10	60
Total	dose dependent	70 min	205 min

Quantification

See Chapter 2, section 2.3 for metabolite quantification protocol.

Statistical Analysis

Six multi-level models (one for metabolite concentration for each experiment) with light treatment and time as fixed independent variables and growth chamber as a random effect were developed for statistical analysis (JMP 7, SAS Institute).

4.4 Results and Discussion

Figure 1 shows the responses of the chemicals during and after treatment with UV-B light. Note: this data is normalized such that the levels reported represent the amount of increase in chemical concentration relative to control plants harvested the same day. Untransformed chemical data for all figures in this chapter are in Appendix J in Tables J.1, J.2, J.3. Visible tissue damage was observed 12 hours after UV-B exposure in the 160 minute treatment. For exposures of 40 minutes or greater, visible tissue damage could be seen in 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

For the single dose experiment, both the dosage and the hours after treatment 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 time after treatment. There was no interaction between the dosage and the harvest, so the metabolite concentration levels followed the same pattern for all the harvests and for all dosages. This may be

visualized by observing the letters in Figure 4.1, with different letters denoting significantly different metabolite concentrations.

The highest levels of hyperforin were seen in the 40 and 80 minute single dose treatments with a maximum at 2.5 times the levels seen in control plants, intermediate concentrations were seen in the 10 and 20 minute treatments with a maximum averaging 2 times the control values, and the 160 minute treatment induced the lowest response with a maximum of 1.25 times the control values. The pattern of response by the treatments was consistent across all the harvests. The hours after treatment was significant and all harvests could be classified into 2 significant levels. The highest concentrations of hyperforin were seen in the 12, 24 and 48 hour harvests (all at the same level statistically despite the dramatic drop in values) averaging 1.5 times control values for all three harvests, and the lowest concentrations shown at the 96 and 144 hour harvests (also the same level statistically) with the average metabolite response being equal to the control values.

The highest levels of pseudohypericin (Figure 4.2) were also found 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 levels at 24, 48 and 96 hours showing values equal to the control and the lowest concentration was found at 144 h after exposure with metabolite concentrations at half the control value.

Hypericin (Figure 4.3) showed the highest increase in concentration compared to the control at 40 and 80 minutes averaging 65 times the control value, an intermediate level at 20 minutes at 40 times the control value and the lowest concentration with the 10 and 160 minute exposures averaging 20 times the control value. The time after treatment that showed the greatest metabolite concentration was

12 hours averaging 42 times the control value, with intermediate values at 24, 48 and 144 hours averaging 10 times the control value and the lowest concentrations was found at 96 hours after exposure and were equal to the control values.

In general, for the first significant block of time after the UV stress was applied, the metabolite response to the following amounts of ultra violet was observed (from most to least): 40 minutes, 80 minutes, 20 minutes and 160 or 10 minutes. A logical reason behind this particular response pattern could not be found.

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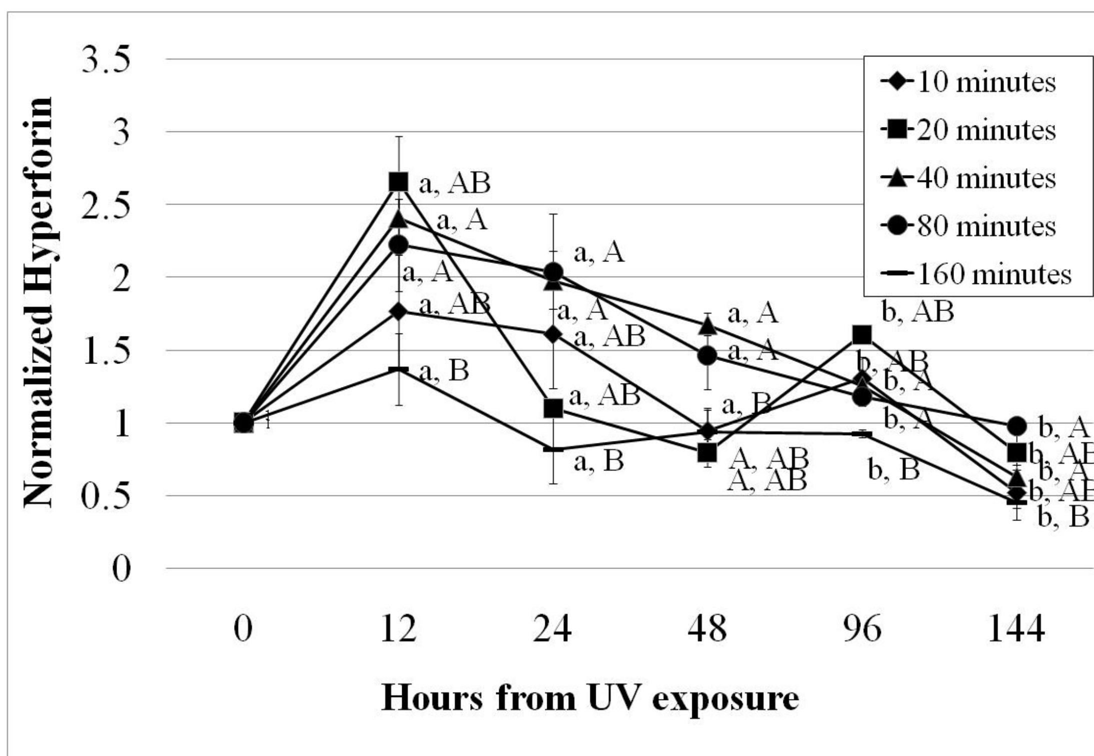

Figure 4.1. Normalized hyperforin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

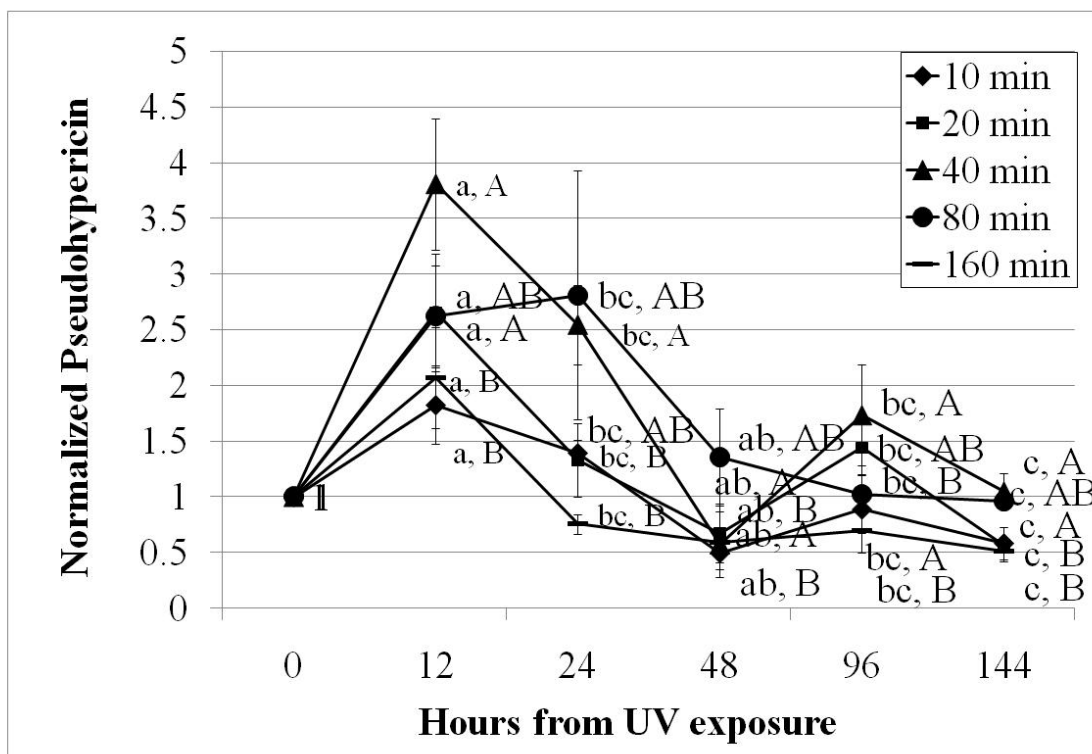

Figure 4.2. Normalized pseudohypericin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

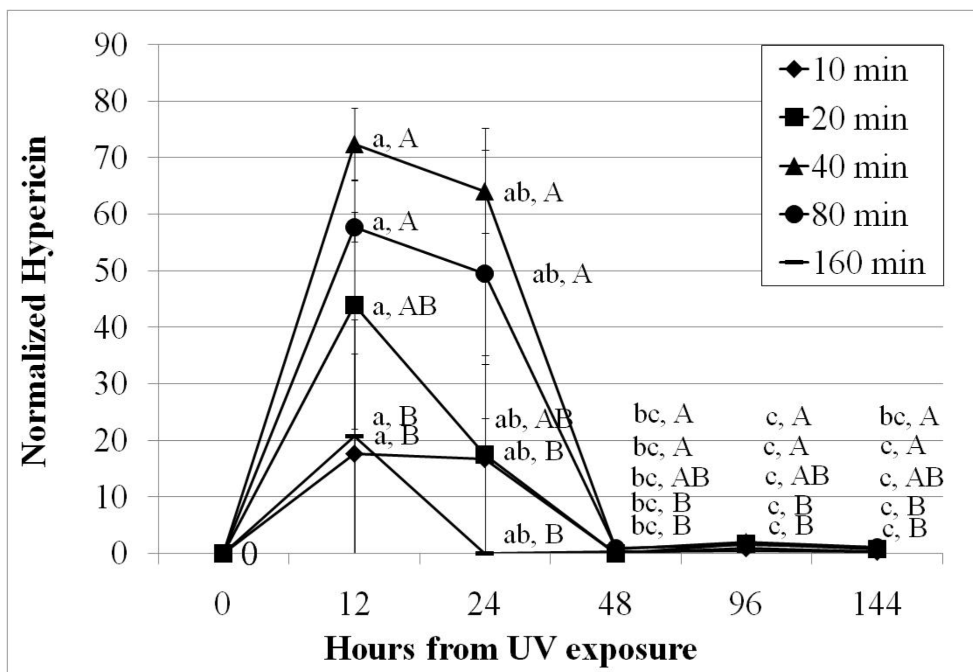

Figure 4.3. Normalized hypericin concentration over time for single dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

Repeated and Progressive Experiment

For hyperforin in the repeated and progressive model, the treatment was not significantly influenced by the UV-B treatment, however the days after treatment was. Day 4 was significantly ($p < 0.01$) higher than all other days with pseudohypericin averaging 1.5 times the control value and days 2 and 6 were significantly lower than all the other days averaging 0.6 times the control and no explanation can be made for this phenomena (Figure 4.4). There were no significant deviations from setpoints for variables such as temperature, light quality and quantity, and nutrient solution composition. Additionally, the plants did not receive insect or disease pressure at any point during this experiment. The remaining days were not different from each other,

and at a level between the previously mentioned levels. For pseudohypericin (Figure 4.5) the light quality treatment was also not significant, however the harvest day was. Highest concentrations were seen in day 4 where the values averaged 1.3 times the control, intermediate concentrations were seen in days 1,3,5,6,7 with average values equal to the control, and lowest concentrations were seen with an average value of 1.3 times the control (statistically the lowest because of the great disparity between the treatments). For hypericin (Figure 4.6), the treatment was significant ($p = 0.0001$) with the progressive treatment being at a higher concentration (averaging .14 times higher) for all the harvests than the repeated treatment. The day of harvest was not significant.

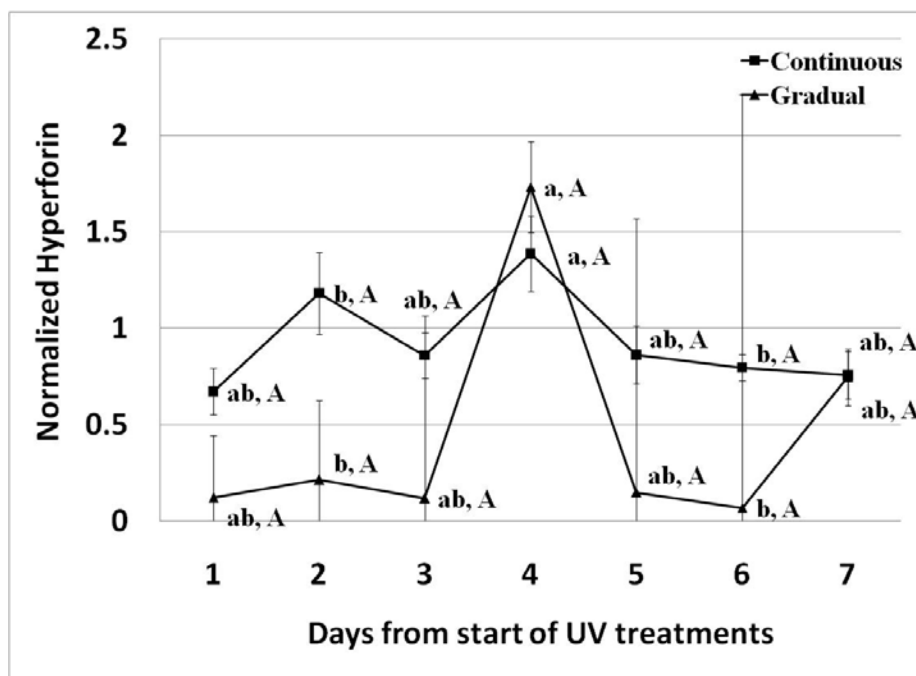

Figure 4.4. Normalized hyperforin concentration over time for repeated and progressive dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

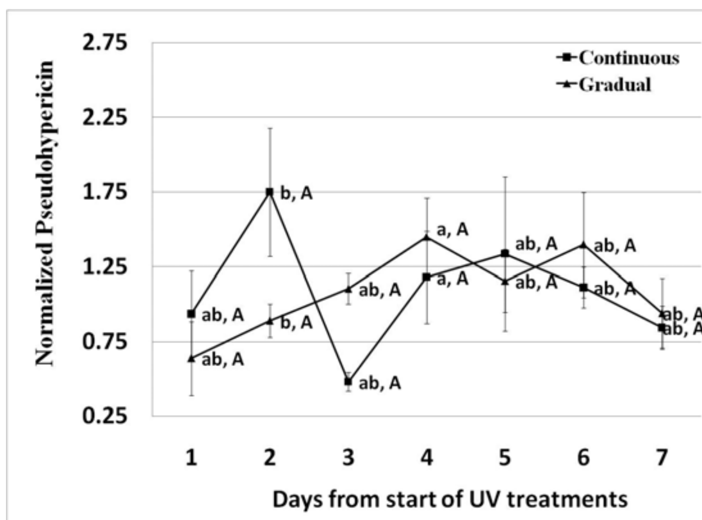

Figure 4.5. Normalized pseudohypericin concentration over time for repeated and progressive dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

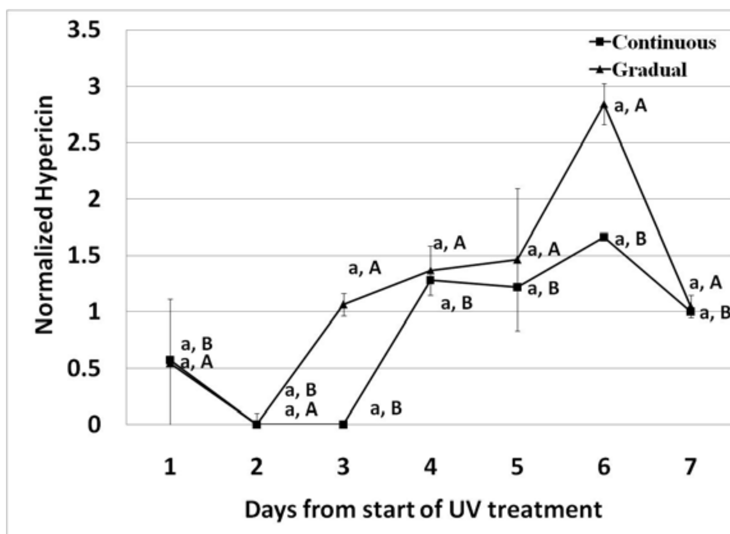

Figure 4.6. Hypericin concentration over time for repeated and progressive dose of supplemental UV-B experiment. Average values represent five plants. Bars represent +/- standard error. Different letters at each time represent significantly different values. Lower case letters represent the overall effect caused by the harvest day and upper case letters represent significantly different treatments.

4.5 Conclusions

In general, the maximum response from all the treatments was demonstrated within 24 hours after the first exposure. Given the expense of maintaining the plants for extra time and the limited additional gain associated with delaying harvest, it is recommended that the UV-B challenge be administered within 24 hours prior to harvest. The exception to this was the metabolite hypericin, where the greatest gain was 4 days after UV-B challenge. However, the final hypericin concentration, while much greater than the control plants, was not greater than the values seen in plants with most flowers open which can be up to 10 times higher than concentrations seen in this experiment.

The maximum response elicited in this experiment was shown in the single dose experiment and included a 250% increase compared to control plants. The increase in metabolite production induced by UV-B light was similar to that seen in peanuts with 200 times increase in resveratrol (Chung et al., 2001). The repeated and progressive treatments did not show significantly greater increases in metabolite concentration than the single dose treatment, and involved more labor to complete. The treatment type was only significant in the induction of increased hypericin concentration. This is the first experiment known to the authors of this study that attempted to elicit an increased metabolite response by repeating exposure to UV-B light.

For the purposes of increasing secondary metabolite production, a single dose of 40 minutes of UV-B light with harvest 12 hours after exposure is optimal. This uses the shortest amount of exposure time and the fastest time to harvest. However, it may be cautioned that compared to the natural levels of metabolites produced when

the plants are at the peak of flowering, the increases induced by the addition of UV-B light do not merit harvesting the plants before flowering (Brechner, unpublished data).

Future work should include the exploration of the response from 0-24 hours after exposure to see if 12 hours yields the greatest response. Additionally, exposing the flowering plants to UV-B to see if an increase beyond the range of the normal high flowering values can be achieved. Finally, surrounding the plant with UV exposure (top and sides) to see if this provides a greater stress and therefore a greater response would be interesting, although not as conducive to commercial production.

4.6 Acknowledgements

I would like to thank Matthew Balestrino for helping with data collection and quantification. I would also like to thank Dr. Donald Krizek for consulting on the UV-B treatment methods.

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CHAPTER 5

Demonstration of inductive photoperiod in *Hypericum perforatum*

5.1 Abstract

Hypericum perforatum was grown under short, 8 hour photoperiods and long, 16 hour photoperiods with the same light integral to determine whether flowering is controlled by photoperiod. Internode numbers were monitored to determine if they may be linked to flower induction. After 122 days from seeding, 100% of the plants under long days were flowering and no flowering was observed under short days.

5.2 Introduction

Hypericum perforatum is a perennial plant that is grown primarily for use as an alternative medicine whose uses include antidepressant, anti-viral, anti-fungal, anti-inflammatory and anti-fungal. Recent novel applications have capitalized on the anti-retroviral and anti-proliferative properties of the herb in the treatment of AIDS and cancer (Kubin et al., 2005). Sales for herbal medicines was $4,410 million in 2005 and *H. perforatum* was in the top ten best selling single herb products, showing $9 million in sales (Blumenthal et al., 2006). It is grown in fields in temperate areas and the aerial portions of the plant are harvested and dried prior to processing.

A number of experiments were controlled environments to demonstrate how environmental factors such as temperature, carbon dioxide concentration and light intensity affect secondary metabolite production. It was found that concentrations of some of the compounds could vary by as much as 30 times that of the control plants when the preceding variables were manipulated (Couciero et al., 2006; Zobayed et al.,

2005; Mosaleeyanon et al., 2005). It was reported maximal production of metabolites has been linked to the flowering stage of this plant. Therefore, it is important to know if flowering is controlled by environmental stress, physiological age, or photoperiod. Photoperiod has been demonstrated to have an effect on flowering (Evtusenko, 1939; Parker and Borthwick, 1939; Thomas, 1948). A difference in the amount of vegetative growth and time to flowering has been established (Evtusenko 1939, Parker and Borthwick, 1939). It has been demonstrated that flowering may be manipulated by controlling the day length (Thomas, 1948). Cholodny (1939) showed a relationship between flowering and internode number. The objectives of this study were to determine whether photoperiod triggers flowering and whether internode number can be related to flowering.

5.3 Materials and Methods

Hypericum perforatum L CV New Stem, (Richter's Herbs, Goodwood ON) was rinsed with reverse osmosis water (electroconductivity 2 uS) to remove germination inhibitors (Campbell, 1985), triple seeded into 1.125 cm rockwool cubes (center hole filled with sifted peatlite). The seedlings were thinned to one plant per cube three weeks after seeding, to select for crop uniformity. The seed trays were placed in 2.4 m x 3 m walk-in growth chambers (Environmental Growth Chambers, Chagrin Falls, OH). The plants received 100 µmol m^{-2} s^{-1} for 16 hours a day for two weeks then light intensity was increased to 400 µmol m^{-2} s^{-1}. The light source was fluorescent lamps (Sylvania, CW, VHO). Temperature in the growth chambers was 24 C +/- 1C. The seedlings were transplanted 30 days after seeding into 15.25 cm containers filled with MetroMix 360 (Scott's Horticultural Products, Inc., Marysville, CA).

The photoperiods studied were 8 and 16 hours. 38 day old plants were placed in special photoperiod chambers constructed in a glass greenhouse. The plants were exposed to photosynthetically active radiation (PAR) from a combination of high pressure sodium lamps and sunlight for 8 hours, after which time a black curtain enclosed the plants to shield them from further light. Plants in the 16 hour condition received an additional 8 hours of light from incandescent light. This insured both light integrals would be the same and not be a confounding factor. Each photoperiodic condition contained 20 plants. Plants were monitored frequently for flowering. At the conclusion of the experiment, the longest stem on each plant was evaluated for number of internodes.

5.4 Results

The first observation of flowering occurred in plants receiving 16 hours of light when the plants were 89 days old and were in the photoperiod treatment for 51 days. Half of the plants were flowering after 55 days and all of the plants were flowering after 84 days in the long photoperiod treatment. Plants in the shorter 8 hour photoperiod treatment did not show signs of flowering at the conclusion of the experiment 122 days from seeding and after 84 days in the photoperiod treatment (Table 5.1).

Table 5.1. Elapsed days to flowering for *Hypericum perforatum* receiving 8 or 16 hours of light at 24 C and 12 mol m^{-2} day^{-1} total light integral.

Days from seeding	Plants flowering per light condition (16 hours, 8 hours)
89	20%,0
93	50%,0
122	100%,0

Figure 5.1. Comparison of plant morphology of *H. perforatum* from 16 hour treatment (left) and 8 hour treatment (right) 93 days after seeding. The photo on the right shows a close-up of the flower buds.

There was no significant difference in internode number between treatments. The average number of internodes for the long photoperiod treatment was 29.7 +/- 2.2. Short photoperiod treatment plants had an average of 29.8 +/-2.7 internodes.

5.5 Discussion and Conclusion

Flowering in this species is linked to both photoperiod and light integral. This study showed that long photoperiods control flowering. No flowering was observed in the short day treatment. Internode number was the same in both treatments indicating little or no effect. Light integral was the same in both treatments demonstrating that flowering is not only controlled by light integral. Flowering was noted as early as 60 days after seeding when plants received an average of 20 mol m^{-2} d^{-1} for 16 hours (Brechner et al., unpublished). In contrast, 89 days after seeding (51 days in inductive photoperiod) were needed for flower buds to form for this condition when light integral averaged 12 mol m^{-2} d^{-1}. The age at which juvenile seedlings are first receptive to photoperiod and the critical photoperiod that induces flowering should be determined in future studies. This study is important because the secondary metabolites that contribute the medicinal properties of this plant are linked to flowering and thus it is important to ensure that flowering occurs when growing *H. perforatum* if concentration of active ingredients is important. In conclusion, a 16 hour photoperiod should be used for all situations where flowering is desired.

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CHAPTER 6

Conclusions

6.1 General

One of the most important observations that was determined in this series of experiments was that concentrations of the secondary metabolites of interest namely, hyperforin, pseudohypericin and hypericin, increase significantly as plants approach the flowering stage in their reproductive cycle. Harvesting plants before they reach full flower, thus, will never optimize the production of these metabolites on a microgram of metabolite per square meter of growing space basis. Neither an increase in light intensity nor light integral alone will significantly increase metabolite production. From light integral experiments, it was determined that an increase in light integral can hasten the induction of flowering. Experiments investigating the effect of UV light on metabolite production demonstrated that the lack of significant amounts of hypericin production that is observed before the plants have begun flower induction is not attributable to the absence of UV light.

It was concluded that *H. perforatum* does not produce a significant amount of hypericin before flowering induction. Optimization of metabolite production would include any treatment that shortens the time between seeding and flowering. Therefore, a recommendation to those who wish to grow this plant in a controlled environment would be to provide the largest light intensity that is possible for a minimum of 16 hours per day, which would provide the largest light integral possible and the shortest time to flowering. Plants should not be harvested before they are in full flower which may be defined as the point at which 50% of the buds in a flower

cluster are open and not more than 50% of the flowers have developed into seed pods. A concise list of the observations and conclusions from each experiment follows.

6.2 Light Intensity

- At a constant light integral of approximately 5 mol d^{-1}, increasing light intensity from 90 to 340 µmol m^{-2} s^{-1} did not increase metabolite concentration or biomass production.
- Counter intuitively, the lowest light intensity (90 µmol m^{-2} s^{-1}) was demonstrated to produce the largest biomass production possibly due to a larger photosynthetic period.
- A mathematical model for biomass production based on light intensity for a fixed light integral of 5 mol d^{-1} was developed.
- No trends in metabolite production for hypericin and pseudohypericin were indicated. The only significant modification in metabolite production was demonstrated by the 90 µmol m^{-2} s^{-1} treatment, where a significantly larger concentration was observed for all harvests, but no trend was observed between harvests.

6.3 Light Integral

- At a constant light intensity of approximately 340 µmol m^{-2} s^{-1}, increasing light integral from 8.6 to 20 mol d^{-1} was shown to significantly increased biomass production.
- Biomass production was linked to the induction of flowering. Flowering is associated with a significant increase in metabolite production. Therefore, the highest light integral that may be achieved is recommended for the rapid production of maximal metabolites per square meter.
- A mathematical model for biomass production at 340 µmol m^{-2} s^{-1} and between increasing light integrals from 8.6 to 20 mol d^{-1} was created.

- Metabolite production was not associated with increased light integral until the plants reached the flowering stage. This would suggest that increasing light integral is not economically worthwhile. However, the increased biomass production and the positive link between biomass production and progress toward the reproductive stage was demonstrated.

6.4 Light Quality

- Greatest biomass was observed in plants that were never exposed to UV, possibly due to a more stable light and temperature environment allowing for more primary production to be allotted toward secondary metabolites associated with defense from insects and disease than compounds such as lignin for support and anthocyanins for defense against UV light.
- It was demonstrated that metabolite production is not directly dependant on the presence of UV light. Furthermore, the presence of both UV-A and UV-B light may add additional stress that induces a depletion of the pool of primary metabolites and thereby decrease biomass production which lengthens the time to flowering.

6.5 Ultra-Violet B Light Supplementation

- Biomass production was not effected significantly, though it must be remembered that the longest duration of the experiments was seven days.
- Maximal metabolite increase was observed 12 hours after supplemental UV exposure and harvest at this interval was determined to be optimal.

Single dose

- It was determined that 40 and 80 minute exposure times increased the metabolite concentration the most compared to control plants. A 40 minute exposure is more practical therefore is the recommended treatment for the manipulation of metabolites.

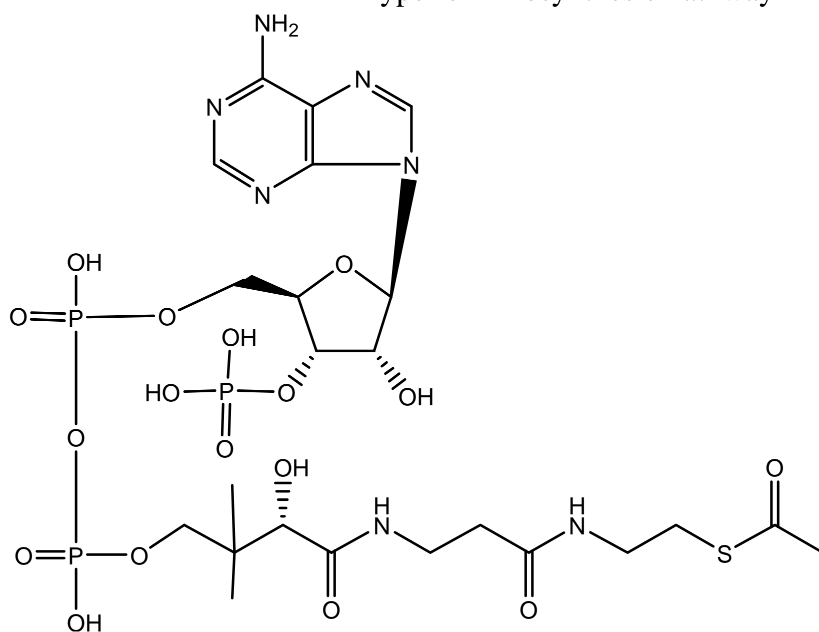
- It was established that a daily repeated or daily increasing amount of supplemental UV-B exposure did not significantly increase metabolite concentration more than a single dose and are therefore not recommended.

6.6 Photoperiod

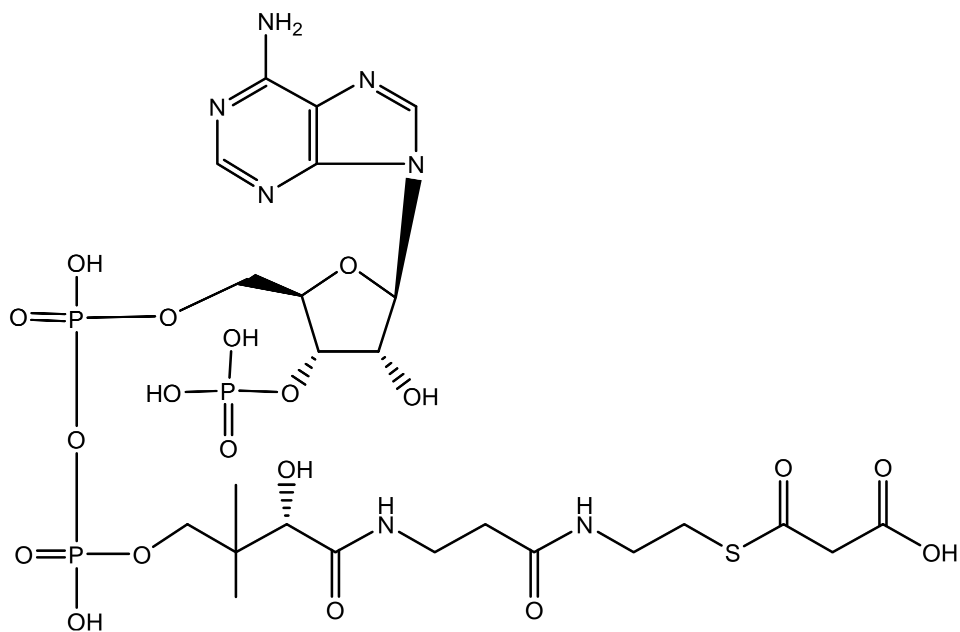
- It was verified that long photoperiods control flowering. There was no flowering with short days and the light intergral was the same as the long day treatment.
- Internodes were the same in both treatments indicating little or no relation between internode number and flowering.

APPENDIX A

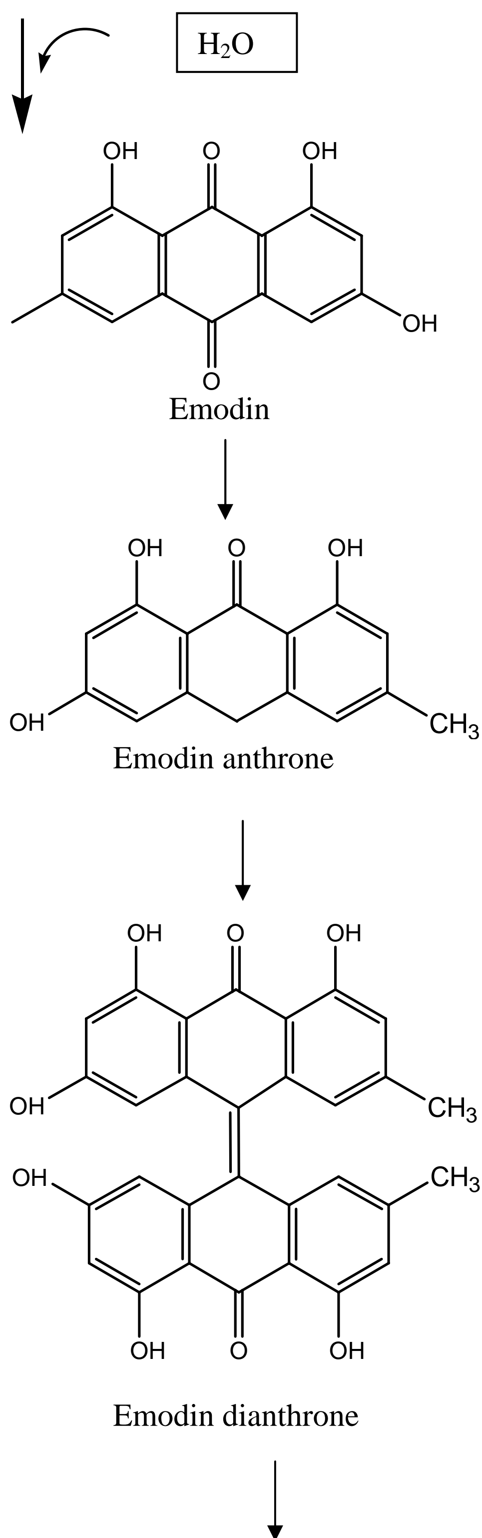
Hypericin Biosynthesis Pathway



1 Acetyl Co-A +



7 Malonyl Co-A


H2O
OH
O
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OH
O
Emodin
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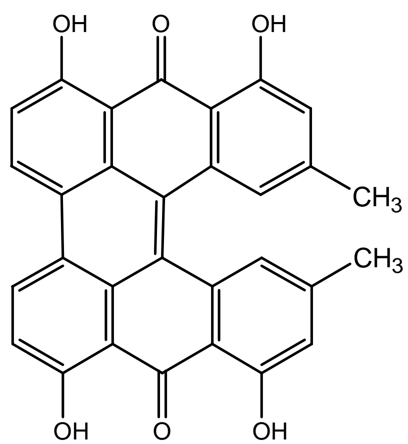
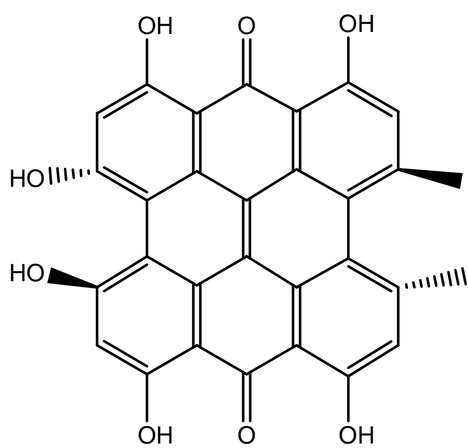


Protohypericin

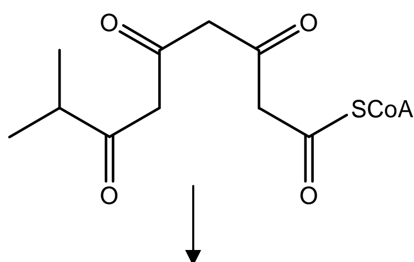


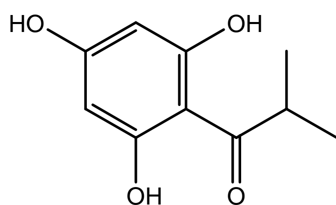
hypericin

APPENDIX B

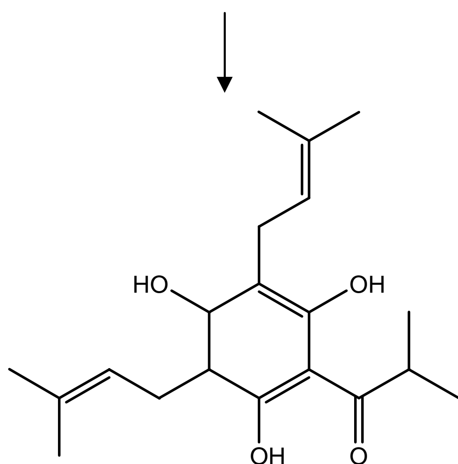
Hyperforin Biosynthesis Pathway

5 Isoprene units formed by deoxyxylulose pathway aka non-mevalonate pathway

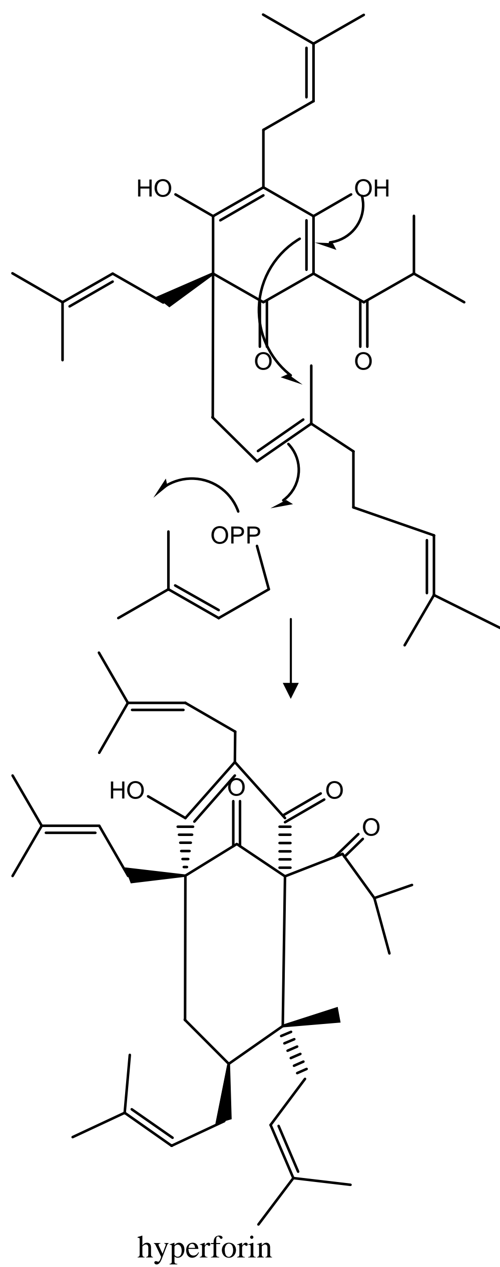




acyl phloroglucinol



dimethyallyl pyrophosphate


HO
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OPP
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hyperforin

APPENDIX C

Herbal Medicine and Western Doctors

When faced with a minor or major health crisis, an increasingly large number of Americans are turning to herbal remedies in lieu of, or in addition to chemically-derived medicines. 65% of the middle-class population and 80% of the lower-class have been found to turn to alternative medicine on a semi-regular basis (Husnu Can Baser, 2005). It is widely accepted that poor people in both developed countries and third-world countries have only herbal medicine available to them due to a lack of funds or organized healthcare.

Many medical doctors trained in the western style of medicine do not actively encourage the use of herbal remedies. They are exposed to the reports on clinical trials in prestigious journals such as the New England Journal of Medicine and have opportunities to attend continuing education workshops summarizing the clinical research findings, but many remain unconvinced that herbal remedies are more effective than the placebo effect. At a continuing education seminar produced by the Cornell Weill Medical School in 2006 entitled, "Understanding Herbal Medicine: What you need to know", the presenter, Marcus McFerren, highlighted the research findings of more than 25 major herbal medicines that aim to treat symptoms as diverse as weight loss, depression, heart health and arthritis. Most of the medicines were found to have demonstrated a negligible positive effect on the targeted condition, and generally were not better than the placebo in double-blind, placebo-controlled studies. When asked at the end of the day which of the many remedies he might recommend to a family member he replied, "As long as it has not been demonstrated to have a negative side effect, I would support any herbal someone wanted to try because the

placebo effect[9] has been demonstrated to be both real and, in some cases, just as effective as both the herbal medicine and the pharmaceutical drug."

Some doctors are so convinced that herbal remedies are not helpful that they are outspoken about the waste of resources that is involved with conducting these studies. Wallace Sampson, an emeritus professor at the Stanford Medical School and editor of Scientific Review of Medicine, wrote such a position piece in the New England Journal of Medicine in 2005. He used the example of studies done with the popular supplement Echinacea (*Echinacea angustifolia)* to demonstrate the possible wastefulness of public funds and scientific effort. Dr. Sampson cited that between the years of 1950 and 1991, over 200 clinical studies were performed on Echinacea, but many of them were not done 'properly' due to a lack of a proper number of subjects, improper randomization and blinding, lack of a control, and inadequate statistical analysis. After admonishing the National Institutes of Health and the National Center for Complimentary and Alternative Medicine for the massive amount of federally-derived money they spent supporting such studies (1.5 billion in 5.5 years), he lamented the deficiency of a 'demarcation of the absurd' or point at which it is acknowledged that further study would be unwise and waste valuable time and money. The only positive use of all of these studies, in his opinion, would be for psychologists to study the phenomenon of herbal medicine and evaluate the 'erroneous thinking and the mechanisms behind the errant social-medical trends such as the alternative medicine movement' (Sampson, 2005).

[9] The placebo effect consists of a physiological change in the body that is due pharmacologically inert dose of medicine that has no active ingredient. It is attributed to a psychosomatic trick of the brain on the body.

Other doctors display an attitude that is a hybrid of Dr. Sampson's and maintain that not either not enough research has been performed on a particular herb or a risk/benefit analysis has not been performed to support many herbal remedies.

Quite a few of the drugs that are commonly used today are derived from or modeled after metabolites found in natural products with the analgesic aspirin from willow tree bark and anti-cancer treatment from the Pacific Yew tree some of the most famous plant-derived products. Dr. Sampson and others do not condemn the search for new products that might prove to be similarly useful, but instead argue for the cessation of spending on those products that have already been extensively tested.

As scientists, we can appreciate the difficulties with trying to isolate the effects of a single variable on a plant or microbe. We can only imagine, however, the increasing levels of complexity associated with a human subject including the effects of diet, exercise, stress levels, emotional states and compliance. I think that the optimists and desperately ill among the American public will continue to support the scientific study of alternative medicine and that, unless a large number of studies reveal overwhelming positive evidence to support the effectiveness of a range of herbal treatments, western-trained doctors will continue to resent the time and money spent studying these remedies. Until herbal medicine is either embraced or rejected by both doctors and patients, serious side effects and herbal-prescription drug negative interactions will occur because of patients lack of disclosure of herbal use to doctors who may deliberatively or inadvertently communicate a ill-will toward herbal remedy use. But that is another story.

APPENDIX D

Method justification

The following rationales were used when determining the protocols used in the preceding experiments:

Growth chamber

It is well established that controlled environment agriculture can produce plant growth that is more consistent than field-grown material. Better temperature and light condition cause greenhouse production to be more consistent than field production, and growth chamber grown plants are more consistent still due to an increased control over light intensity and light integral (Both, 1995).

Type of hydroponic system

Others have used nutrient film technique to grow this crop (Murch et al., 2002), however reliability issues encourage the use of a deep pond system when possible. This crop was found to grow acceptably in a deep pond system. Initial trials were conducted using speedling trays. However, the large plant density would have necessitated thinning the plants almost daily and this seemed a waste of seed. Furthermore, a speedling-type system uses a large amount of media and requires extensive sanitation. Since *H. perforatum* was able to grow as quickly in a raft-type system, this was chosen.

Seedling production method

Cultivar

Of the many commercially available cultivars, *H. perforatum* L. New Stem was used for two reasons. The first was because of a reported resistance to the fungal disease Anthracnose, and the second was because many other authors used this cultivar and continuing this trend would allow for a better comparison of metabolite concentrations.

Washing seed

In 1985, Campbell demonstrated that young seed contains a growth inhibitor that must be rinsed off prior to seeding or decreased germination will occur.

Covering seed

The seed needs to be exposed to light to encourage germination, but since germination can take up to two weeks, a cover was needed to ensure that seeds remained moist.

Transplanting period

Seed germination can take up to 3 weeks. Before transplanting could occur, seed germination and thinning must be finished. For this reason, transplanting occurred 3 weeks after plants were seeded. If more than one seed germinated per rockwool cube, the extra seedlings were removed before transplantation. During this thinning process, seedlings were selected for uniformity.

Temperature

The study by Zobayed et al. 2005 suggested that hypericin production could be optimized at 25C. It must be noted that the same study demonstrated that hyperforin

production is optimized at 30C, however due to the comparatively larger amount of hyperforin produced at all stages in the life of the plant and the elusiveness of hypericin production, the optimal temperature for hypericin production was chosen.

Transition period to higher light

Mosaleeyanon et al., 2005 demonstrated the necessity to transition seedlings from a low light intensity such as might be seen in tissue culture or used during germination, to a higher intensity in stages. At least three days were allotted for plants to adjust to each addition of 100 µmol m^{-2} s^{-1} in experiments conducted in growth chambers.

Nutrient solution composition

Renewal rate

A complete renewal of nutrient solution was considered, but due to the decreasing width of the ponds and the fixed shape of the floats, this was abandoned. Removal of the solution would have left the roots dangling in the air because the sides of the ponds had slightly sloped sides that led to a slight decrease in the pond width that would prevent the lowering of the float to the bottom of the pond. If the roots were allowed to dangle in the air, it would possibly cause cavitation within the vascular tissues, and thus interference with plant growth. An ICP analysis of the solution after 8 weeks showed that very few nutrients were below their setpoints.

Plant growth parameters

Temperature

Temperature was maintained at 25C which was also used for seedling production.

Photoperiod

From preliminary investigations, it was determined that this plant was sensitive to photoperiod and perhaps needed a long day to flower. Literature investigations showed a universal use of a 16 hour photoperiod, prompting our decision to also use such a photoperiod. Our experiment to verify that a 16 hour photoperiod is important in flowering induction may found in Chapter 5.

Nutrient Solution Composition

Historically, the CEA program at Cornell University has determined that the nutrient solution recipe known as 'Sonneveld' (Sonneveld et al., 1992), which is modified from work done by Hoagland et al., (1920) is an excellent all-purpose fertilizer. Furthermore, concentrations that are half the original mix are sufficient for plant growth and development. For these reasons, ½ strength Sonneveld nutrient solution was utilized and nutrient deficiency symptoms were not observed, and thus the solution composition was maintained throughout the experiments. No attempt at further nutrient solution optimization were performed.

Harvest interval

Initially, plants were harvested every week, but as it became clear that flowering would not occur after 2 months, harvests were adjusted to once every two weeks so that enough biomass would be available for the final harvests, and so that the number of samples to be analyzed in the laboratory would be manageable. Since maximum metabolite production has been observed in flower tissue (Murch et al., 2002), experiments were continued, when possible, through flowering and until the seed pods were mature.

Harvested material

Top 10 cm were analyzed by HPLC for metabolite concentration as was suggested in the protocol we followed. This method seemed one of the most repeatable, as other protocols were very specific (i.e. sample the fourth leaf from growing tip), but not very practical usually due to extremely small sample volume and weight.

Experiment duration

The original intent was to continue each experiment until plants were at least in full flower, and possibly until seed pods were developed if time allowed. However, due to the poor growth and development that was a result of the very low light integrals found in the light intensity and one treatment in the light integral experiments, investigations were terminated after a little more than 100 days as it could not be determined how much more time would be necessary to allow to plants to flower.

HPLC method

pH

It was found that lowering the pH allows for the separation of the hyperforin and pseudohypericin peaks (Fourneron and Nait-Si, 2006). Also Liu et al. (2005) demonstrated that at a pH of 2, hyperforin never completely degrades and, in fact, only ever degrades to ½ the original amount upon exposure to light.

Sonication

Direct sonication has been shown to be slightly more effective due to the increased effect of cavitation and intensification of mass transfer, but it requires that each sample be processed separately. The 2006 study by Smelerovic et al. demonstrated that the continuation of indirect sonication after 15 minutes produced negligible additional metabolite extraction. Our protocol used 30 minutes of indirect sonification and provided an acceptable combination of maximum metabolite extraction and minimum laboratory usage.

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APPENDIX E

Experimental Timeline

All experiments described in preceding chapters were conducted between September 2006 and December 2008. Additional experiments presented in appendices were conducted between 2006 and 2007.

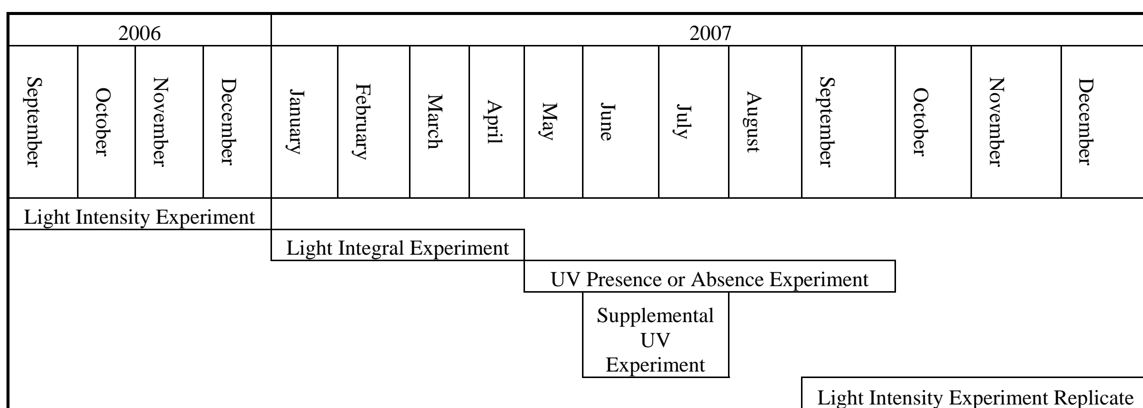

Figure E.1. Timeline for experiments presented in main body of dissertation.

APPENDIX F

Growth Chamber Description

The growth chambers were manufactured by Environmental Growth Chambers (EGC) in 1965 and do not have a model number. Temperature and fluorescent lighting was controlled by the computer that directs the heating, cooling and lighting in all the chambers. Supplemental incandescent lighting for day length extension was controlled by time clocks. The dimensions of the chamber were 3.5m x 2.6 m x 2.1m. Three identical growth chambers were used once seedlings were placed into experimental conditions.

New fluorescent lamps were installed in May 2007. All the lights remained on for over 100 hours to allow for the decay that happens during this period.

Temperature was controlled by air intake vents along the bottom of the side walls of the chamber, and returned through vents along the top of the side walls of the chambers.

Figure F.2. Photo side view of ponds showing light barrier and photoperiod control incandescents.

Figure F.3. Photo of silver light barriers with a cohort of plants.

APPENDIX G

Suggested protocol for reporting plant production and quantification for secondary metabolite studies

As seen in Tables 1.2-1.5, many studies do not report experimental conditions fully making comparison between publications difficult. The International Committee for Controlled Environments has published guidelines to assist in the standardization of reporting to avoid such problems in the literature published about plants grown in Controlled Environments. It is suggested that all researchers who wish to report the results of their experiments follow these guidelines regardless of where the plants are grown (field production, or controlled environment production). The guidelines are summarized in Table G.1. In addition, it is recommended that the following details be mentioned when reporting quantification methods:

<u>Quantification</u>

- State the amount of plant material harvested.
- State which plant parts were harvested.
- State the elapsed time between harvest and when samples were processed or frozen.
- Detail which quantification method was used (HPLC, TLC etc.).
- State solvent(s) used.
- If sonicated, mention if in-sample or a sonicating bath was used.
- If working with light-sensitive compounds, detail light conditions used during sample preparation and analysis.

Table G. 1. Minimum guidelines for reporting experiments with secondary metabolites.

Parameter	Units
Temperature	
Air temperature	C
Root temperature	C
Relative Humidity	%
Radiation	
Light Quantity (PAR)	µmol m^{-2} s^{-1} and mol d^{-1}
Light Quality – type of light, sunlight, fluorescent, HPS, MH, LED, mixed Sunlight plus supplemental. etc	
Direction of source of light	
Photoperiod	hours
Carbon dioxide concentration	ppm
Air movement – wind speed if possible	m s^{-1}
Watering	
pH	pH
Electrical conductivity	µS cm^{-2}
Nutrition	molar ratio of salts or recipe used
Plant density	
Plant cultivar	
Plant weight (Fresh or dry)	g

APPENDIX H

Wild-collected *H. perforatum* metabolite data

During the summer of 2007 samples of wild-type H. perforatum were taken and analyzed for metabolite production. The increase and subsequent decrease in metabolite concentration that may be seen in pseudohypericin and hypericin (Figures H.2 and H.3) was also observed by Coucerio et al., 2006.

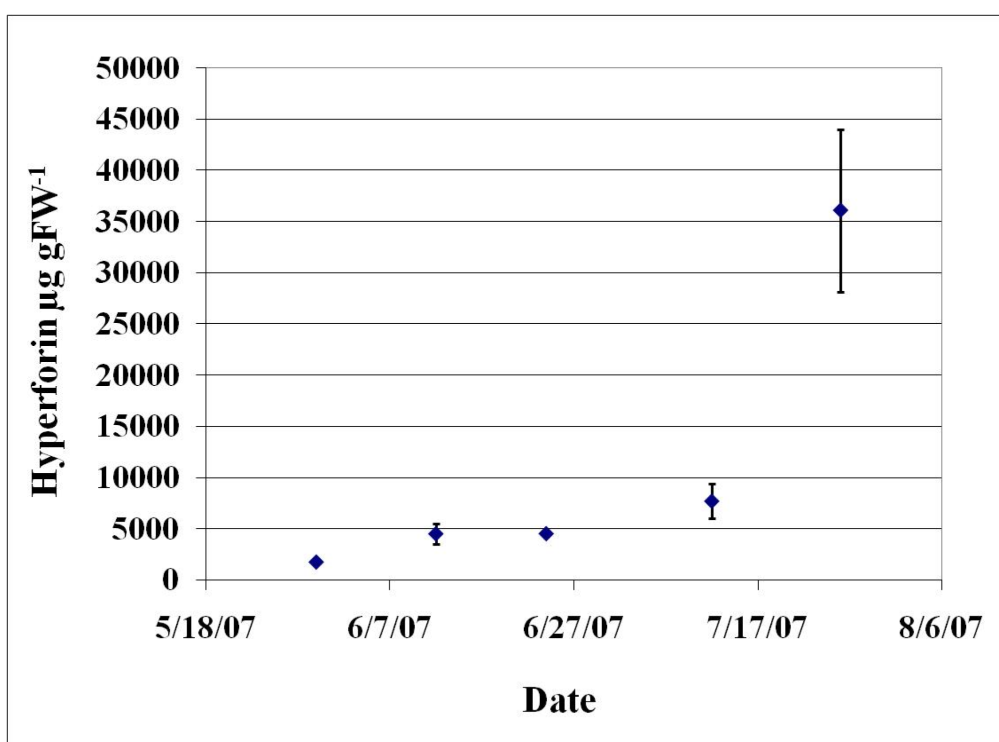

Figure H.1 Hyperforin concentration of wild-collected *H. perforatum* during the Summer of 2007 in Ithaca, NY. Points represent the average of 5 replicates +/- standard error.

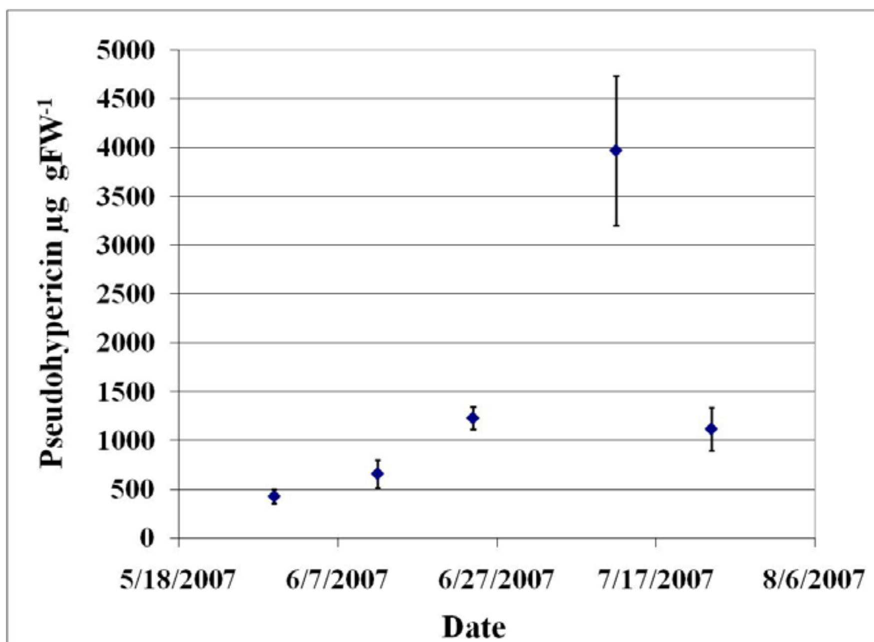

Figure H.2. Pseudohypericin concentration of wild-collected *H. perforatum* during the Summer of 2007 in Ithaca, NY. Points represent the average of 5 replicates +/- standard error.

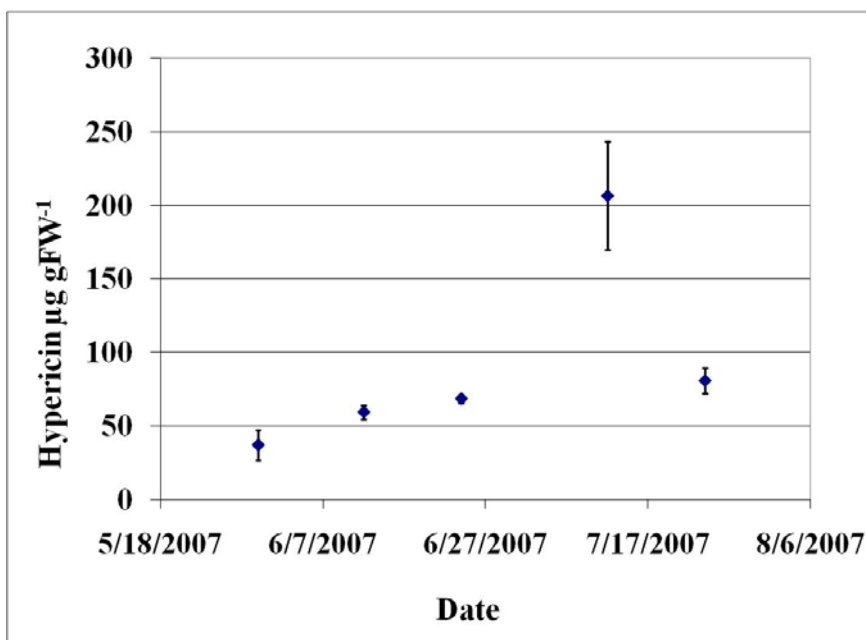

Figure H.3. Hypericin concentration of wild-collected *H. perforatum* during the Summer of 2007 in Ithaca, NY. Points represent the average of 5 replicates +/- standard error.

APPENDIX I

Light Intensity, Integral and Quality Average Metabolite Data

Light Intensity I

Table I.1. Hyperforin concentration in µg/gFW for the first light intensity experiment. Mean represents 18 or 27 plants sampled and pooled into two or three samples for chemical analysis +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [A]	1014 ± 119	991 ± 186	1015 ± 125	1289 ± 207
160 umol m^{-2} s^{-1} [A]	649 ± 11	562 ± 129	868 ± 92	1360 ± 257
340 umol m^{-2} s^{-1} [A]	574 ± 262	225 ± 317	455 ± 115	816 ± 34

Table I.2. Pseudohypericin concentration in µg/gFW for the first light intensity experiment. Mean represents 18 or 27 plants sampled and pooled into two or three samples for chemical analysis +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [B]	491 ± 284	462 ± 115	829 ± 156	645 ± 151
160 umol m^{-2} s^{-1} [A]	863 ± 363	746 ± 36	658 ± 241	1300 ± 72
340 umol m^{-2} s^{-1} [AB]	713 ± 266	969 ± 214	323 ± 225	1240 ± 160

Table I.3. Hypericin concentration in µg/gFW for the first light intensity experiment. Mean represents 18 or 27 plants sampled and pooled into two or three samples for chemical analysis +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding			
Treatment	49 [b]	78 [b]	91 [b]	104 [a]
90 umol m^{-2} s^{-1} [A]	ND ± 0	10 ± 20	ND ± 0	20 ± 40
160 umol m^{-2} s^{-1} [B]	20 ± 30	ND ± 0	ND ± 0	60 ± 10
340 umol m^{-2} s^{-1} [C]	ND ± 0	ND ± 0	ND ± 0	60 ± 10

Light Integral

Table I.4. Hyperforin concentration in µg/gFW for the light integral experiment. Mean represents 27 plants sampled and pooled into three samples for chemical analysis +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding				
Treatment	51	65	71	79	108
20 mol m^{-2} d^{-1}	2540 ± 194	3170 ± 856	2920 ± 881	3270 ± 137	4540 ± 707
13 mol m^{-2} d^{-1}	2300 ± 439	2110 ± 278	1970 ± 68	2690 ± 466	2310 ± 391
8.6 mol m^{-2} d^{-1}	1620 ± 93	1830 ± 207	1560 ± 237	3550 ± 382	1010 ± 352

Table I.5. Pseudohypericin concentration in µg/gFW for the light integral experiment. Mean represents 27 plants sampled and pooled into three samples for chemical analysis +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding				
Treatment	51	65	71	79	108
20 mol m^{-2} d^{-1}	1110 ± 150	960 ± 207	715 ± 268	966 ±73	2834 ± 465
13 mol m^{-2} d^{-1}	1130 ± 380	850 ± 146	701± 202	850 ± 69	773 ± 361
8.6 mol m^{-2} d^{-1}	1000 ± 450	617 ± 140	667 ± 72	877 ± 233	660 ± 319

Table I.6. Hypericin concentration in µg/gFW for the light integral experiment. Mean represents 27 plants sampled and pooled into three samples for chemical analysis +/- standard deviation. ND denotes below the level of detection.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding				
Treatment	51	65	71	79	108
20 mol m^{-2} d^{-1}	52 ± 4	50 ± 28	55 ± 31	51 ± 3	104 ± 13
13 mol m^{-2} d^{-1}	59 ± 31	ND ± 0	51 ± 29	54 ± 29	63 ± 37
8.6 mol m^{-2} d^{-1}	54 ± 34	ND ± 0	ND ± 0	50 ± 31	55 ± 32

Light Quality

Table I.7. Hyperforin concentration in µg/gFW for the light quality experiment. Mean represents 5 plants +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding					
Treatment	38	54	68	84	102	115
Without UV	4030 ± 1510	3760 ± 1040	6230 ± 2290	5290 ± 1330	7340 ± 2800	12200 ± 4680
UV-A and UV-B	2640 ± 275	2990 ± 1040	3980 ± 1270	3640 ± 1960	4390 ± 1420	5790 ± 2470
UV-A only	2060 ± 340	3290 ± 1590	4330 ± 750	2750 ± 1130	2460 ± 780	2450 ± 647

Table I.8. Pseudohypericin concentration in µg/gFW for the light quality experiment. Mean represents 5 plants +/- standard deviation.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding					
Treatment	38	54	68	84	102	115
Without UV	428 ± 192	756 ± 272	1530 ± 825	2370 ± 854	2110 ± 1050	3200 ± 1030
UV-A and UV-B	436 ± 20	381 ± 85	379 ± 71	650 ± 697	2133 ± 300	1233 ± 751
UV-A only	403 ± 174	772 ± 214	562 ± 103	843 ± 559	1024 ± 506	536 ± 157

Table I.9. Hypericin concentration in µg/gFW for the light quality experiment. Mean represents 5 plants +/- standard deviation. ND denotes below level of detection.

	Chemical concentration from each harvest (µg/gFW) Days from Seeding					
Treatment	38	54	68	84	102	115
Without UV	ND	33 ± 29	73 ± 24	99 ± 24	113 ± 43	162 ± 46
UV-A and UV-B	ND	10 ± 24	ND	40 ± 67	104 ± 12	96 ± 31
UV-A only	ND	42 ± 24	19 ± 26	55 ± 31	72 ± 20	56 ± 6

APPENDIX J

UV Supplementation average data

Table J.1. Non-normalized hyperforin concentration for Hypericum perforatum exposed to UV-B light for a single time (single dose, time in minutes), for a continuous dose (10 minutes per day) or for a progressive dose (10 minutes on day one, increasing daily). Mean composition of five replicates of sampled leaf tissue +/- standard deviation. N/A represents a time when a harvest was not performed.

	Chemical concentration (ug/gFW) for each harvest Hours (top) or Days (bottom) from UV exposure						
Treatment	**12**	**24**	**48**	**72**	**96**	**120**	**144**
Control	3760 +/- 1570	2540 +/- 1890	3520 +/- 280	2080 +/- 550	2980 +/- 1080	2950 +/- 599	3890 +/- 1820
Single Dose							
10	6630 +/- 2550	4080 +/- 1640	3320 +/- 879	n/a	3880 +/- 785	n/a	2020 +/- 1280
20	10000 +/- 2050	2790 +/- 275	2790 +/- 568	n/a	4780 +/- 119	n/a	3080 +/- 1070
40	9060 +/- 842	5030 +/- 876	5900 +/- 476	n/a	3740 +/- 539	n/a	2440 +/- 313
80	8360 +/- 2070	5160 +/- 1780	5150 +/- 1430	n/a	3500 +/- 321	n/a	3790 +/- 220
160	5150 +/- 1620	2080 +/- 1050	3300 +/- 993	n/a	2760 +/- 130	n/a	1760 +/- 279
Repeated	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Continuous	252 +/- 780	3010 +/- 934	3020 +/- 711	2880 +/- 708	2570 +/- 764	2340 +/- 349	2930 +/- 836
Progressive	2220 +/- 250	2240 +/- 384	4520 +/- 675	3600 +/- 1470	2900 +/- 1090	3320 +/- 747	2900 +/- 745

Table J.2. Non-normalized pseudohypericin concentration for Hypericum perforatum exposed to UV-B light for a single time (single dose, time in minutes), for a continuous dose (10 minutes per day) or for a progressive dose (10 minutes on day one, increasing daily). Mean composition of five replicates of sampled leaf tissue +/- standard deviation. N/A represents a time when a harvest was not performed.

	Chemical concentration (ug/gFW) for each harvest Hours (control and single dose) or days (repeated) from initial UV-B exposure						
	12	**24**	**48**	**72**	**96**	**120**	**144**
Control	428 +/- 73	485 +/- 91	1460 +/- 164	1030 +/- 233	884 +/- 377	1140 +/- 672	1220 +/- 446
Single Dose							
10	781 +/- 259	676 +/- 94	722 +/- 363	n/a	784 +/- 329	n/a	712 +/- 306
20	1140 +/- 394	644 +/- 277	983 +/- 670	n/a	1270 +/- 366	n/a	691 +/- 76
40	1630 +/- 437	1230 +/- 300	839 +/- 742	n/a	1530 +/- 691	n/a	1280 +/- 346
80	1120 +/- 338	1302 +/- 937	1980 +/- 1100	n/a	898 +/- 264	n/a	1170 +/- 72
160	886 +/- 336	365 +/- 76	868 +/- 335	n/a	616 +/- 306	n/a	624 +/- 192
Repeated	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Continuous	706 +/- 379	848 +/- 359	702 +/- 161	1210 +/- 547	1180 +/- 789	1260 +/- 266	1030 +/- 310
Progressive	481 +/- 323	430 +/- 93	1610 +/- 262	1490 +/- 468	1020 +/- 320	1590 +/- 695	1150 +/- 494

Table J.3. Non-normalized hypericin concentration for Hypericum perforatum exposed to UV-B light for a single time (single dose, time in minutes), for a continuous dose (10 minutes per day) or for a progressive dose (10 minutes on day one, increasing daily). N/A represents a time when a harvest was not performed. ND denotes below the level of detection.

	Chemical concentration (ug/gFW) for each harvest						
	Hours (control and single dose) or days (repeated) from initial UV-B exposure						
	12	**24**	**48**	**72**	**96**	**120**	**144**
Control	ND	63 +/- 5	57 +/- 9	39 +/- 35	44 +/- 39	63 +/- 11	65 +/- 12
Single Dose							
10	17 +/- 31	16 +/- 29	ND	n/a	37 +/- 33	n/a	21 +/- 37
20	43 +/- 38	17 +/- 30	ND	n/a	67 +/- 11	n/a	50 +/- 11
40	72 +/- 11	64 +/- 13	43 +/- 39	n/a	79 +/- 26	n/a	66 +/- 14
80	57 +/- 5	49 +/- 44	60 +/- 52	n/a	62 +/- 3	n/a	62 +/- 3
160	20 +/- 36	0 +/- 0	17 +/- 30	n/a	17 +/- 31	n/a	17 +/- 30
Repeated	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Continuous	19 +/- 34	ND	73 +/- 22	48 +/- 43	74 +/- 14	63 +/- 5	15 +/- 16
Progressive	18 +/- 32	ND	67 +/- 9	78 +/- 17	57 +/- 7	126 +/- 76	66 +/- 18

APPENDIX K

Biomass Data from Light Intensity, Light Integral and Light Quality Experiments

Table K.1. Biomass data in grams fresh weight for the light intensity experiment. Mean represents 27 plants +/- standard deviation.

	Fresh weight for each harvest (g) Days From Seeding									
Treatment	43	49	56	63	70	78	84	91	100	104
Low Light	0.06 ± 0.03	0.32 ± 0.18	0.55 ± 0.22	0.86 ± 0.44	1.44 ± 0.7	2.77 ± 1.02	3.00 ± 0.18	4.28 ± 0.93	5.22 ± 0.75	6.63 ± 1.49
Medium Light	0.04 ± 0.02	0.18 ± 0.06	0.25 ± 0.07	0.51 ± 0.15	0.87 ± 0.33	1.18 ± 0.5	1.79 ± 0.64	2.36 ± 0.74	3.14 ± 0.84	4.69 ± 0.24
High Light	0.03 ± 0.01	0.07 ± 0.03	0.13 ± 0.05	0.27 ± 0.07	0.37 ± 0.14	0.46 ± 0.12	0.72 ± 0.06	1.05 ± 0.07	1.43 ± 0.38	1.55 ± 0.29

Table K.2. Biomass data in grams fresh weight for light integral experiment. Mean represents 27 plants +/- standard deviation.

	Fresh weight for each harvest (g) Days from seeding						
Treatment	45	51	58	65	71	79	85
8.6 mol	0.276 ± 0.11	0.34 ± 0.11	0.96 ± 0.25	1.86 ± 0.58	3.3 ± 0.78	5.45 ± 1.67	7.23 ± 2.41
13 mol	0.119 ± 0.28	0.62 ± 0.17	1.53 ± 0.49	3.85 ± 1.26	6.11 ± 2.05	9.54 ± 2.82	7.56 ± 3.19
20 mol	0.172 ± 0.59	1.17 ± 0.39	2.61 ± 1.22	6.09 ± 2.44	10.44 ± 4.84	13.07 ± 7.27	15.05 ± 5.96

Table K.3. Biomass data in grams dry weight for light integral experiment. Mean represents 27 plants +/- standard deviation.

	Dry weight for each harvest (g) Days from Seeding					
Treatment	51	58	65	71	79	85
8.6 mol	0.75 ± 0.08	1.28 ± 0.11	1.69 ± 0.03	3.33 ± 0.26	7.46 ± 1.09	9.33 ± 1.77
13 mol	1.43 ± 0.26	5.29 ± 0.37	3.72 ± 0.11	6.78 ± 0.23	11.27 ± 0.37	8.45 ± 1.4
20 mol	3.07 ± 0.76	5.53 ± 0.43	7.47 ± 0.71	13.08 ± 1.99	19.35 ± 1.81	21.46 ± 1.84

Table K.4. Biomass data in grams fresh weight from light quality experiment. Mean represents 27 plants +/- standard deviation.

	Fresh weight for each harvest (g) Days from seeding					
Treatment	38	54	68	84	102	115
Without UV	0.537 ± 0.078	12.78 ± 1.12	42.17 ± 7.91	91.86 ± 12.4	245.9 ± 16.6	228.8 ± 30.1
UV-A only	0.453 ± 0.121	4.135 ± 0.978	14.29 ± 0.606	27.76 ± 2.16	105 ± 23.8	114.4 ± 9.32
UV-A and UV-B	0.479 ± 0.159	6.338 ± 1.65	28.34 ± 3.13	50.27 ± 7.17	88.6 ± 20.6	76.28 ± 17.2

Table K.5. Biomass data in grams dry weight from light quality experiment. Mean represents 27 plants +/- standard deviation.

	Dry weight for each harvest (g) Days from seeding					
Treatment	38	54	68	84	102	115
Without UV	0.107 ± 0.022	2.893 ± 0.329	9.841 ± 2.23	19.67 ± 2.21	69.01 ± 4.19	71.41 ± 11.7
UV-A only	0.083 ± 0.023	0.796 ± 0.185	2.603 ± 0.189	6.322 ± 0.932	23.14 ± 4.17	28.22 ± 2.42
UV-A and UV-B	0.086 ± 0.007	0.796 ± 0.163	5.78 ± 0.487	7.74 ± 0.89	26.34 ± 6.32	22.9 ± 5.45

APPENDIX L

Light Intensity Replicate Graphs and Comments

As seen in Figure L.1, biomass values from both replicates are very similar and could be combined. However, Figures L.2 – L10 demonstrates that replicates may not be combined on a metabolite basis. Just after transplantation of seedlings into the hydroponic system, plants were infested with thrips and chemical control methods are not allowed in the growth chambers. Biological controls were attempted, but it is possible that the damage the seedlings sustained was responsible for the increased and varied amounts of metabolites seen in replicate 2. Further plant stresses occurred when the chamber that housed the low light treatment in replicate 2 overheated periodically (See logger data graph, Appendix I) causing the plants to be temperature stressed. Clearly, this plant cannot endure periodic biological and temperature stresses without simultaneous secondary metabolite changes.

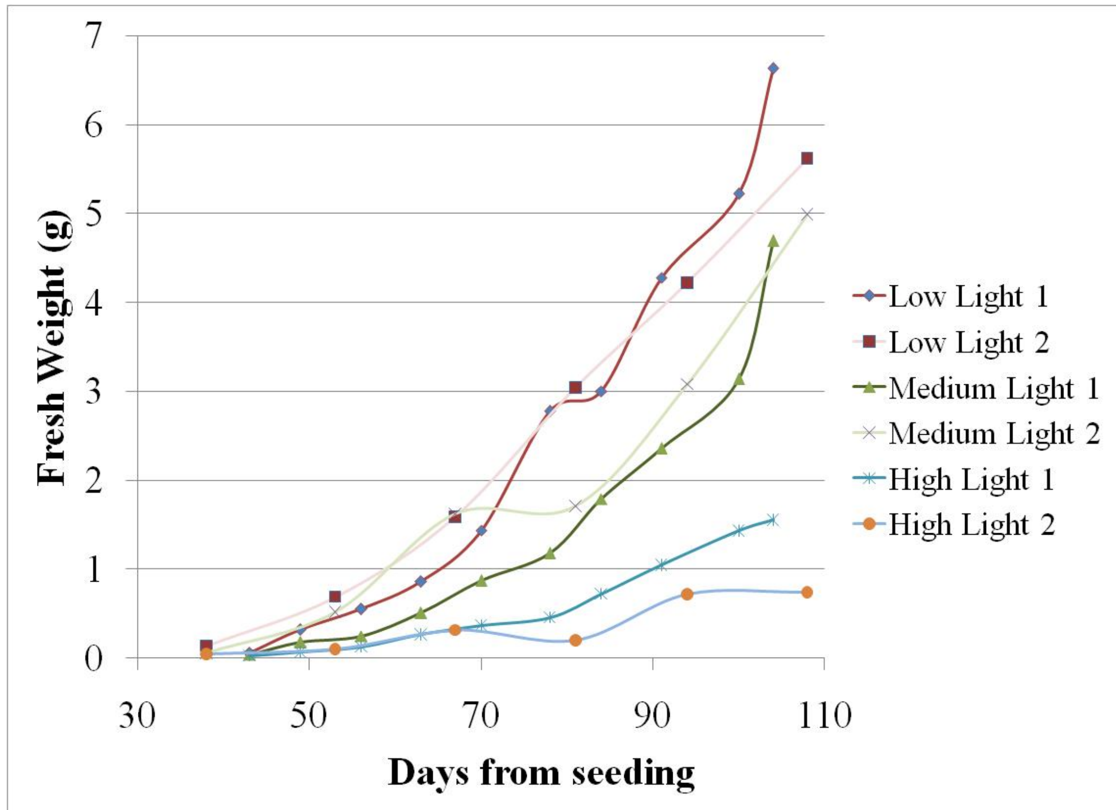

Figure L.1. Mean biomass from both replicates for all light conditions.

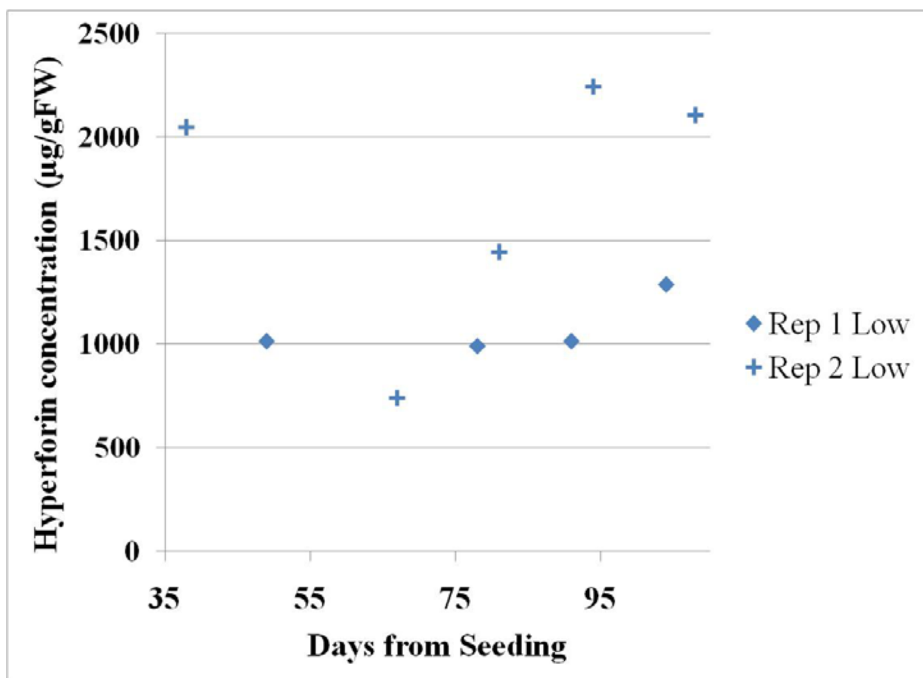

Figure L.2. Mean hyperforin from both replicates for the low light intensity condition.

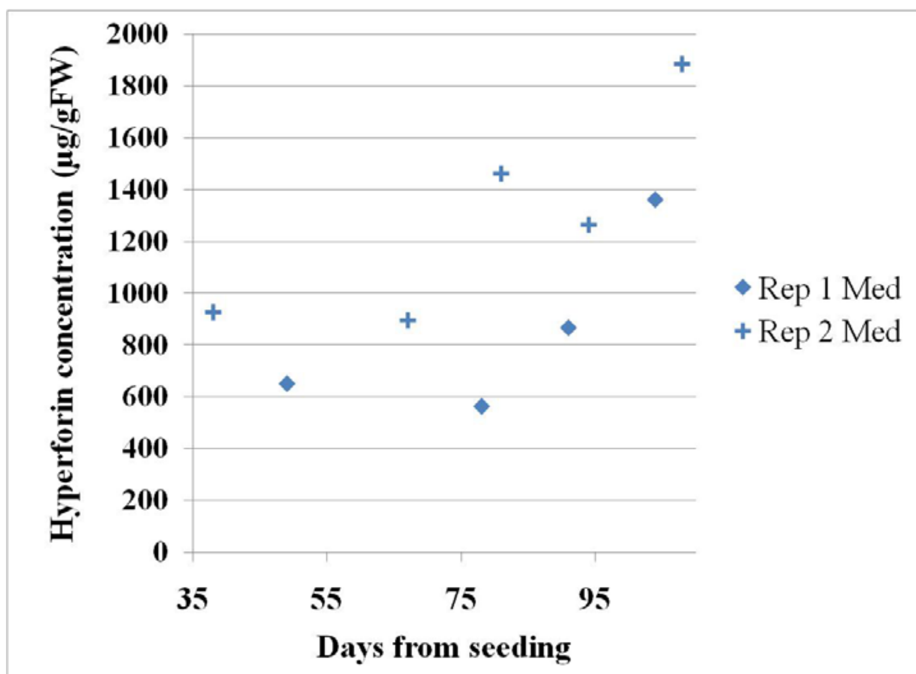

Figure L.3. Mean hyperforin from both replicates for the medium light intensity condition.

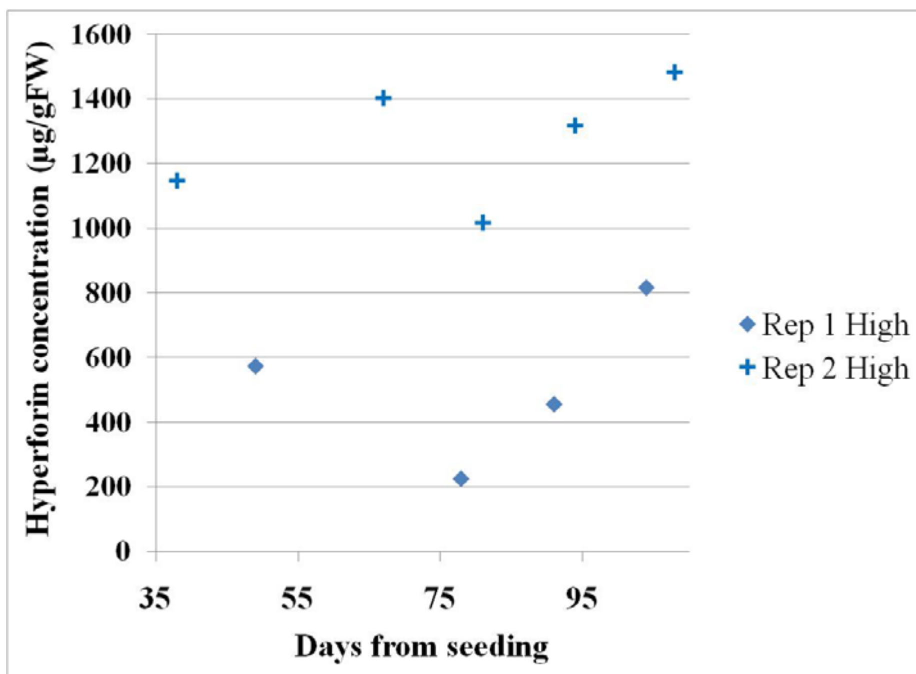

Figure L.4. Mean hyperforin from both replicates for the high light intensity condition.

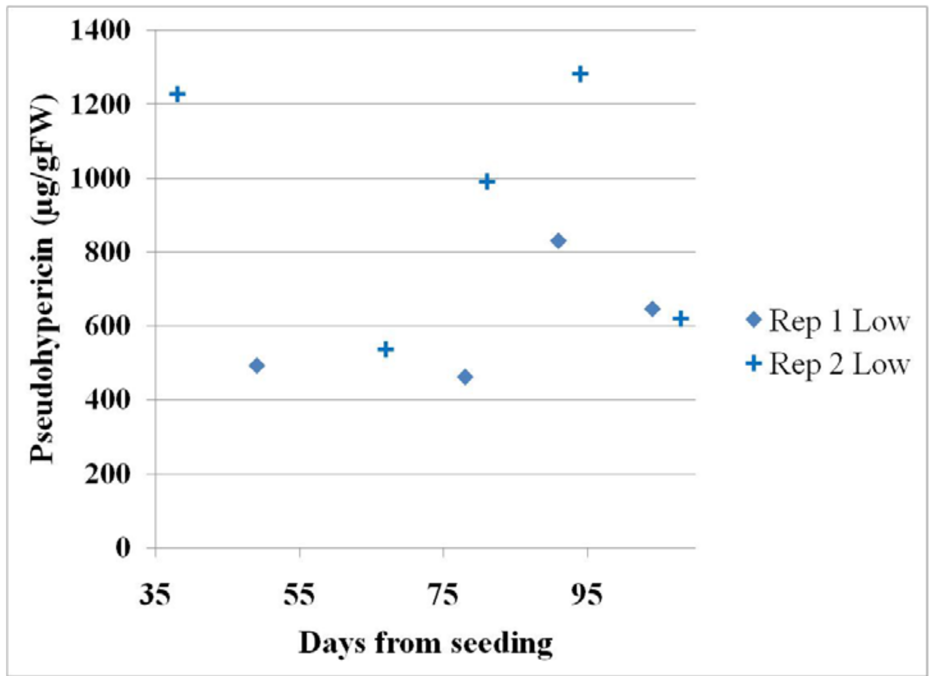

Figure L.5. Mean pseudohypericin from both replicates for the low light intensity condition.

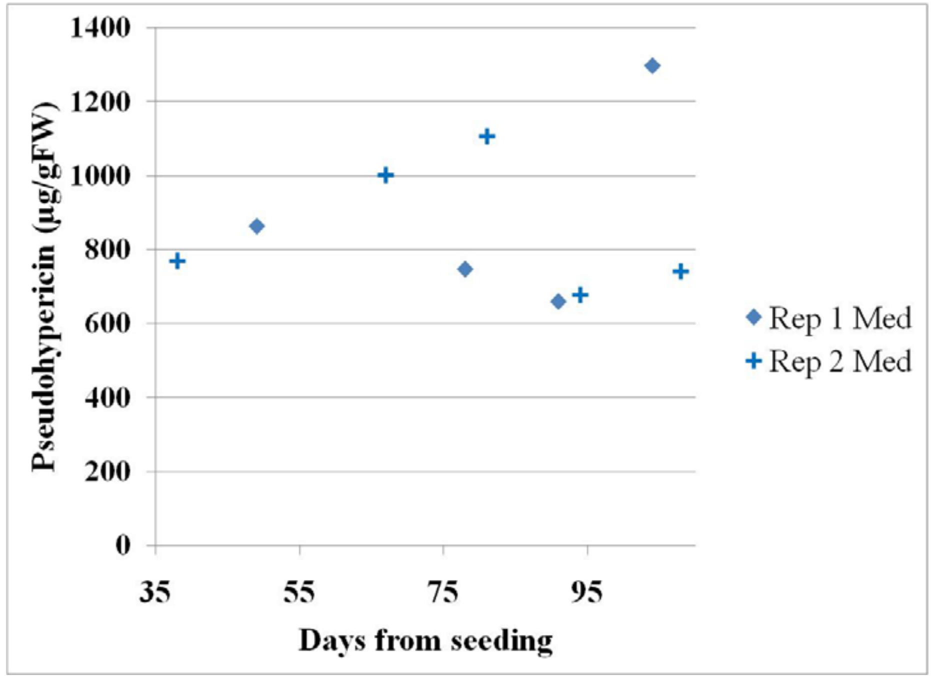

Figure L.6. Mean pseudohypericin from both replicates for the medium light intensity condition.

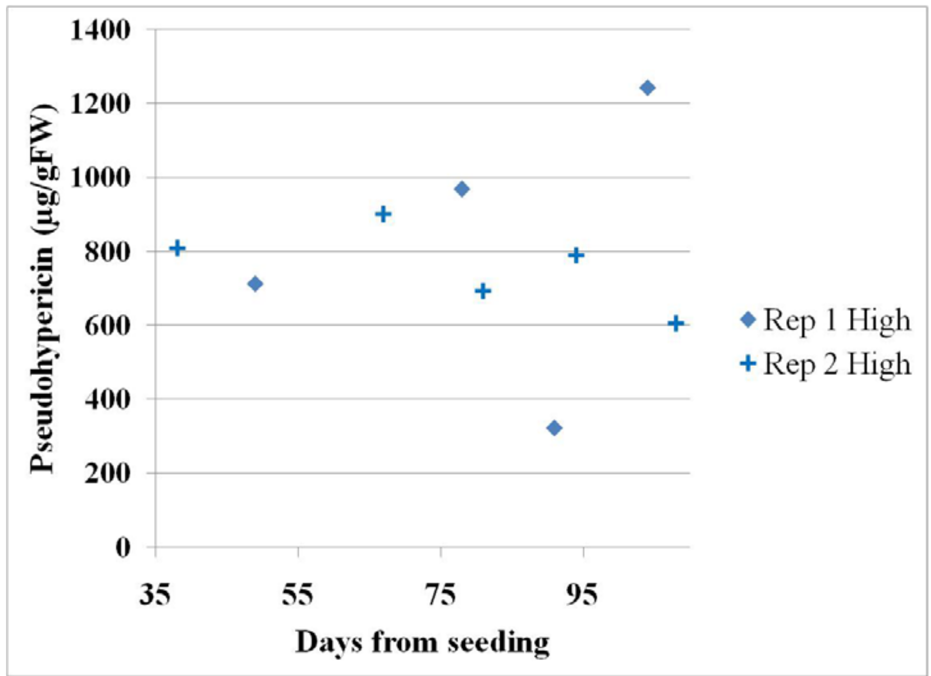

Figure K.7. Mean pseudohypericin from both replicates for the high light intensity condition.

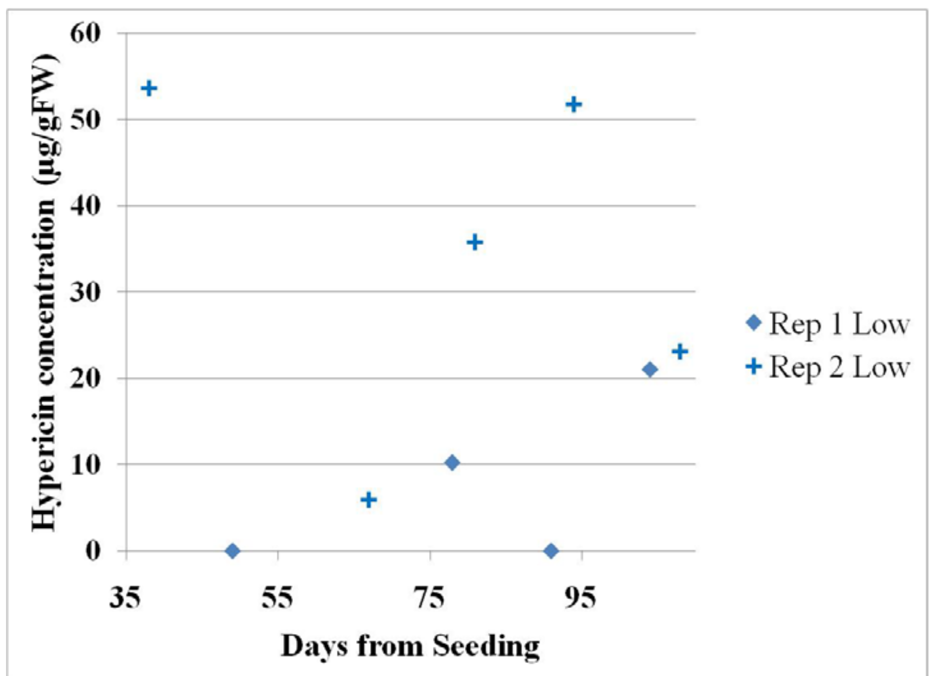

Figure L.8. Mean hypericin from both replicates for the low light intensity condition.

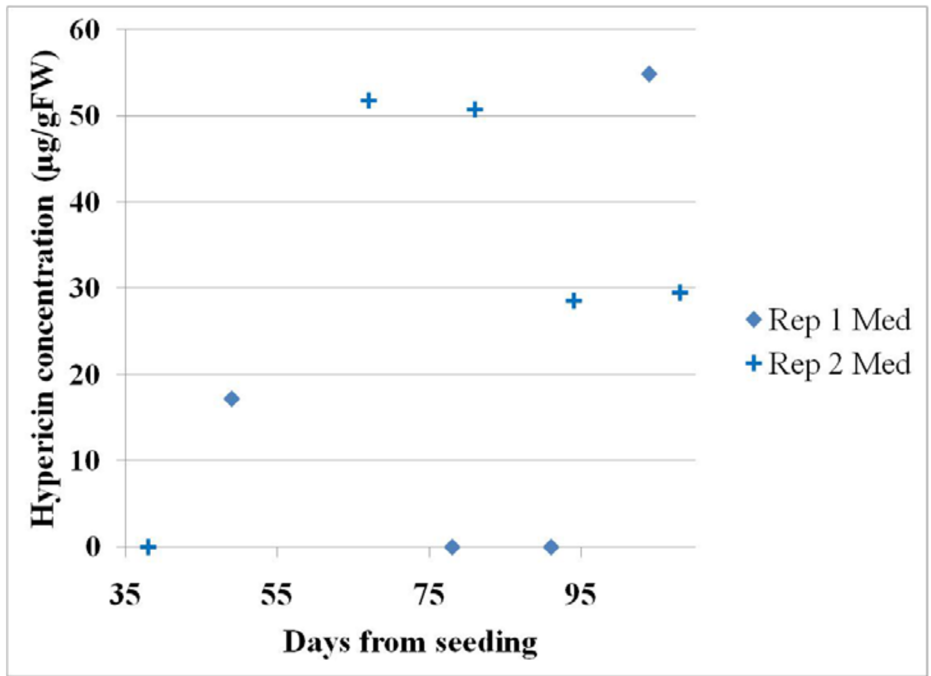

Figure L.9. Mean hypericin from both replicates for the medium light intensity condition.

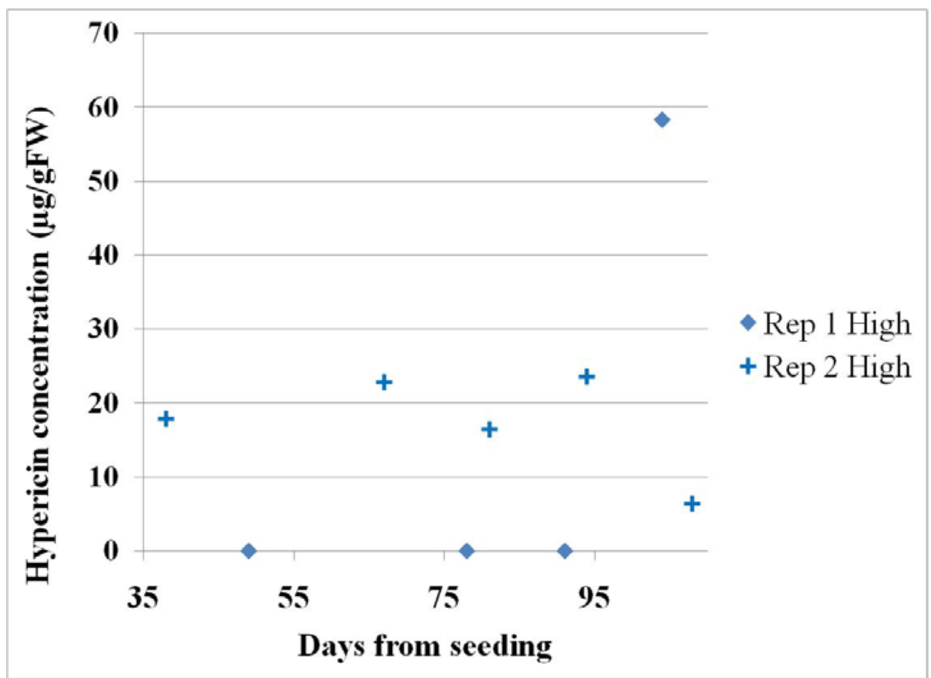

Figure L.10. Mean hypericin from both replicates for the high light intensity condition.

APPENDIX M

Summary of Metabolites Including Experiments Included in Dissertation

Table M.1. Summary of metabolite concentrations as a percent dry weight of experiments mentioned in the literature review in Chapter 1. This table also includes metabolite data from experiments reported in this dissertation.

			Metabolite concentrations		
			Hyperforin % DW	Pseudohypericin % DW	Hypericin % DW
Greenhouse	Murch et al.	2002	2-6	0.5-1.7	0.02-0.1
	Zobayed et al.	2004	1.00	0.08	0.01
	Zobayed and Saxena	2004	0.31	0.05	0.01
	Brechner et al. (UV exposure)	2008	1-2	0.25-0.5	0-0.35
Growth Chamber	Briskin et al.	2000	not measured	Reported as 'hypericins'	0.1-0.4
	Briskin and Gawienowski	2001	not measured	Reported as 'hypericins'	0.05 - 0.24
	Zobayed and Saxena	2004	3.00	0.09	0.015
	Mosaleeyanonn et al.	2005	2.67	0.075	0.033
	Zobayed et al.	2005	0.875	0.31	0.123
	Couceiro et al.	2006	2-4	0.05-0.125	0.025-0.085
	Zobayed et al.	2007	3-6	0.05-0.26	0.002-0.14
	Brechner et al. (IntensityI)	2007	0.125-0.75	0.25-0.625	0.036
	Brechner et al. (Integral)	2008	0.83	0.5-1.4	0.025-0.05
	Brechner et al. (UV exposure)	2008	2-6	0.5-1.6	0-0.08
Field	Southwell and Bourke	1991	not measured	Reported as 'hypericins'	0.02-0.47
	Osinska and Weglarz	2000	not measured	not measured	0.035-.0.183
	Sirvent et al.	2002	not measured	0.0019-0.85	0.0003-0.12
	Poutaraud and Girardin	2004	0.65-3.2	Reported as 'hypericins'	0.7-3
	Gray et al.	2003	1.2-2.9	0.05-0.18	0.04-0.125
	Buter et al.	1998	not measured	40	0.25-0.5
	Mosaleeyanon et al.	2005	0.67	0.07	0.03
	Brechner et al. (UV exposure)	2007	1.25-3	0.25-1	0-0.5
In Vitro	Sirvent and Gibson	2002	0.035	0.050	0.007
	Kirakosyan et al.	2003	not measured	0.05-0.3	0.025-0.2
	Murch et al.	2003	0.1	0.2	0.01
	Pasqua et al.	2003	2.22-7.41	0.02-0.2	0.007-0.015
	Zobayed et al.	2003	0.2-0.48	0.015-0.05	0.0004-0.003
	Zobayed et al.	2004	0.1-0.8	0.025-0.125	0.002-0.0007
	Zobayed and Saxena	2004	0.300	0.0490	0.00200