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CULTIVATION OF MEDICINAL PLANTS IN GREENHOUSE HYDROPONICS

by

Dana Simeunovic

A Thesis

**Submitted to the faculty of Graduate Studies and Research
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor**

Windsor, Ontario, Canada

2002

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ABSTRACT

Interest in medicinal plants as an alternative crop for greenhouse growers was the impetus for this project, as little information exists on the commercial production of medicinal plants in hydroponics under greenhouse conditions. This research project was designed to 1) identify medicinal plants with commercial potential, 2) evaluate their suitability for greenhouse hydroponic growth, 3) determine optimal concentrations and ratios of nutrient solutions, and 4) manipulate nitrogen levels as a possible regulator of leaf tissue production and of secondary metabolite production in two medicinally important plants, *Hypericum perforatum* and *Tanacetum parthenium*. Once nutrient regimes had been optimized to give maximum yield of both target tissues and concentration of target compounds (hence yield per plant of secondary compounds) additional experimental manipulations were carried out to determine if management practices such as leaf removal or flower bud removal could enhance target tissue yields and secondary metabolite production in *Hypericum perforatum* and *Tanacetum parthenium*.

For the first part of this study, three nutrients solutions (modifications of the Hoagland solution) with progressively increasing levels of N, K, Ca and Mg were used. Four promising medicinal plant species (*Achillea millefolium*, *Tanacetum parthenium*, *Leonurus sibiricus*, and *Linum usitatissimum*) were grown in these solutions. In all cases the intermediate solution yielded the best results; differences were statistically significant for *Linum usitatissimum* and *Leonurus sibiricus*.

Leaves of *Hypericum perforatum* and *Tanacetum parthenium* contain the medicinal constituents of interest (hypericin and hyperforin; and parthenolide, respectively). Since nitrogen is known to contribute greatly to vegetative growth of plants, a second experiment was conducted in which only nitrogen (N) varied (60, 120, 180, and 242 mg/L). In *Hypericum perforatum*, the lowest N regime yielded the greatest concentration and yield of hypericin. In *Tanacetum parthenium*, parthenolide concentrations were greatest in the highest N treatment.

Simulated herbivory has been shown to cause some plants to overcompensate in growth and to induce secondary metabolite production. Simulated herbivory in *Hypericum perforatum* caused neither overcompensation in growth nor induction of secondary metabolites. In contrast, simulated herbivory (flower bud removal) in *Tanacetum parthenium* caused an increase in both leaf production and parthenolide yield compared to controls.

DEDICATION

This thesis is dedicated to my family for their encouragement, support, and faith in me.

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CHAPTER 1

GENERAL INTRODUCTION

Medicinal plants

It has been estimated that perhaps 90% of the world's population currently relies on raw herbs and unrefined extracts as medicines (Small and Catling 1999). Estimates of the number of medicinal plants in use vary: Tyler (1993) estimated that close to 13,000 species of plants have been used medicinally by various cultures around the world; Deans and Svoboda (1990) state that a list of over 20,000 medicinal plant had been published; and Small and Catling (1999) quote an estimate as large as 70,000. Eisenberg *et al.* 1998, reported that the use of "alternative therapies" in the United States increased from 33.8% use in 1990 to 42.1% in 1997. In particular, use of herbal medicine increased from 2.5% in 1990 to 12.1% in 1997 in the U.S. (Eisenberg *et al.* 1998). About 23% of Canadians have used herbal medicines (Small and Catling 1999), and it has been estimated that the Canadian market for herbal medicines in 1995 was \$150 million and expected to increase at 15% per year thereafter (Cottrell 1996).

Health Canada is currently in the process of setting guidelines for the manufacturing and sale of medicinal plants (<http://www.hc-sc.gc.ca>). Presently, herbal medicines are not considered drugs, but foods, and regulated by guidelines developed within the Food Directorate under the Food and Drug Act. In addition, a separate department has recently been formed, called the Natural Health Products Directorate (NHPD) which will serve as a regulatory framework for the manufacturing and sale of natural health products (NHPs), including herbs. To reflect this distinction, a third

category of plant products has been proposed under the Food and Drug act which will be specific for NHPs. Products that are identified as members of this third category will be considered neither as foods nor drugs, but as distinct NHPs (<http://www.hc-sc.gc.ca>).

Medicinal qualities of plants are due to the chemicals that they produce. As plants grow, they produce a variety of compounds that can be classified as either primary metabolites or secondary metabolites (Taiz and Zeiger 1998). Primary metabolites include chlorophyll, lipids, amino acids, proteins, nucleic acids and carbohydrates, which are essential for plant growth, reproduction, and energy supply (Taiz and Zeiger 1998). Also, they support the organisms that feed on them (Taiz and Zeiger 1998). Secondary metabolites include a wide array of substances, many of which are bioactive molecules conferring therapeutic properties (Cox and Balick 1994) and that have apparently evolved as chemical defences against herbivory and infection (Picman 1986, Taiz and Zeiger 1998). There are three principle groups of secondary metabolites: terpenes, phenolic compounds, and nitrogen-containing products (Taiz and Zeiger 1998). Many of these chemicals have been studied extensively and a portion of them have led to the production of many modern pharmaceuticals. Approximately 50 - 60% of pharmaceutical drugs are either of natural origin or synthesized from natural products (Verlet 1990, Balandrin *et al.* 1993).

Herbal medicines possess several advantages over synthetic drugs or pharmaceuticals, some of which may be responsible for their increased popularity:

1. Most herbal medicines are less toxic (less side-effects) than their synthetic counterparts (Murray 1995, Wheatley 1997).

2. Along with decreased toxicity, the interactive nature of compounds in whole herbs may result in a greater medicinal effect (Small & Catling 1999). However it must be noted that herbal medicines are not all safe and should be treated with the same care as prescription drugs (Ernst 1998).
3. Herbal medicines are relatively inexpensive (Small and Catling 1999).
4. Herbal medicines provide an excellent reservoir of effective responses to pathogens which have become drug resistant (Small and Catling 1999).
5. Some diseases are not responsive to present-day pharmaceuticals; the large number of medicinal plants may include some as yet unrecognized cures or treatments (Small and Catling 1999).
6. As use grows, safety, toxicity, and clinical effectiveness data are being gathered for herbal medicines at an increasing rate (Murray 1995).
7. Herbal medicines provide commercial value and expand trade opportunities (Murray 1995, Small and Catling 1999).

Undoubtedly, the cultivation of herbs may provide a significant economic opportunity for farmers (Small and Catling 1999). Currently, wild-crafting or gathering of plants from their natural wild habitat, is a common method of medicinal plant collection (Small and Catling 1999). However, as popularity of particular herbs grows, unsustainable collection may affect their natural survival as evidenced by the over-harvesting of Canadian ginseng (Small and Catling 1999). Hence, cultivation is becoming increasingly important. Cultivation has several advantages, including: proximity of herbal supply, uniform maturation, consistency of chemical concentrations, being certified as organic, or serving as additional cash crops for farmers (Small and

Catling 1999). Ultimately, cultivation can preserve wild sources while diversifying Canadian agriculture.

Another problem concerning medicinal plants is contamination. Several herbal products have been found to be contaminated with any of lead, zinc, mercury, arsenic, aspirin, paracetamol, triamcinolone, mefenamic acid, diazepam, thallium, or pesticide residues, and often multiple contaminants are present in a single herbal preparation (Ernst 1998; Abou-Arab and Abou Donia 2001). Moreover, misidentification of plants and their subsequent inclusion in medicinal products have resulted in poor clinical responses and sometimes toxic effects (Ernst 1998).

For quality control, herbal extracts must contain a set level of active constituents known to be medicinally active. Recent chemical analyses of commercially available products, such as extracts of feverfew (*Tanacetum parthenium*) and St. Johnswort (*Hypericum perforatum*), for active constituents (parthenolide and hypericin, respectively) were shown to be below their labelled claim or were non detectable (Heptinstall *et al.* 1992, Constantine and Karchesy 1998). Variability in the level of active constituents can occur due to plant genetic variation, season, temperature, geographic location, nutrient availability, plant age, plant part used, and harvesting and processing techniques (Jensen *et al.* 1995, Murray 1996, Hendriks *et al.* 1997, Buter *et al.* 1998, Denke *et al.* 1999).

At present, most medicinal plants are grown under field conditions, but it is difficult to control growing conditions and maintain consistent product yields under such circumstances. Variations in chemical constituents and yield of plants may be minimized and optimized through greenhouse hydroponic cultivation. According to Jensen and

Collins (1985), the main advantages of greenhouse hydroponics include: absence of soil borne diseases, indifference to soil chemistry and suitability, reduced land area requirements, shortened growth cycle, conservation of water, minimization of weed problems, retention of fertilizer, reduced transplant shock, indifference to ambient temperature, independence of season, and growing conditions can be altered quickly to suit specific species or growth stages. Some disadvantages to greenhouse hydroponics are: high initial set-up cost, a degree of skill and competence in plant science is required, disease and pests can spread quickly (especially when plants share the same nutrient solution) and not all plant species are suitable for hydroponic growth (Jensen and Collins 1985).

Outline of research objectives

This study was conducted to identify medicinal plants with commercial potential, evaluate their suitability for greenhouse hydroponic growth, determine optimal concentrations and ratios of nutrient solutions, and optimize growth and yield of target tissues. Experiments were also conducted to more specifically assess the influence of nitrogen on plant growth and levels of bioactive chemical constituents in two medicinally important plants, *Hypericum perforatum* and *Tanacetum parthenium*. In addition, experiments were conducted to determine if management practices such as leaf removal and flower bud removal would affect target tissue yields and secondary metabolite production in *Hypericum perforatum* and *Tanacetum parthenium*.

Hypericum perforatum

Hypericum perforatum (St. Johnswort) is a member of the Hypericaceae family. The genus *Hypericum* is comprised of about 350 species (Mitich 1994). *Hypericum perforatum* is an upright perennial, 20 to 100 cm in height (Mitich 1994). Stems are multi-branched, with simple, oblong-ovate, opposite, dark green leaves (Crompton *et al.* 1988). The leaves are perforated with many clear glands on the leaf surface and with dark glands along the leaf margins (Fields *et al.* 1990). Flowers are numerous, golden yellow with dark glands, perfect, 5-petaled, borne in cymes (Crompton *et al.* 1988) (see Appendix Figure A.1).

Since ancient times, *Hypericum perforatum* has been utilized as a medicinal plant for its astringent, diuretic, and sedative properties, in the treatment of diarrhea, dysentery, jaundice, nervous irritation, hemoptysis, hard tumors and bruises (Mitich 1994).

Recently, clinical trials have demonstrated that St. Johnswort extracts are an effective and tolerant treatment for mild to moderate depression (Sommer and Harrer 1994, Woelk *et al.* 1994, Mueller 1998) and equally efficacious as compared to tricyclic antidepressants such as amitriptyline and imipramine, but with fewer side effects (Vorbach *et al.* 1994, 1997, Wheatley 1997). *Hypericum* extracts have been shown to inhibit 5-Hydroxytryptophan (5-HT), norepinephrine, and dopamine reuptake, to inhibit monoamine oxidase and interleukin-6, and to upregulate 5-HT_{1A} and 5-HT₂ postsynaptic receptors (Greeson *et al.* 2001). St. Johnswort extracts have also been shown to have antiviral, antibacterial and antiglioma activities (Barbagallo and Chisari 1987, Takahashi *et al.* 1989, Lopez-Bazzocchi *et al.* 1991, Couldwell *et al.* 1994).

The therapeutic extracts of *Hypericum perforatum* contain a variety of chemical constituents (Nahrstedt and Butterweck 1997) and are often made solely from the aerial portion of the plant. Recent studies have shown hyperforin, a phloroglucinol, found in the flowers and leaves of *Hypericum perforatum* (Umek *et al.* 1999) to have antidepressant effects (Chatterjee *et al.* 1998, Laakmann *et al.* 1998).

Other studies have shown hypericin, a naphodianthrone, found in the dark glands of the leaves, stems and flowers of *Hypericum perforatum* (Fields *et al.* 1990, Repcak and Martonfi 1997, Briskin *et al.* 2000) to play a role in St. Johnswort's antidepressant activity (Butterweck *et al.* 1997, Butterweck *et al.* 1998). located

Tanacetum parthenium

The herb, feverfew, *Tanacetum parthenium* (L.) Shultz-Bip (Asteraceae), has been used for centuries as a medicinal plant (Knight 1995). *Tanacetum parthenium* is a bushy perennial, 30 to 92 cm in height. Its leaves are pinnately divided into coarsely-toothed, ovate divisions. The daisy-like capitula are many, in open terminal clusters with yellow center disks and radiating white florets. Depending on the cultivar, leaf colour ranges from dark green, to light green, to yellow (Hobbs 1989). Flowers also differ depending on cultivar, with one or more rows of ray florets, which can be spaced or overlapped. Some varieties completely lack ray florets (Hobbs 1989) (see Appendix Figure A.2).

Since ancient times, *Tanacetum parthenium* has been used for the treatment of fever, migraine, arthritis, anemia, earache, stomachache, toothache, dyspepsia,

dysmenorrhea, psoriasis and insect bites (Knight 1995; Murray 1995, Heptinstall and Awang 1998).

Recently, clinical trials have demonstrated that dried feverfew leaves can prevent migraines, reduce frequency of migraine attacks, decrease associated nausea and vomiting, and minimize sensitivity to noise and light (Johnson *et al.* 1985, Murphy *et al.* 1988, Palevitch *et al.* 1997). Although the physiological mechanism of migraine prophylaxis has not yet been elucidated, extracts of feverfew are known to inhibit serotonin release, platelet aggregation, and prostaglandin synthesis (Pugh and Sambo 1988, Groenwegen and Heptinstall 1990, Marles *et al.* 1992). The chemical constituent thought to be responsible for these activities is parthenolide, a sesquiterpene lactone (Pugh and Sambo 1988, Groenwegen and Heptinstall 1990, Marles *et al.* 1992). This substance is found in the glandular trichomes of leaves, flowers and seeds of *Tanacetum parthenium* (Banthorpe *et al.* 1990, Hendriks *et al.* 1997, Cutlan 2000).

CHAPTER 2

CULTIVATION OF MEDICINAL PLANTS, *ACHILLEA MILLEFOLIUM*, *TANACETUM PARTHENIUM*, *LEONURUS SIBIRICUS*, AND *LINUM* *USITATISSIMUM* IN GREENHOUSE HYDROPONICS

Introduction

In North America, the use of natural products has increased dramatically in recent decades (Craker 1999, Saxena *et al.* 1999). In 1997, Canadians spent approximately \$340 million on herbal products (Saxena *et al.* 1999). The herbal market represents opportunities for increased domestic production of medicinal plants (Craker 1999).

In Canada, herbal products are imported from as many as 42 different countries, making it difficult to guarantee that the plants utilized have not been sprayed with pesticides or contaminated with mercury and other fungicides (Adams 1996). Many of the cultivated herbs in Egypt are considered of high quality because of their active constituents, which has encouraged their export from Egypt (Abou-Arab and Abou Donia 2001). However, Abou-Arab and Abou Donia (2001) found that a variety of medicinal plants collected from various sources in Egypt contained malathion at levels exceeding the maximum permissible levels recognized by the Egyptian Organization for Standardization and Quality Control. For instance, Abou-Arab and Abou Donia (2001), found *Matricaria chamomila* to have concentrations of malathion, and dimethoate and residues of lindane, aldrin, dieldrin, DDT, chlordane, and endrin all exceeding the maximum permissible levels.

Another problem concerning medicinal plants is wild-crafting. Canada's most prized medicinal plant, ginseng (*Panax quinquefolius*), contributes approximately \$100 million annually to the Canadian economy (Small and Catling 1999). The over-

harvesting of this native Canadian species for its commercial use has caused it to be designated as “threatened” (Small and Catling 1999). Another Canadian native species, Pacific yew (*Taxus brevifolia*), which furnishes the anticancer drug taxol, has also decreased in abundance and natural supplies can no longer meet market demand (Small and Catling 1999). Moreover, when a herb is collected from the wild, there is a greater probability of misidentification that is harder to detect once the material is dried for processing (Murray 1995).

A possible solution to these problems is to cultivate medicinal plants in greenhouses providing optimal conditions for plant growth and ensuring toxin-free and properly identified species collection. There is a growing interest from Canadian farmers to diversify their crops and some are interested in meeting the new and expanding market for medicinal plants. However, even though there is a significant amount of information on the scientific and clinical evidence of the benefits of medicinal plants, very little is known about their cultivation, particularly in greenhouse hydroponics.

The Institute of Hydroponic Problems, in Armenia, has grown Aloe (*Aloe arborescens*), Valerian (*Valerian officinalis*), motherwort (*Leonurus quinquelobatus*) and other medicinal plants in both open-air hydroponics and soil under the climatic conditions of the Arafat valley, Armenia, and found that under soilless conditions, plant target tissues and active constituents were higher than in comparable plants grown in soil (Mairapetyan and Tadevosyan 1999). At present, most medicinal plants are grown under field conditions, but it is difficult to control growing conditions and maintain consistent product yields under such circumstances. For example, Buter *et al.* (1998) found *Hypericum perforatum* dry matter yield to vary between growing sites and Denke *et al.*

(1999) found *Hypericum perforatum* fresh mass at harvest to differ between years at the same growing sites.

The objectives of this study were to identify alternative crops that may have greenhouse potential, evaluate their suitability for greenhouse hydroponic growth, determine optimal concentrations and ratios of nutrients in hydroponic solutions, optimize growth and yield of target tissues, and assess pest susceptibility. Three nutrient solutions, varying in their proportions of nitrogen, potassium, calcium, magnesium, and electrical conductivity were examined for their effects on various growth parameters in yarrow (*Achillea millefolium*), feverfew (*Tanacetum parthenium*), Siberian motherwort (*Leonurus sibiricus*), and flax (*Linum usitatissimum*) grown in a deep root hydroponic system under greenhouse conditions.

Materials and Methods

Experimental plants

Table 2.1 outlines the characteristics of the four medicinal plants used in this study. For a line drawing of each species refer to Appendix figures A2 - A5. Seeds of *Achillea millefolium*, *Leonurus sibiricus*, *Linum usitatissimum* (cv. Omega), and *Tanacetum parthenium* were obtained from Richters Herbs (Goodwood, Ontario, Canada) and placed on top of moist rockwool plugs (2.5 cm x 2.0 cm), with one seed per plug. The seeds were then covered with a thin layer of peat moss and placed in a greenhouse compartment under the following conditions: 20 °C, 60 % RH, and supplemental lighting. All plugs received the same standard nutrient solution during

Table 2.1. Brief descriptions of *Achillea millefolium*, *Tanacetum parthenium*, *Leonurus sibiricus*, and *Linum usitatissimum*.

Latin name	Common name	Family	Life-habit	Parts used	Indications
<i>Achillea millefolium</i>	Yarrow	Asteraceae	Perennial	Leaves Flowers	Anti-inflammatory Anti-hepatotoxic
<i>Tanacetum parthenium</i>	Feverfew	Asteraceae	Perennial	Leaves Flowers	Migraine prophylactic
<i>Leonurus sibiricus</i>	Siberian motherwort	Labiatae	Perennial	Leaves Flowers	Anti-arrhythmic Anti-spasmodic on uterine muscle
<i>Linum usitatissimum</i>	Flax "Omega"	Linaceae	Annual	Seeds	Treats dysmenorrhea Treats menopausal symptoms Anti-inflammatory

propagation. Table 2.2 lists the sowing, transplant, germination, and harvest dates, and germination rate (per 100 seeds) of each species.

Experimental setup

The experiment was carried out at the Greenhouse and Processing Research Centre (Agriculture and Agri-Food Canada, Harrow, Ontario, Canada). Small plants (with roots emerging from the bottom of the rockwool plug) were lodged into matching-sized holes (2 cm diameter) in styrofoam floats. The floats were placed in 53 L Rubbermaid™ tubs filled with nutrient solution (see below). Air stones (30.5cm), connected to an airline (pressure was maintained at 4 PSI) by PVC tubing (4.8mm diameter and 70 cm long), provided aeration and water movement. Plant roots were completely immersed in nutrient solution. Treatments consisted of three different nutrient regimes (I, II, III) maintained by the “Harrow Fertigation Manager”® (Papadopoulos and Liburdi, 1989), a nutrient delivery system, which allowed for programmable fertigation. The three nutrient solutions (Table 2.3) varied in their nitrogen (N), potassium (K), calcium (Ca), and magnesium (Mg) contents and were modifications of standard nutrient solutions found, in preliminary experiments, to support healthy tomato growth in greenhouses at the Greenhouse and Processing Crops Research Centre. In addition, these nutrient concentrations were close to the nutrient ranges suggested by Alberta Agriculture, Food and Rural Development for the growth of medicinal plants (<http://www.agric.gov.ab.ca>). The micronutrient concentrations in all three solutions were the same. There were four replicate tubs per treatment, in a randomized block design. Each tub contained either 8 plants of *Linum usitatissimum*, 6 plants of *Achillea millefolium*, 6 plants of *Tanacetum parthenium*, or 4 plants of *Leonurus*

Table 2.2. Sowing, germination, transplant, and harvest dates and germination rate of *Achillea millefolium*, *Tanacetum parthenium*, *Leonurus sibiricus*, and *Linum usitatissimum* in 1999.

Species	Sowing date	Germination period	% germination	Transplant date	Harvest date
<i>Achillea millefolium</i>	July 8	July 12 – 14	66	July 23	December 21 - 26
<i>Tanacetum parthenium</i>	July 21	July 26 - August 3	93	August 10	January 4 – 8
<i>Linum usitatissimum</i>	August 19	August 23 - 26	93	September 7	January 11 - 14
<i>Leonurus sibiricus</i>	October 18	October 22 - 27	77	November 22	April 13 - 15

Table 2.3. Concentration of macronutrients and electrical conductivity of the three nutrient regimes.

Macronutrients	Nutrient Treatment		
	I (mg/L)	II (mg/L)	III (mg/L)
Nitrogen (from NO₃)	75	115	133
Nitrogen (from NH₄)	5	5	7
Phosphorus	40	40	40
Potassium	214	321	365
Calcium	120	150	180
Magnesium	30	40	50
Electrical Conductivity (mS/cm)	1.6	2.1	2.5

sibiricus. Every four days, water levels in the tubs were “topped-up” by adding either tap water or the appropriate nutrient solution, alternatively, to replace what was absorbed by the plants. Plants were grown at 21 °C, and 75 % RH under natural photoperiod (without supplemental lighting).

The pests observed on each plant during the course of the study were documented, as were the biocontrol agents used. Biocontrol agents were put into the greenhouse compartment within one week of the first sighting of a pest. The effectiveness of the biocontrol was rated according to the following: low (no control of pests), moderate control (some decrease of pest population observed), good (few pests observed), and excellent (no pests apparent).

Data collection

Depending on species, the plant measurements taken at harvest were: number of flowers, flower buds and fruit; total leaf area per plant; and plant height. Refer to Table 2.2 for harvest dates. Plant tissues were separated and placed in paper bags, labeled, and dried at 35-40 °C to constant mass. Individual plant roots could not be separated and sorted, due to root entanglement between plants within tubs; however total root mass per tub was easily determined, so average mass per plant in a tub could be calculated. Two weeks later, the dry masses of leaf, stem, buds, flowers and belowground (caudex + root) tissues were recorded.

Statistical analyses

Statistical analyses were conducted using the SYSTAT 9.0 statistical package (SPSS Inc., 1999). Nested analyses of variance were conducted to determine whether there were significant differences among nutrient treatments with respect to various

growth parameters and to determine if there were differences between tubs within specific treatments. One-way ANOVAs were used to distinguish differences in belowground dry mass (caudex + root) per tub among nutrient treatments. Tukey post-hoc multi-comparison tests were then used where appropriate to examine the detail of the differences among nutrient treatments for each growth parameter.

Results

Achillea millefolium

The nested ANOVA results for *Achillea millefolium* plant height, stem dry mass, number of flowers, total reproductive tissue dry mass (flowers + buds + peduncles), green leaf dry mass, brown leaf dry mass, total leaf dry mass (green leaves + brown leaves), and total aboveground dry mass are summarized in Table 2.4. No significant differences due to nutrient treatment were observed for the above parameters. However, significant differences ($p \leq 0.05$) between tubs within a specific treatment were found for height and reproductive weight. In addition, no significant differences were observed for belowground tissue per tub among treatments. Nested ANOVAs also revealed no statistically significant differences among treatments on the proportion of biomass allocated to reproductive tissue, leaf tissue, stem tissue, and belowground tissue.

Tanacetum parthenium

Table 2.5 summarizes the nested ANOVA results for *Tanacetum parthenium*. No significant differences were found between nutrient treatments for most parameters. However, root dry mass was observed to be significantly different between treatments ($p \leq 0.001$), with the lowest nutrient regime producing the greatest root mass, but only significantly greater than the highest nutrient regime (Figure 2.1). Results of Nested

Table 2.4. Results of nested analysis of variance to determine the effects of nutrient treatment on various *Achillea millefolium* growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Plant height (cm)	2	270.439	0.952	0.395	8	760.255	2.676	0.019
Stem dry mass (g)	2	27.301	0.874	0.426	8	64.171	2.054	0.066
Number of flowers	2	143026.676	0.443	0.645	8	624253.902	1.935	0.082
Total reproductive tissue dry mass (g)	2	10.427	1.005	0.376	8	26.041	2.510	0.027
Green leaf dry mass (g)	2	91.411	0.452	0.639	8	219.537	1.086	0.388
Brown leaf dry mass (g)	2	13.834	1.571	0.218	8	4.918	0.558	0.806
Total leaf dry mass (g)	2	183.875	0.718	0.493	8	180.331	0.706	0.686
Aboveground dry mass (g)	2	51.031	0.199	0.820	8	496.889	1.934	0.074

Table 2.5. Results of nested analysis of variance to determine the effects of nutrient treatment on various *Tanacetum parthenium* growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Stem dry mass (g)	2	5.643	1.429	0.249	9	5.644	1.430	0.202
Green leaf dry mass (g)	2	151.288	0.734	0.485	9	193.325	0.937	0.501
Brown leaf dry mass (g)	2	40.897	1.933	0.155	9	22.326	1.055	0.411
Total leaf dry mass (g)	2	279.612	0.899	0.413	9	267.226	0.859	0.566
Aboveground dry mass (g)	2	365.911	0.842	0.437	9	381.555	0.878	0.551
Leaf area (cm²) per plant	2	5972372.857	0.516	0.600	9	4059684.656	0.351	0.952

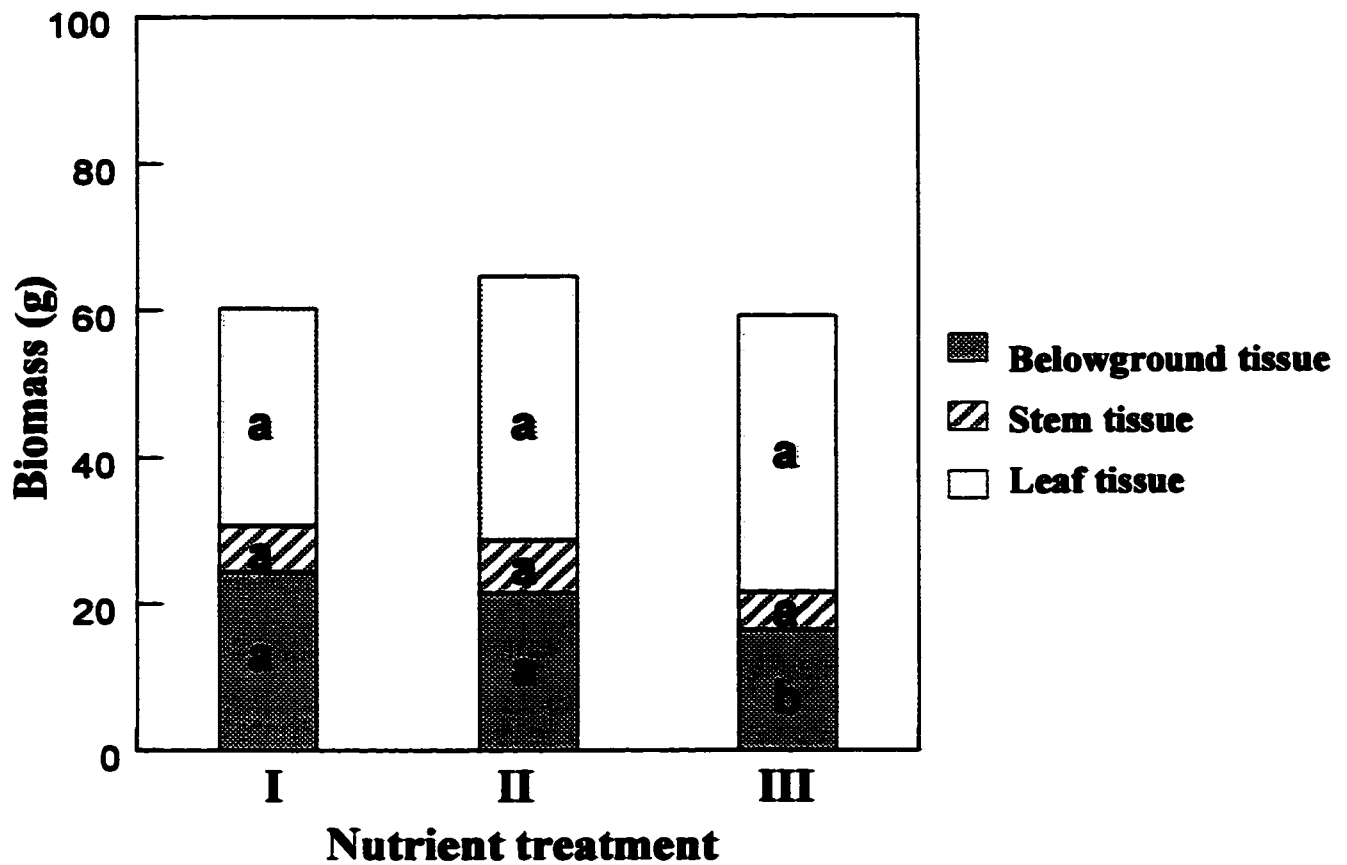


Figure 2.1. Effects of nutrient treatment on the biomass (g) of leaf, stem and belowground tissue in *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other.

ANOVAs indicated the proportion of biomass allocated to leaf tissue and belowground tissue differed significantly between treatments (both $p \leq 0.001$). Allocation to leaf tissue showed an upward trend with increased nutrient concentrations, whereas allocation to root tissue decreased with an increase in nutrients (Figure 2.2).

Leonurus sibiricus

Nested ANOVAs on the proportion of biomass allocated to leaf, stem, and belowground tissue for *Leonurus sibiricus* resulted in significant differences between treatments ($p \leq 0.01$, 0.05, 0.01, respectively). Plants in nutrient treatment II allocated significantly more to leaf tissue and stem tissue, than plants from treatment I. In contrast, plants in treatment I allocated significantly more to belowground tissue compared to plants in treatment II (Figure 2.3).

Table 2.6 shows the nested ANOVA results of various plant growth analyses for *Leonurus sibiricus*. Nutrient treatments had a significant effect on stem dry mass ($p \leq 0.05$), green leaf dry mass ($p \leq 0.01$), total leaf dry mass (green leaves + brown leaves) ($p \leq 0.05$), and total aboveground dry mass ($p \leq 0.01$). Stem dry mass, green leaf dry mass total leaf dry mass (Figure 2.4) and aboveground dry mass produced from plants grown in Treatment I were significantly lower than for plants in Treatment II. No significant differences in root dry mass were observed between treatments, according to ANOVA results.

Limnanthes alba

Nested ANOVAs were performed to compare the effects of nutrient treatment on the proportion of biomass allocated to reproductive tissue (viable and nonviable flower buds and fruit), vegetative tissue (stem + leaf), and belowground tissue (caudex + root) in

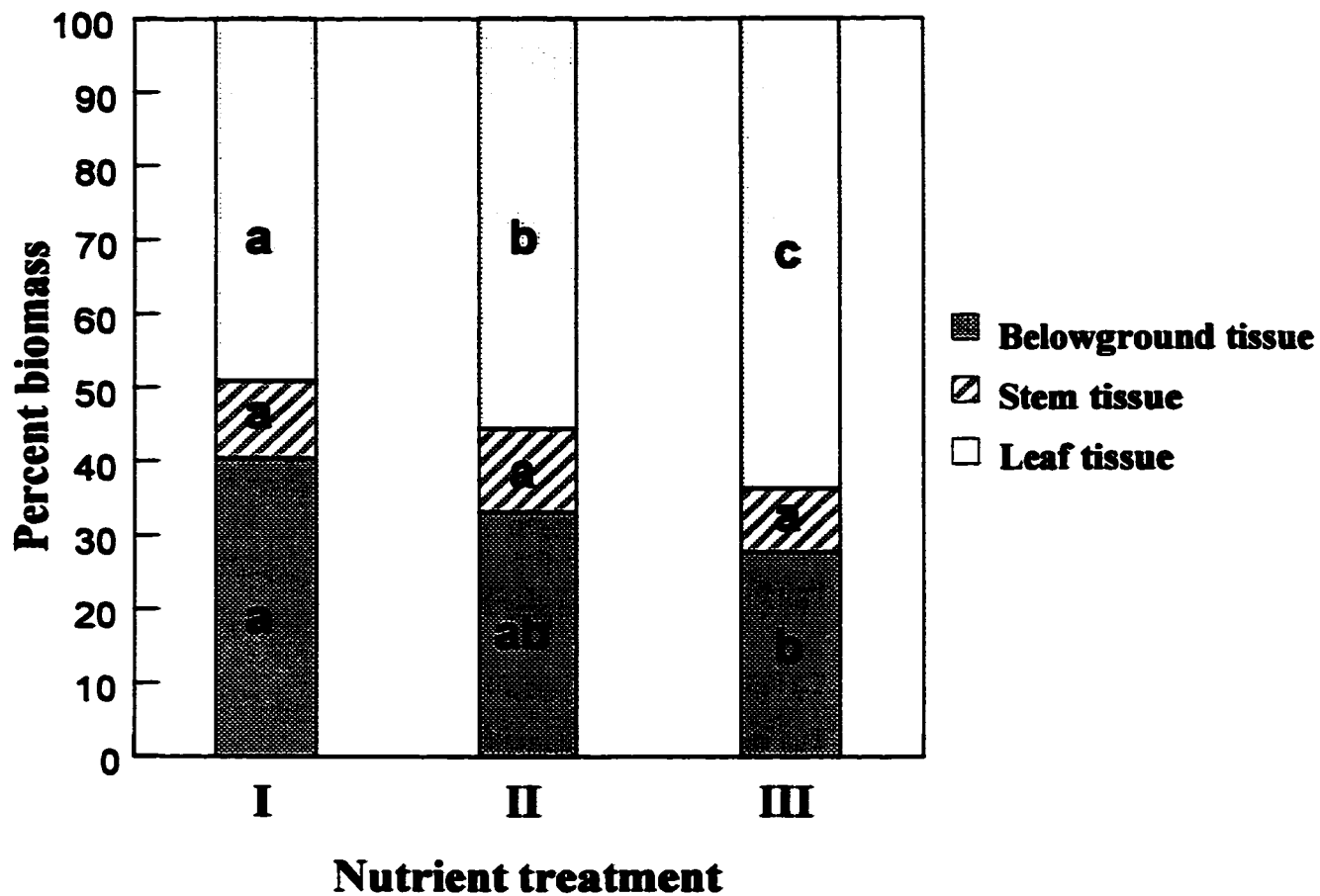


Figure 2.2. Proportional allocation of biomass to leaf, stem and belowground tissue in *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other.

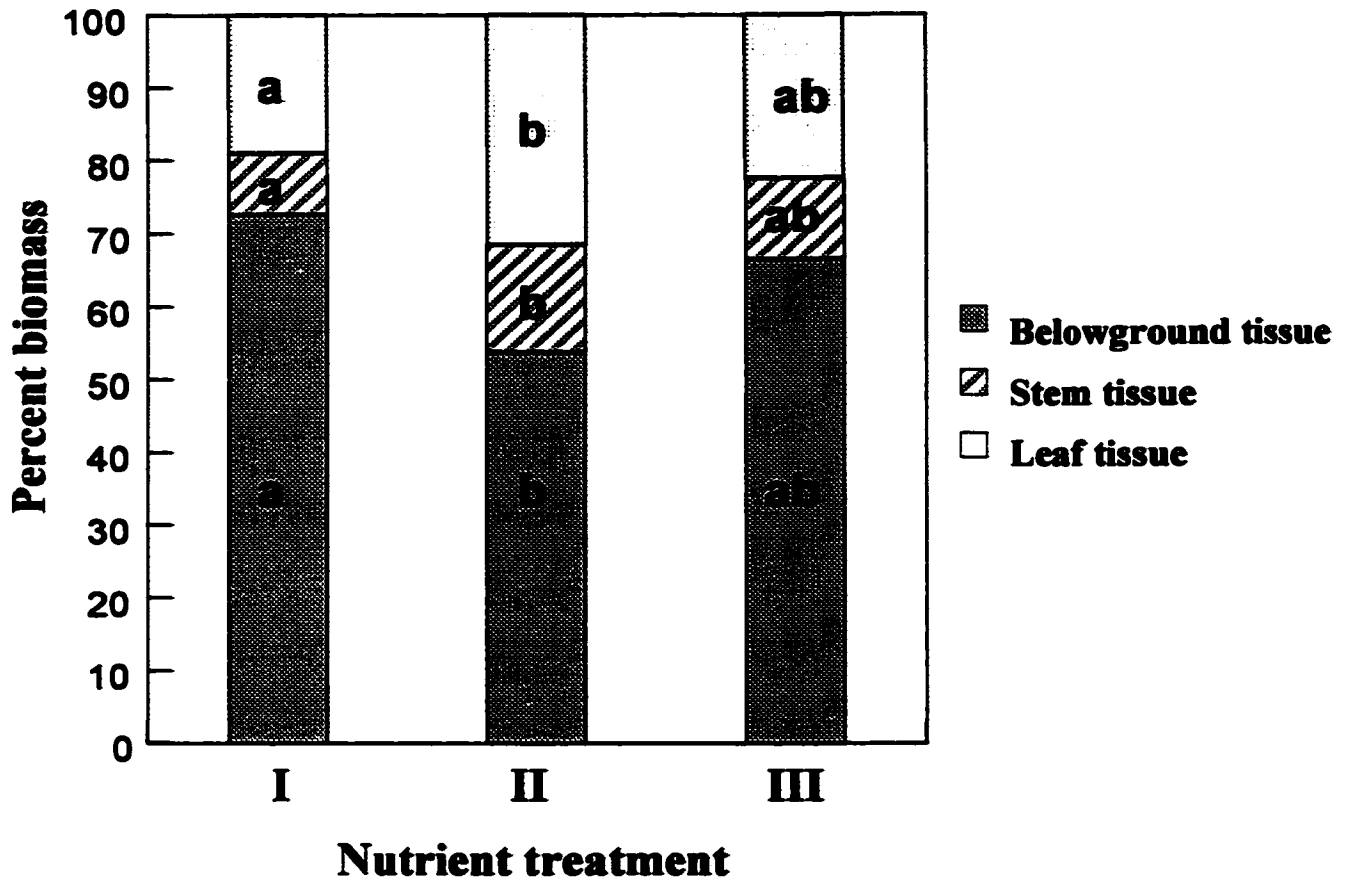


Figure 2.3. Proportional allocation of biomass to leaf, stem and belowground tissue in *Leomurus sibiricus*. Between treatments, bars with the same letter are not significantly different from each other.

Table 2.6. Results of nested analysis of variance to determine the effects of nutrient treatment on various *Leonurus sibiricus* growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Plant height (cm)	2	5023.450	3.004	0.064	9	1785.803	1.068	0.412
Stem dry mass (g)	2	155.459	4.640	0.017	8	45.671	1.363	0.250
Green leaf dry mass (g)	2	701.007	6.379	0.004	9	20.854	0.190	0.994
Brown leaf dry mass (g)	2	35.471	0.248	0.782	9	121.247	0.846	0.580
Total leaf dry mass (g)	2	824.156	4.980	0.013	9	37.859	0.229	0.988
Aboveground dry mass (g)	2	1800.195	5.495	0.009	9	147.362	0.450	0.897

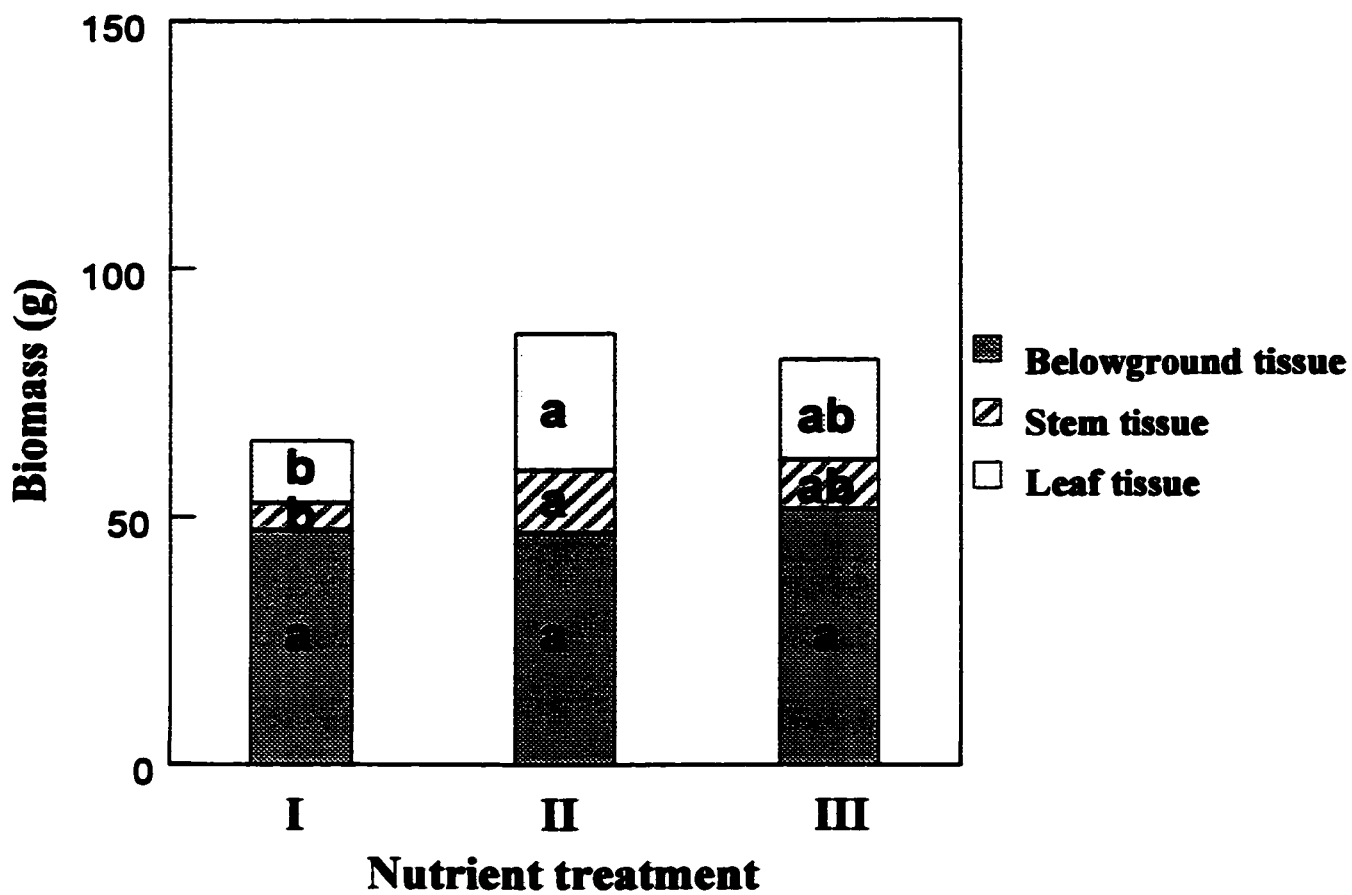


Figure 2.4. Effects of nutrient treatment on the biomass (g) of leaf, stem, and belowground tissue in *Leonurus sibiricus*. Between treatments, bars with the same letter are not significantly different from each other.

Limum usitatissimum (all $p \leq 0.000$). Plants in treatment II allocated significantly more biomass to reproductive tissue and vegetative tissue than plants in treatments I and III, and plants in treatment I allocated significantly more biomass to reproductive tissue and stem and leaf tissue compared to plants in treatment III. Plants in treatment III allocated more biomass towards belowground tissue compared to plants in treatments I and II. (Figure 2.5).

Table 2.7 summarizes the nested ANOVA results of various plant growth analyses for *Limum usitatissimum*. The buds and fruit of this plant are of particular interest, since they will eventually lead to the production of the medicinally important seeds. Thus, these structures were separated and classified as either viable (healthy) or nonviable (dried up, with no potential to yield seeds). Nutrient treatments had significant effects on plant height ($P \leq 0.05$), vegetative tissue dry mass, viable bud dry mass, nonviable bud dry mass, total bud (viable + nonviable) dry mass, viable fruit dry mass, nonviable fruit dry mass, total (viable + nonviable) fruit dry mass, total reproductive tissue (buds + fruit) dry mass, number of viable buds, number nonviable buds, number total buds (viable and nonviable buds), number viable fruit, number nonviable fruit, and number of total fruit (viable and nonviable fruit) dry mass (all $p \leq 0.001$). Belowground tissue was also found to differ between treatments ($p \leq 0.001$). All of the above parameters (except nonviable bud dry mass, viable fruit dry mass, and number of nonviable buds) were significantly higher in plants in Treatment II, compared to plants in Treatments I and III. The nonviable bud dry mass was greatest in plants from Treatment II, but only significantly different than plants in Treatment III. The number of nonviable buds and dry mass of viable fruit was significantly lower in plants from Treatment III compared to

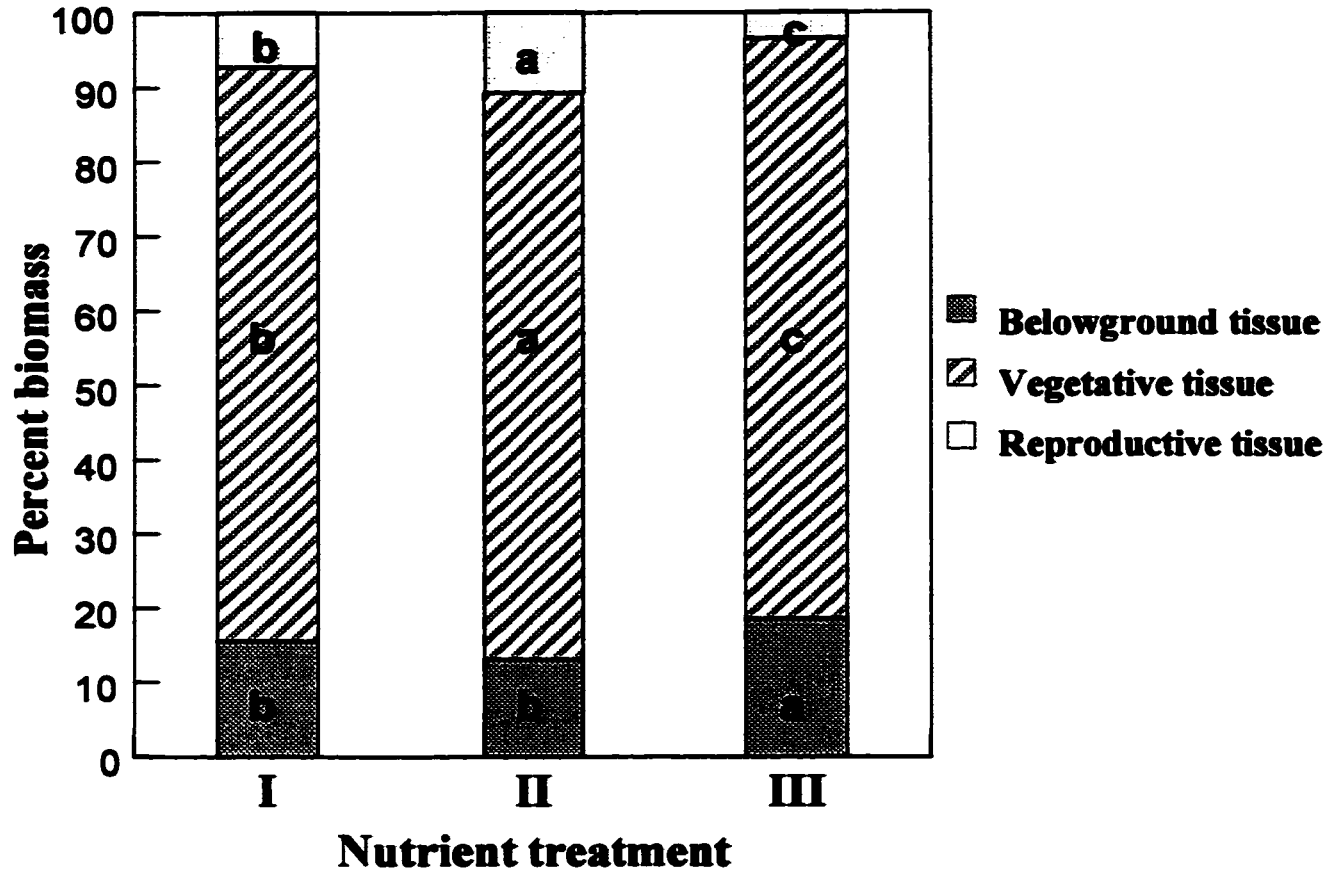


Figure 2.5. Proportional allocation of biomass to reproductive tissue (viable and nonviable flower buds + fruit), vegetative tissue (stem + leaf), and belowground tissue in *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

Table 2.7. Results of nested analysis of variance to determine the effects of nutrient treatment on various *Linum usitatissimum* growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Plant height (cm)	2	1209.899	3.348	0.042	9	679.869	1.881	0.072
Combined stem and leaf dry mass (g)	2	762.021	37.080	0.000	9	96.620	4.702	0.000
Viable bud dry mass (g)	2	0.804	33.768	0.000	9	0.202	8.460	0.000
Nonviable bud dry mass (g)	2	2.397	34.311	0.000	9	1.006	14.398	0.000
Total bud dry mass (g)	2	5.807	42.404	0.000	9	1.550	11.314	0.000
Viable fruit dry mass (g)	2	1.171	19.308	0.000	9	0.381	6.283	0.000
Nonviable fruit dry mass (g)	2	15.224	32.718	0.000	9	3.883	8.345	0.000
Total fruit dry mass (g)	2	23.287	29.971	0.000	9	6.036	7.769	0.000
Total reproductive tissue dry mass (g)	2	52.733	27.368	0.000	9	9.329	10.149	0.000
Total aboveground dry mass (g)	2	821.353	34.839	0.000	9	116.153	4.927	0.000

Table 2.7 (continued). Results of nested analysis of variance to determine the effects of nutrient treatment on various *Linum usitatissimum* growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Number of viable buds	2	32544.097	28.586	0.000	9	11546.139	10.142	0.000
Number of nonviable buds	2	131108.514	37.512	0.000	9	30549.454	8.741	0.000
Total number of buds	2	265657.681	42.361	0.000	9	53833.898	8.584	0.000
Number of viable fruit	2	6798.167	25.228	0.000	9	1544.537	5.732	0.000
Number of nonviable fruit	2	15692.764	45.450	0.000	9	8491.009	8.446	0.000
Total number of fruit	2	87548.014	43.514	0.000	9	16181.269	8.043	0.000

those from Treatments I and II (Figures 2.6 - 2.13). Significant variability between tubs for all above growth parameters, except plant height, within specific treatments was also detected for *Linum usitatissimum* plants.

The various pests that were observed on *Achillea millefolium*, *Tanacetum parthenium*, *Leonurus sibiricus*, and *Linum usitatissimum* over the course of these studies and biocontrol agents used are summarized in Table 2.8.

Discussion

Achillea millefolium and Tanacetum parthenium

The yield of the target marketable tissues (leaves and flowers) of *Achillea millefolium* and *Tanacetum parthenium*, as well as other growth parameters examined, were not significantly affected by the different nutrient regimes and their contrasting electroconductivities (ECs). High concentrations of nitrogen, known to enhance vegetative tissue at the expense of reproductive tissue (Papadopoulos 1998), did not influence leaf, flower, or stem production in these plants. Ehret and Ho (1986) found that ECs of 2, 4 and 6 mS/cm in nutrient solutions did not affect total dry mass in tomato plants. *Tanacetum parthenium* plants did, however, allocate a greater percentage of biomass to leaf tissue and less to belowground tissue, as nutrient concentrations increased. Similarly, Reuss *et al.* (1983) found the biomass investment in green leaf production in *Kyllinga nervosa*, grown hydroponically, to increase with increasing nitrogen concentrations, whereas, allocation to root production increased with low concentrations of nitrogen.

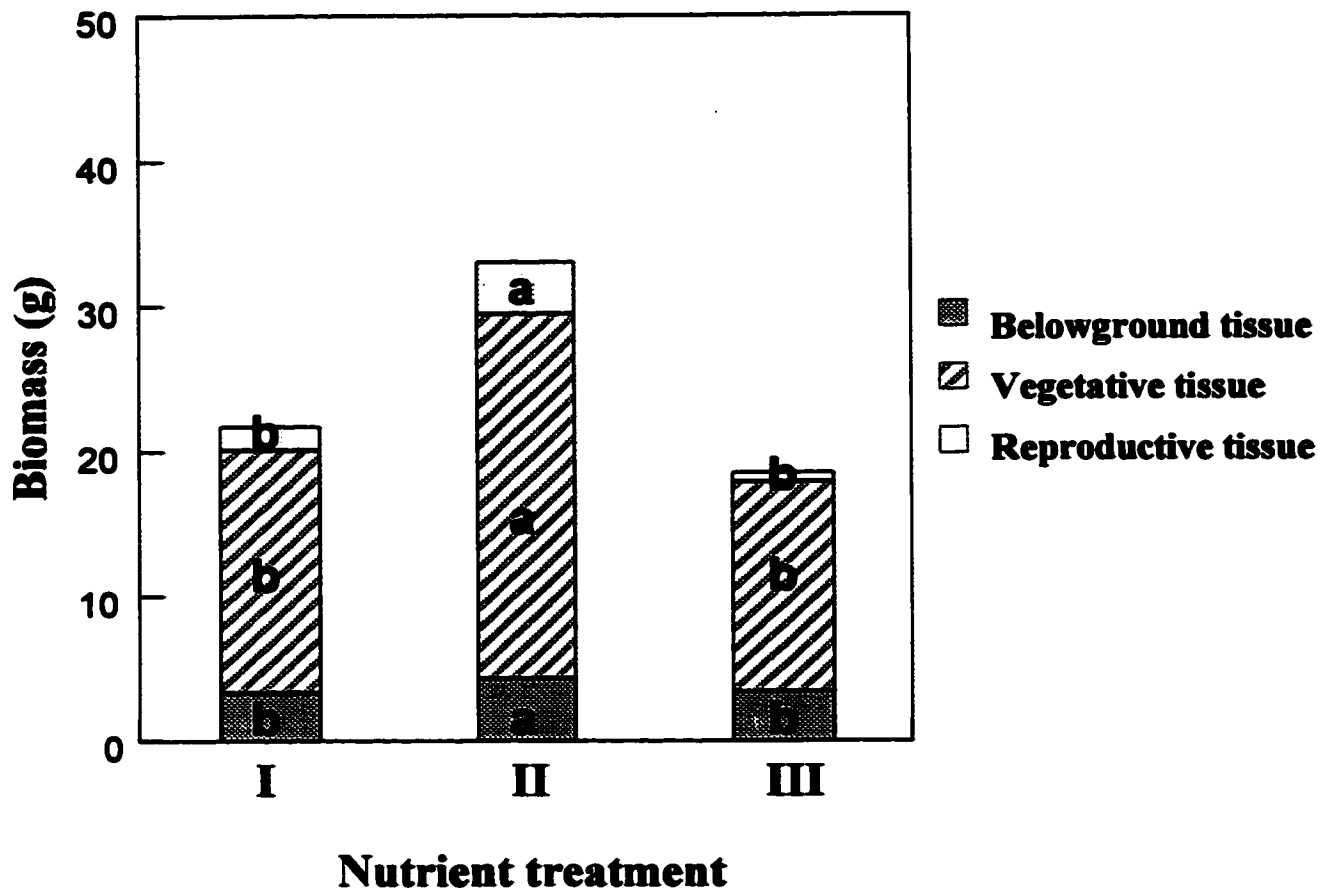


Figure 2.6. Effects of nutrient treatment on the biomass (g) of reproductive tissue (viable and nonviable flower buds + fruit), vegetative tissue (stem + leaf), and belowground tissue in *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

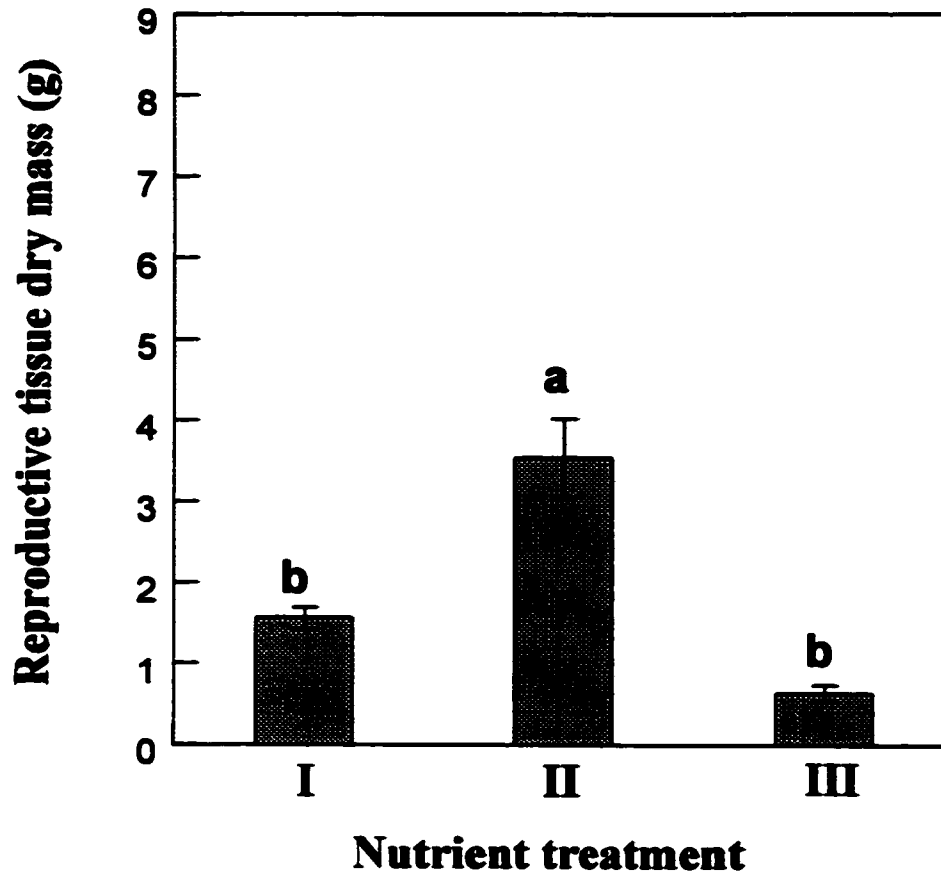


Figure 2.7. Effects of nutrient treatment on the dry mass (\pm SE) of reproductive tissue (viable and nonviable buds and fruit) of *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

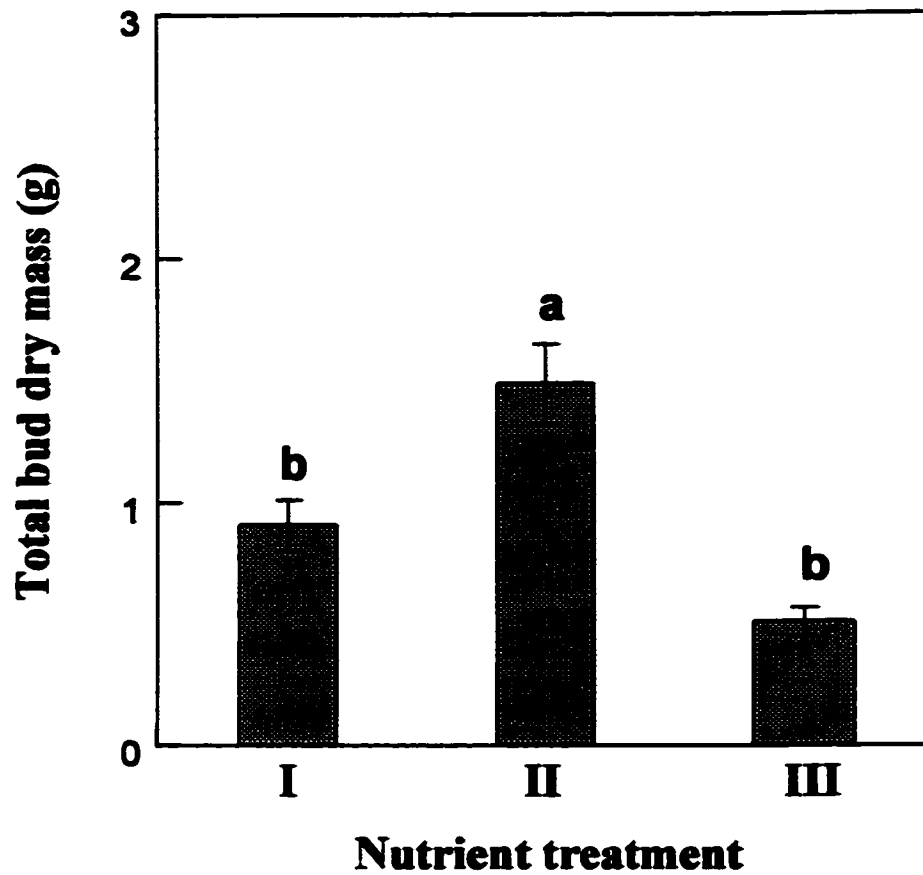


Figure 2.8. Effects of nutrient treatment on the total (viable + nonviable) bud dry mass (\pm SE) of *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

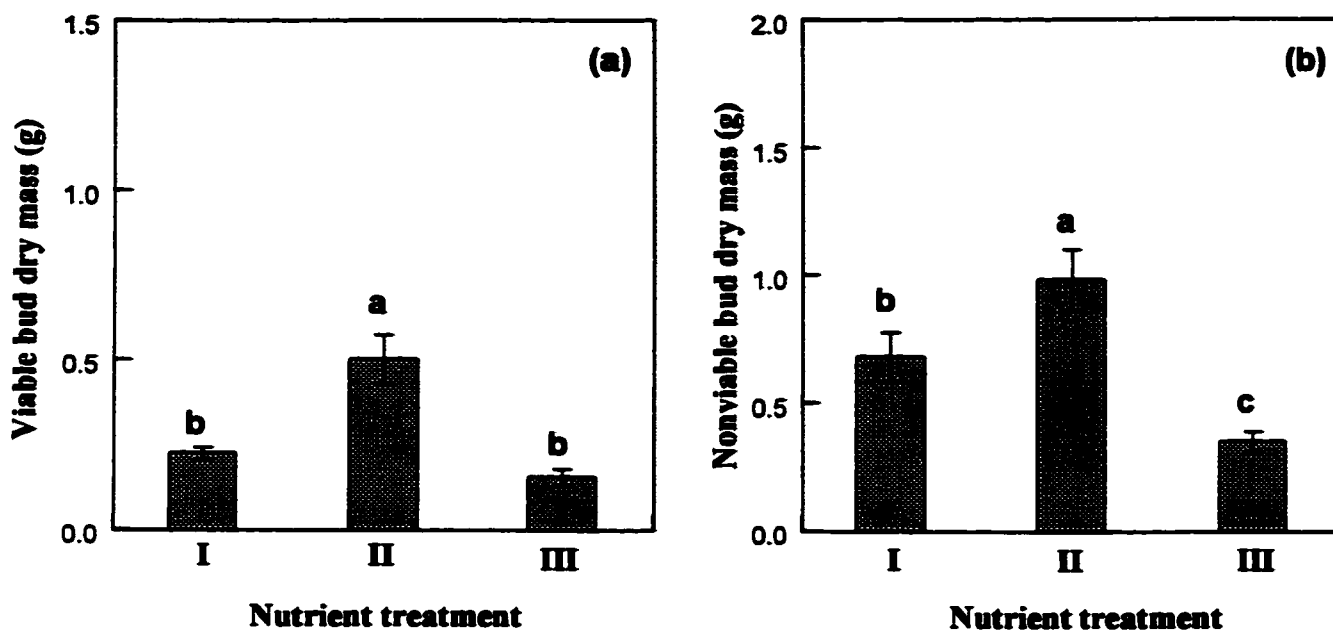


Figure 2.9 (a) and (b). Effects of nutrient treatment on (a) viable bud dry mass (\pm SE) and (b) nonviable bud dry mass (\pm SE) of *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

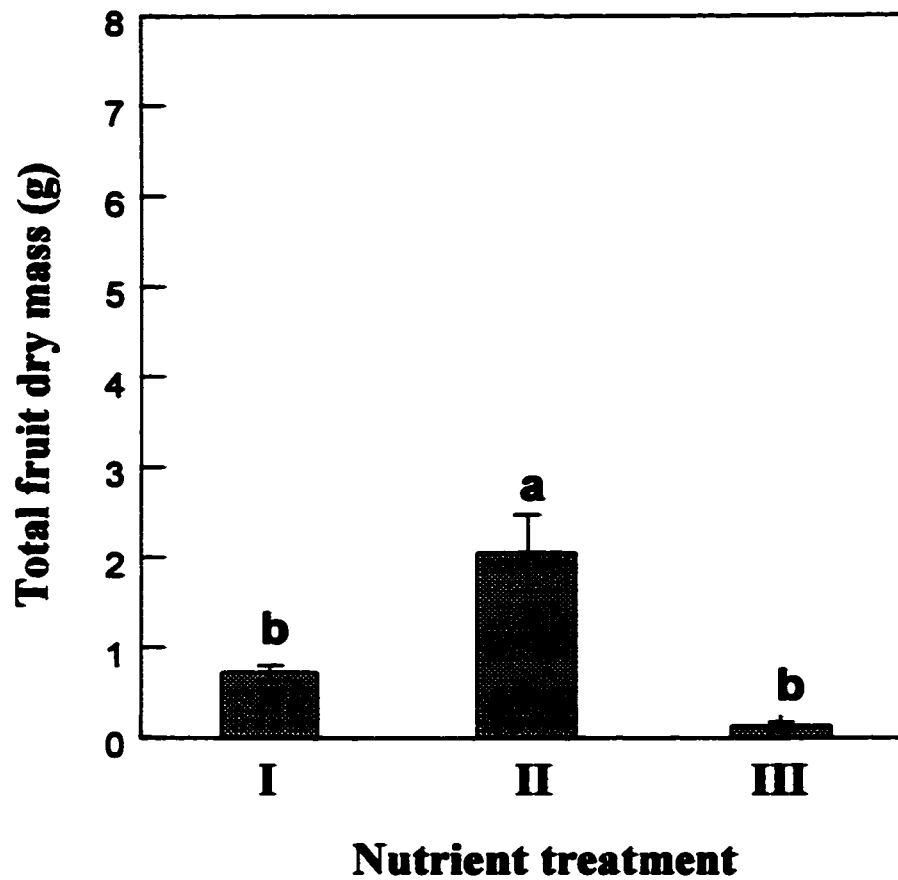


Figure 2.10. Effects of nutrient treatment on the total (viable + nonviable) fruit dry mass (\pm SE) of *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

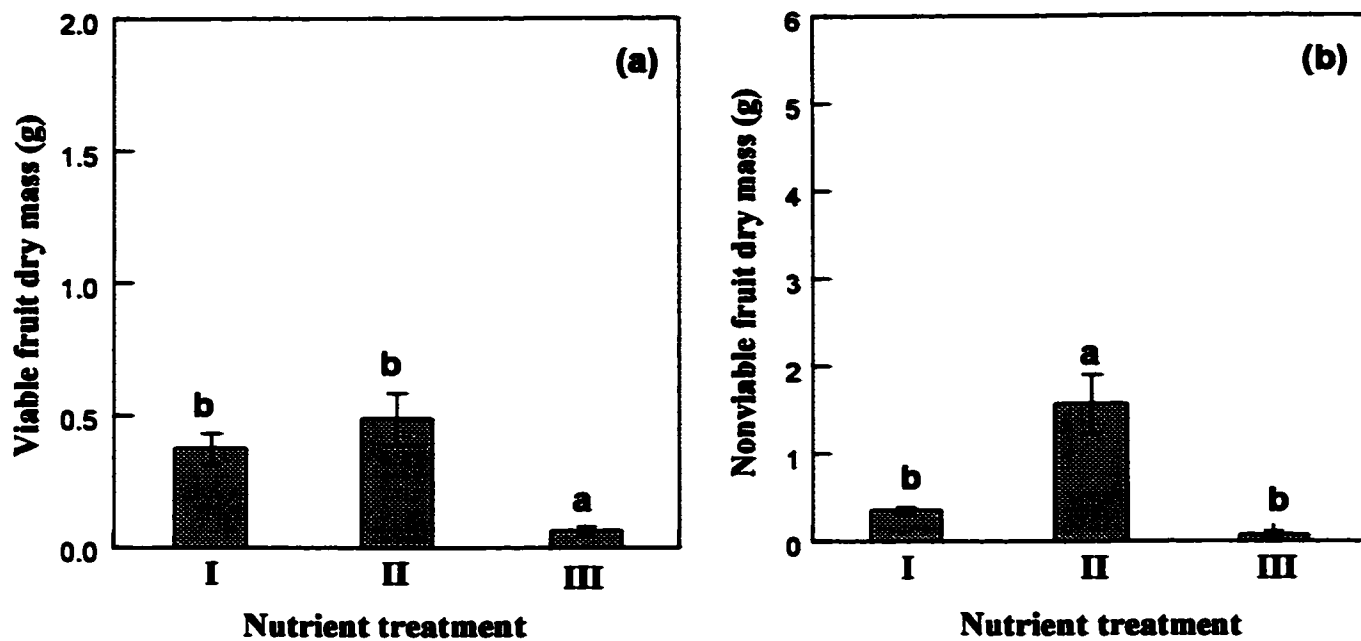


Figure 2.11 (a) and (b). Effects of nutrient treatment on (a) viable fruit dry mass (\pm SE) and (b) nonviable fruit dry mass (\pm SE) of *Limum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

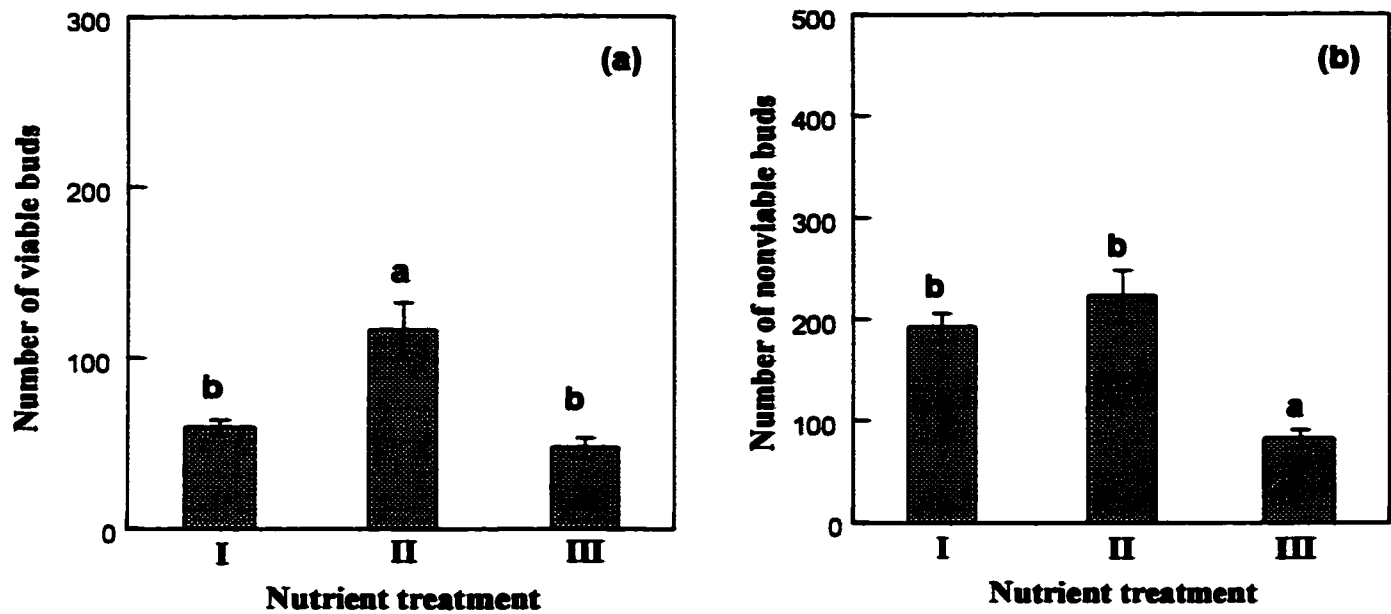


Figure 2.12 (a) and (b). Effects of nutrient treatment on the number of (a) viable buds (\pm SE) and (b) nonviable buds (\pm SE) of *Linum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

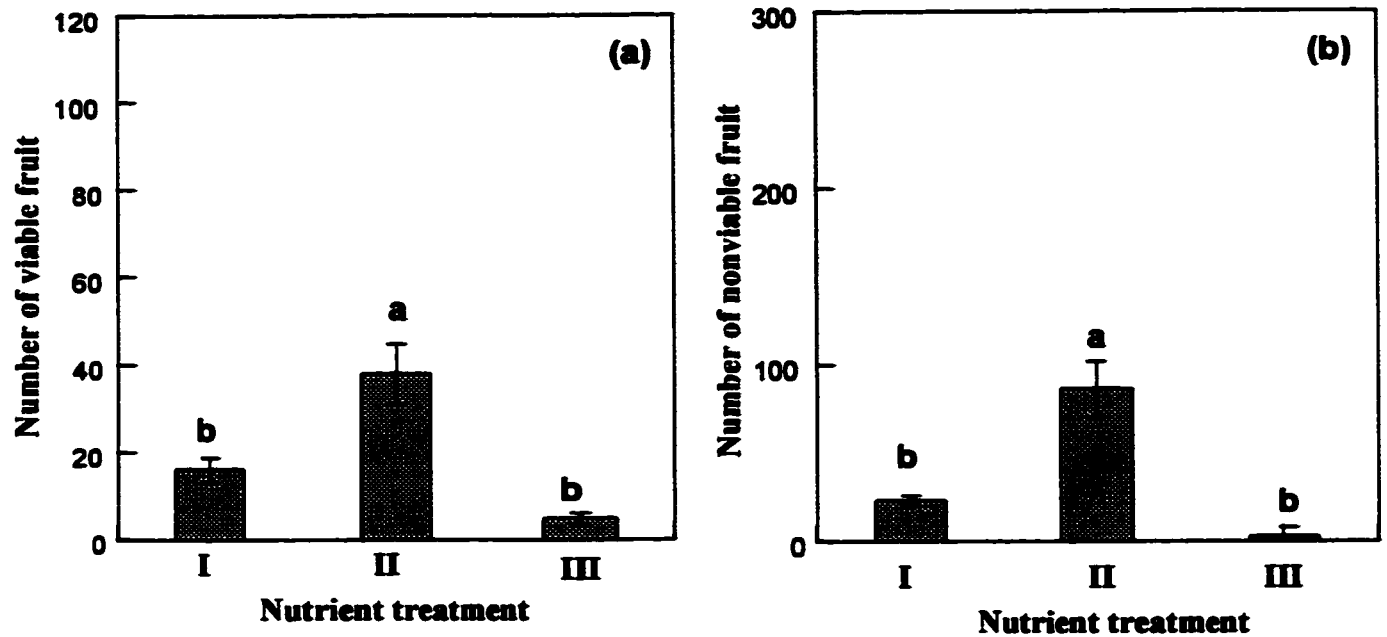


Figure 2.13 (a) and (b). Effects of nutrient treatment on the number of (a) viable fruit (\pm SE) and (b) nonviable fruit (\pm SE) of *Limum usitatissimum*. Between treatments, bars with the same letter are not significantly different from each other.

Table 2.8. Pests that appeared on *Achillea millefolium*, *Tanacetum parthenium*, *Leonurus sibiricus*, and *Linum usitatissimum* during the course of the studies and biocontrol agents used.

Plant species	Pest	Biocontrol agent	Effectiveness¹
<i>Achillea millefolium</i>	<i>Myzus persicae</i> (green peach aphid)	<i>Aphidius colemani</i> (parasitic wasp)	**
<i>Tanacetum parthenium</i>	<i>Frankliniella occidentalis</i> (western flower thrip)	<i>Amblyseius cucumeris</i> (predatory mite)	***
<i>Leonurus sibiricus</i>	<i>Trialeurodes vaporariorum</i> (greenhouse whitefly)	<i>Encarsia formosa</i> (parasitic wasp)	**
<i>Linum usitatissimum</i>	<i>Trialeurodes vaporariorum</i> (greenhouse whitefly) <i>Frankliniella occidentalis</i> (western flower thrip) <i>Tetranychus urticae</i> (two-spotted spider mite)	<i>Encarsia formosa</i> (parasitic wasp) <i>Amblyseius cucumeris</i> (predatory mite) <i>Phytoseiulus persimilis</i> (predatory mite)	*

1. Effectiveness of biocontrol

- * low (no control)
- ** moderate (decrease of pests)
- *** good (few pests)
- **** excellent (no pests apparent)

The significant differences observed between tubs within a specific treatment for height and dry mass of reproductive tissue for *Achillea millefolium* may have been due to the location of tubs in the greenhouse compartment. Tubs closest to the compartment's evaporation and cooling vent had plants with shorter plant heights and lower reproductive tissue dry masses. In contrast, plants in tubs closer to the west side (with possibly greater sunlight exposure) of the greenhouse compartment had plants which were taller and produced more reproductive tissue.

Leonurus sibiricus

Leonurus sibiricus grown in the intermediate treatment allocated a greater percentage of biomass to vegetative tissues than plants in the low nutrient treatment. Plants in the low nutrient treatment allocated more towards belowground tissues. This observation follows the same reasoning as above for *Tanacetum parthenium*.

The green leaf dry mass, stem dry mass, and total aboveground dry mass were all highest in *Leonurus sibiricus* plants from Treatment II (containing intermediate nutrient and EC levels), but only significantly higher than plants from Treatment I, which had the lowest N, K, Ca, and Mg concentrations. Munsli (1992) observed dry matter production in field grown *Mentha arvensis* to increase with higher applications of nitrogen and phosphorus. Herrera *et al.* (1997) found that shoot dry mass in *Angelica archangelica*, *Thymus vulgaris*, and *Marrubium vulgare* grown under greenhouse conditions increased as N, P, K, Ca, Mg, and EC increased in compost fertilizer applications. The total green leaf dry mass in plants from Treatment II (EC of about 2.1 mS/cm) was 2.5 times that of plants from Treatment I (EC of about 1.6 mS/cm) and 1.4 times that of plants in Treatment III (EC of about 2.5 mS/cm).

Leonurus sibiricus plants in Treatment I exhibited nutrient deficiency symptoms such as chlorosis, with the oldest leaves turning light green first, and eventually the whole plant turning light green-white by week 12. This may have been due to low N and K concentrations, and deficiencies of these ions in treatment I (Papadopoulos 1998).

Linum usitatissimum

It is crucial that *Linum usitatissimum* plants receive the proper nutritional requirements for bud, flower, fruit, and ultimately seed development, as the seeds are major contributors to the medicinal activity of this herb.

The variability detected between tubs within the three nutrient treatments for *Linum usitatissimum* plants may have been due to tub location. Tubs located in the corner with the least amount of sunlight, generally had significantly lower bud and fruit weights, and bud and fruit numbers.

Plants grown in both Treatment I (containing the lowest nutrient levels and EC) and Treatment III (with the highest nutrient concentrations and EC) yielded significantly less reproductive tissue than plants in Treatment II. The lower bud and fruit production in plants from Treatment I may be a result of insufficient supplies of N, K, Ca and Mg in the nutrient solution. Potassium and calcium have been shown to affect flower production in roses and fruit production in tomatoes (Papadopoulos 1998, Gislerod 1999). These *Linum usitatissimum* plants exhibited decreased growth with dieback of meristematic tissues, which is characteristic of calcium deficiency (Gislerod 1999). The plants also showed other visible nutrient deficiency symptoms such as necrotic spots on leaf tips and leaf chlorosis. Chlorosis first appeared on the lower (older) leaves, suggesting a mobile element such as nitrogen or potassium to have been too low in the

nutrient solution to support normal vegetative and reproductive growth (Papadopoulos 1994). The petioles and peduncles of these plants were also weak and brittle, with leaves and fruit abscising and dropping off easily.

Plants grown in Treatment III, which had the highest EC and contained the highest N, K, Ca, and Mg concentrations, showed very low bud and fruit production. High electrical conductivity can have two main effects on plants: toxicity, due to the excessive accumulation of particular ions; and nutritional imbalances, caused by an excess of a particular ion (Pasternak 1987). The high N concentrations may have accentuated K deficiency or, high K concentrations may have inhibited Ca and Mg uptake by the plant (Papadopoulos, 1994), affecting plant growth and fruit production. These plants also experienced severe chlorosis and had some dead meristematic tissue, suggesting that Ca was unable to reach the buds, fruits and flowers (Gislerod 1999).

The greatest vegetative tissue dry mass occurred in *Linum usitatissimum* grown in Treatment II. These plants produced more flowering branches compared to plants in Treatments I and III, which was evident by their higher bud and fruit production. Although these plants were highly vegetative, having many stems and dark green leaves, and lacking visible nutrient deficiency symptoms, they did not reach their maximum reproductive potential, since they produced high levels of nonviable buds and nonviable fruit.

In all of the nutrient treatments, *Linum usitatissimum* plants, experienced high pest infestation throughout their growth, which may have contributed to the reduction in reproductive success. The plants may have allocated more energy towards defense rather

than to reproductive tissues (Bazzaz *et al.* 1987) or the feeding insects may have removed large amounts of sugars from the phloem.

Conclusion

In conclusion, increasing the nutrient levels did not significantly affect the dry yields of target tissues in *Achillea millefolium* and *Tanacetum parthenium* at harvest, and thus there would be no benefit from increased nutrients in this hydroponic system for these two crops. On the other hand, *Leonurus sibiricus* and *Linum usitatissimum* both increased the production of their target tissues in response to a moderate increase in nutrients. *Leonurus sibiricus* grown in the intermediate nutrient treatment yielded the greatest amount of dry leaf tissue (target tissue), producing 2.5 to 1.4 times more leaf tissue than treatments I and III. The intermediate nutrient treatment also had a positive effect on the tissues of interest in *Linum usitatissimum*, producing the highest yields of flower buds and fruit. *Linum* plants grown in this nutrient regime, produced 2.3 to 5.6 times more reproductive tissue than plants grown in the low and high nutrient treatments, respectively. Although the intermediate nutrient levels furnished *Linum* plants with the highest amounts of desired tissue, it also yielded the greatest amount of nonviable buds and fruit.

CHAPTER 3

EFFECTS OF NITROGEN ON VEGETATIVE GROWTH AND LEVELS OF SECONDARY METABOLITES IN *HYPERICUM PERFORATUM* AND *TANACETUM PARTHENIUM*

Introduction

Since ancient times, *Hypericum perforatum* and *Tanacetum parthenium* have been utilized as medicinal plants (Mitich 1994, Knight 1995). Today, *Hypericum perforatum* (St. Johnswort) and *Tanacetum parthenium* (feverfew) are among the most commonly used and intensely researched medicinal herbs (O'Hara *et al.* 1998). Recently, clinical trials have demonstrated St. Johnswort extracts to be an effective treatment for mild to moderate depression (Sommer and Harrer 1994, Woelk *et al.* 1994, Mueller 1998). These extracts contain a variety of chemical constituents (Nahrstedt and Butterweck 1997) and are usually made from the aerial portion of the plant. Studies have shown that the carbon-based secondary metabolites, hyperforin and hypericin, play important roles in the antidepressant activity of St. Johnswort (Butterweck *et al.* 1998, Chatterjee *et al.* 1998).

Hyperforin is found in the leaves and in the flowers of *Hypericum perforatum* (Umek *et al.* 1999). Hypericin is located in the dark glands of the leaves, stems and flowers of *Hypericum perforatum* (Fields *et al.* 1990, Repcak and Martonfi 1997, Briskin *et al.* 2000). Most commercial *Hypericum* preparations are standardized with respect to their hypericin content to contain at least 0.3 % hypericin (LaValle *et al.* 2000). Constantine and Karchesy (1998) found that a number of St. Johnswort preparations on the American market varied in their percentage of hypericin from 0.141% to 0.355% dry mass.

The concentration of hypericin varies within the plant, with the greatest concentration in flowers followed, in order, by: seed capsules, top leaves, bottom leaves, and stems (Southwell and Campbell 1991, Denke *et al.* 1999). In addition, genetic and environmental aspects affect hypericin concentrations in *Hypericum perforatum* (Jensen *et al.* 1995, Buter *et al.* 1998, Briskin *et al.* 2000). Hypericin has been shown to vary in concentration with leaf morphology (Southwell and Campbell 1991), accession, geographic location, (Buter *et al.* 1998), temperature (Jensen *et al.* 1995), and nitrogen fertilization (Denke *et al.* 1999).

In addition, there has been an increasing interest in the levels of the carbon-based secondary metabolite, parthenolide, found in the leaves of *Tanacetum parthenium*. Parthenolide, the predominant sesquiterpene in *Tanacetum parthenium*, is thought to be responsible for the plant's migraine prophylactic activities (Johnson *et al.* 1985, Murphy *et al.* 1988, Pugh and Sambo 1988, Groenwegen and Heptinstall 1990, Marles *et al.* 1992, Palevitch *et al.* 1997).

Hendriks *et al.* (1997) found that the percentage of parthenolide in *Tanacetum parthenium* grown under greenhouse soil conditions was highest at an early stage of development (just before stem formation) and gradually decreased as growth continued. However, the quantity of parthenolide per individual plant increased with growth and was greatest when the plant was in full bloom. In addition, parthenolide content has been shown to vary among different feverfew cultivars grown under identical greenhouse conditions (Cutlan *et al.* 2000).

In order to achieve consistency of *Tanacetum parthenium* preparations, Health Canada has proposed that feverfew products must be in the form of dried leaf material,

formulated into tablets or capsules, containing no less than 2000 µg/g parthenolide. Abourashed and Khan (2000) found that a number of feverfew preparations on the Canadian and European market varied greatly in parthenolide content, ranging from 0 to 3600 µg/g dry mass of parthenolide. Similarly, Heptinstall *et al.* (1992) found commercial feverfew products from the United Kingdom and Canada varied significantly in their parthenolide content, from undetectable up to as much as 10 000 µg/g dry mass of parthenolide. Therefore, there is an interest in understanding the factors that affect feverfew leaf productivity and its parthenolide levels.

Nutrient availability has been shown to affect the yield of various plants (Munsi 1992, Papadopoulos 1998), and to affect carbon allocation to carbon-based chemical defences such as sesquiterpenes and phenols (Mihaliak and Lincoln 1985, Mihaliak and Lincoln 1989, Briskin *et al.* 2000) which exhibit antiherbivore activity on insects and mammals (Picman 1986, Arnason *et al.* 1983). It has been suggested that parthenolide and hypericin are produced as defensive compounds in *Tanacetum parthenium* and *Hypericum perforatum*, respectively (Knight 1995, Fields *et al.* 1990).

If *Hypericum perforatum* and *Tanacetum parthenium* were grown in a greenhouse under controlled climatic conditions and with manipulation of management practices such as optimization of nutrient conditions, we propose that an increase in yield and consistency of product quality would result. Munsi (1992) found dry matter accumulation of field grown *Mentha arvensis* increased with increased applications of nitrogen. In tomato cultivation, the most common technique used to control crop growth is by manipulating nitrogen and water supply to the plant (Papadopoulos 1998). Nitrogen is a mineral element that is required in the greatest amount by a plant and contributes

more toward vegetative growth than to reproductive tissues (Papadopoulos 1998, Taiz and Zeiger 1998). Nitrogen is an essential component of amino acids, nucleic acids, and chlorophyll (Mauseth 1998). Therefore a deficiency can greatly reduce plant growth, and if such a deficiency persists chlorosis may result (Taiz and Zeiger 1998).

Resource availability has been hypothesized to be a major factor influencing the quality and quantity of secondary metabolites, with slow-growing, nutrient-limited species producing more defenses than fast growing-species in nutrient-rich environments (Coley *et al.* 1985). In addition, the Carbon/Nutrient Balance hypothesis proposes that secondary metabolites are affected by nutrient availability. When nutrients, such as nitrogen are limiting, leaf non-structural carbohydrates increase and carbon-based secondary metabolites accumulate (Bryant *et al.* 1983).

Here we examined the effects of nitrogen concentration on various growth parameters such as leaf yield and production of hypericin and hyperforin in the leaves of *Hypericum perforatum*, and leaf yield and production of leaf parthenolide in *Tanacetum parthenium* grown in a deep root hydroponic system under greenhouse conditions.

Materials and Methods

Experimental plants

Seeds of *Hypericum perforatum* (cv. Anthos) and *Tanacetum parthenium* were obtained from Richters Herbs (Goodwood, Ontario, Canada). On June 14 and June 23, 2000, seeds of *Tanacetum parthenium* and *Hypericum perforatum*, respectively, were sprinkled on top of moist rockwool plugs (2.5 cm x 2.0 cm); the seeds were then covered with a thin layer of peat moss and placed in a Conviron CMP 3244 growth chamber

under the following conditions: 80% RH, 16 hours of light at 20 °C, and 8 hours of darkness at 18 °C. Seedlings were thinned to one per plug.

Experimental setup

The experiment was set up on July 22, 2000 for *Tanacetum parthenium* and July 25, 2000, for *Hypericum perforatum* at the Greenhouse and Processing Crops Research Centre (Agriculture and Agri-Food Canada, Harrow, ON, Canada). Plants (about 5 cm tall with roots emerging from the bottom of the rockwool plug) were lodged into matching-sized holes (2 cm diameter) in styrofoam floats. Floats were placed in 53 L Rubbermaid tubs filled with nutrient solution. Each tub was equipped with air stones (30.5 cm) connected to an airline (pressure was maintained at 4 PSI) by PVC tubing (4.8 mm diameter and 70 cm long), to provide aeration and water movement around the roots. Plant roots were completely immersed in nutrient solution.

Treatments consisted of four nitrogen (N) levels (N₆₀, N₁₂₀, N₁₈₀, N₂₄₂, representing 60 mg/L, 120 mg/L, 180 mg/L, and 242 mg/L of N, respectively). The four nutrient solutions used varied only in their N content (Table 3.1) and were modifications of solution II from Chapter 2 found to support healthy herb growth. For a detailed description of the specific fertilizers and amounts refer to Appendix Table A.1. There were six replicate tubs per treatment, with six plants per tub, in a randomized block design. On August 25, 2000, tubs were emptied and refreshed with the appropriate nutrient solution. For the remainder of the experiment, water levels were maintained by adding tap water to replace what was absorbed by the plants. Plants were grown at 21 °C, and 83% RH under natural photoperiods (without supplemental lighting).

Table 3.1. Concentrations of macronutrients and electrical conductivity of the four nitrogen treatments.

Macronutrients	Nitrogen treatment			
	N ₆₀	N ₁₂₀	N ₁₈₀	N ₂₄₀
	---mg/L---			
Nitrogen (from NO₃)	55	115	175	235
Nitrogen (from NH₄)	5	5	5	7
Phosphorus	40	40	40	40
Potassium	360	360	360	360
Calcium	150	150	150	150
Magnesium	40	40	40	40
Electrical conductivity (mS/cm)	2.4	2.4	2.4	2.6

Nutrient deficiency symptoms such as necrosis or chlorosis observed on both species were graded as either 0 (no leaf necrosis), 1 (low leaf necrosis), 2 (moderate leaf necrosis), or 3 (severe leaf necrosis). The pests that occurred on each plant during the course of the study were documented and rated as either 0 (no pests apparent), + (few pests observed), ++ (moderate pest infestation), or +++ (severe pest infestation).

Data collection

A destructive harvest of the plants was conducted in mid-October, 2000. Plant tissues were separated and placed in paper bags, labeled, and dried at 35-40 °C to constant mass. Plant growth measurements for *Tanacetum parthenium* taken at harvest were: number of flowers and leaves; total leaf area; number of secondary stems; number of nodes on the primary stem; plant height; and primary stem height. Two weeks later, the dry masses of leaf, stem, flower, caudex, and root tissues were recorded for both *Hypericum perforatum* and *Tanacetum parthenium*.

Extraction and determination of target secondary metabolites

Hypericin

Dried plant samples from each treatment were ground to a fine powder using a Wiley-Mill grinder (Arthur H. Thomas Co., Philadelphia). A portion of the powder (0.2 g) was extracted four times with 10 mL 70% acetone, vortexed, then placed for 5 minutes at 60 °C in a Branson model 5210 ultrasonic bath (Branson, Danbury, CT). Then, the sample was centrifuged for 5 minutes and the supernatant of each aliquot was collected. For each sample, four successive aliquots were pooled, and the extract volume was adjusted to 40 mL with 70% acetone. Samples (1 mL) were filtered through a 0.2 µm PTFE membrane (Chromatographic Specialties, Brockville, Canada); then, 5 µL of

sample was injected into the HPLC system. This procedure was replicated two times for each powdered leaf sample per plant.

Hyperforin

The same drying, milling, and preparation procedures were followed as for the measurement of hypericin, above, except that hyperforin was extracted with 100% ethanol.

Parthenolide

Dried plant samples from each treatment were ground to a fine powder using a Wiley-Mill grinder (Arthur H. Thomas CO., Philadelphia). A portion of the powder (0.5 g) was extracted three times with 10 mL methanol, vortexed, then placed for 15 minutes at 60 °C in a Branson model 5210 ultrasonic bath (Branson, Danbury, CT). After that, the sample was centrifuged for 5 minutes and the supernatant of each aliquot was collected. For each sample, three successive aliquots were pooled, and the extract volume was adjusted to 30 mL with methanol. Samples (1.5 mL) were filtered through a 0.2 µm PTFE membrane (Chromatographic Specialties, Brockville, Canada), then 5 µL of sample was injected into the HPLC system. To ensure reliable results, this procedure was replicated three times for each powdered sample.

HPLC apparatus and chromatographic conditions

A Beckman HPLC system equipped with an autosampler (Module 502, with a 5 µL loop), solvent delivery system (Module 126), and a photo diode array detector (Module 168) was used. Separation was accomplished on a 4 µm LiChrospher 100 RP-18, 75 x 4.6 mm analytical cartridge with a 5 µm LiChrospher RP-18, 4 x 4.6 mm guard

cartridge (E. Merck/BDH Inc., Toronto, Canada). Chromatographic data were analyzed using System Gold Software (Beckman System Gold, version 8.10, Fullerton, CA).

Hypericin and hyperforin

The mobile phases consisted of solvent A: 50 mM Na₂HPO₄, lowered to pH 7.1 with H₃PO₄, and B: acetonitrile. All solvents were degassed using a pump (KNF Neuberger model UN820.3 FTP, Fisher Scientific, Ottawa, Canada). Gradient elution was run at a flow rate of 1.1 mL/min: 10-25 % B in 5 min; 25-90 % B in 5 min; 90 % B held for 2.5 min; 90-10 % B in 2.5 min. Detection wavelengths were set at 587 nm for hypericin, and 290 nm for hyperforin.

Parthenolide

The mobile phase consisted of solvent A: water, and solvent B: acetonitrile. All solvents were degassed using a pump (KNF Neuberger model UN820.3 FTP, Fisher Scientific, Ottawa, Canada). Isocratic elution was run at a flow rate of 1.1 mL/min using 45% B over 15 minutes, with UV detection at 210 nm.

Method validation

Hypericin and hyperforin elute at about 10 and 10.5 minutes, respectively. Parthenolide elutes at approximately 3 minutes. The peak identities of all compounds were confirmed by the relative retention times and by spectral analyses of the experimental samples compared to injections of purified commercial standards (Sigma-Aldrich Canada Ltd., Oakville, ON). Hypericin, hyperforin, or parthenolide quantification in samples were based on the response factor (slope of standard curve) calculated from known amounts of pure standard for each chemical. Results are reported in µg/g.

Calibration, precision, and accuracy

Hypericin

Standard solutions used for calibration were prepared by dissolving 15 mg hypericin in a volumetric flask and bringing the volume up to 100 ml with 70% acetone. Solutions with concentrations of 0.150, 0.075, 0.0375, 0.0188, 0.0094, 0.0047, and 0.000 mg/mL were generated from the stock solution by serial dilution with 70% acetone.

A standard solution for the determination of accuracy and precision was prepared by placing 2 mg hypericin in a volumetric flask and bringing the volume up to 100 mL with 70 % acetone. This standard was injected at the beginning and end of each run, and after every 7 injections of samples. Standard solutions used for calibration were also injected at the beginning of each run.

Hyperforin

A quality control solution containing 62 ug/mL hyperforin was injected at the beginning and end of each run, and after every 7 injections of standard. The hypericin standards were also injected at the beginning of each run.

Parthenolide

Standard solutions used for calibration were prepared by dissolving 40 mg parthenolide in a volumetric flask and bringing the volume up to 100 mL with acetonitrile. Solutions with concentrations of 0.400, 0.200, 0.100, 0.050, 0.025 and 0.000 mg/mL were generated from the stock solution by serial dilution with acetonitrile.

A standard solution for determination of accuracy and precision was prepared by placing 5 mg parthenolide in a volumetric flask and bringing the volume up to 100 mL with acetonitrile. This standard was injected at the beginning and end of each run, and

after every 7 injections of samples. The standard solutions used for calibration were also injected at the beginning of each run.

Statistical analyses

Statistical analyses were conducted using the SYSTAT 9.0 statistical package (SPSS Inc., 1999). Nested analyses of variance were conducted to determine whether there were significant differences among nitrogen treatments with respect to various growth parameters and to determine if there were differences between tubs within specific treatments. Tukey post-hoc multiple-comparison tests were then used to examine the detail of the differences among nitrogen treatments for each growth parameter.

Results

Hypericum perforatum

Table 3.2 summarizes the results of the nested ANOVAs for various plant parameters. Nitrogen treatments had significant effects on: caudex dry mass ($p \leq 0.05$), root dry mass ($p \leq 0.01$), belowground (caudex + root) dry mass ($p \leq 0.01$), leaf hypericin concentration ($p \leq 0.001$), and leaf hypericin yield ($p \leq 0.01$). There were no statistically significant differences between treatments for leaf dry mass, stem dry mass, aboveground dry mass, total plant dry mass, leaf hyperforin concentrations, and leaf hyperforin yields. There were no significant differences between replicate tubs, within nitrogen treatments (right hand portion of Table 3.2).

Table. 3.2. Results of nested analysis of variance to determine the effects of nitrogen treatment on various *Hypericum perforatum* growth parameters.

Growth parameter	Source of variation: nutrient solution			Source of variation: tub(nutrient solution)				
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Leaf dry mass (g)	3	39.399	2.577	0.057	20	6.615	0.433	0.983
Stem dry mass (g)	3	5.064	0.599	0.617	20	3.924	0.464	0.975
Caudex dry mass (g)	3	0.162	3.673	0.014	20	0.051	1.147	0.313
Root dry mass (g)	3	58.384	4.119	0.008	20	10.197	0.719	0.800
Aboveground dry mass (g)	3	100.732	2.357	0.075	20	13.826	0.323	0.997
Belowground dry mass (g)	3	64.184	4.182	0.007	20	11.238	0.732	0.786
Total plant dry mass (g)	3	183.070	1.771	0.156	20	32.206	0.312	0.998
Leaf [hypericin] ($\mu\text{g/g}$)	2	143239.615	23.571	0.001	9	6627.561	1.091	0.475
Leaf hypericin yield (μg)	2	1.197×10^7	16.485	0.004	9	517120.167	0.712	0.689
Leaf [hyperforin] ($\mu\text{g/g}$)	3	231734.470	0.262	0.852	17	1230807.223	1.389	0.285
Leaf hyperforin yield (μg)	3	2.874×10^8	1.530	0.257	17	2.455×10^8	1.307	0.323

Plants in the N₂₄₂ treatment also had a significantly lower caudex dry mass than plants in the N₆₀ treatment. Also, root dry mass and belowground (caudex + root) dry mass were significantly higher in plants grown in the N₆₀ treatment compared to plants in the N₁₂₀ and N₂₄₂ treatments, although they did not differ significantly from N₁₈₀ (Figure 3.1).

Nested ANOVAs were also performed to compare the effects of nitrogen treatment on the proportion of biomass allocated to leaf tissue, stem tissue, and belowground tissue. The proportion of biomass allocated to leaf and belowground tissue were found to be significantly different among treatments (with $p \leq 0.001$ and 0.001 , respectively) (Figure 3.2). Plants in the N₆₀ treatment allocated significantly more biomass to root tissue compared to plants in all other N treatments. Also, the N₆₀ plants allocated significantly less to leaf tissue compared to plants in the N₁₂₀ and N₁₈₀ treatments, although they did not differ significantly from plants in the N₂₄₂ treatment.

The highest concentrations of leaf hypericin ($483.98 \pm 41.18 \mu\text{g/g}$ per plant) and of leaf hypericin yields ($4900.20 \pm \mu\text{g}$ per plant) were found in plants grown in the N₆₀ treatment, with concentrations significantly higher than in plants of both the N₁₆₀ and N₂₄₂ treatments (Figure 3.3 a, b). Only the N₆₀, N₁₈₀, and N₂₄₂ treatments were included in these analyses. Leaf hyperforin concentrations and leaf hyperforin yields ($2525.70 \pm 175.13 \mu\text{g/g}$ and $29626.89 \pm 2619.83 \mu\text{g}$ over all treatments, respectively) did not differ statistically between treatments.

In *Hypericum perforatum*, neither symptoms of nutrient deficiency nor pests were apparent during the course of the study.

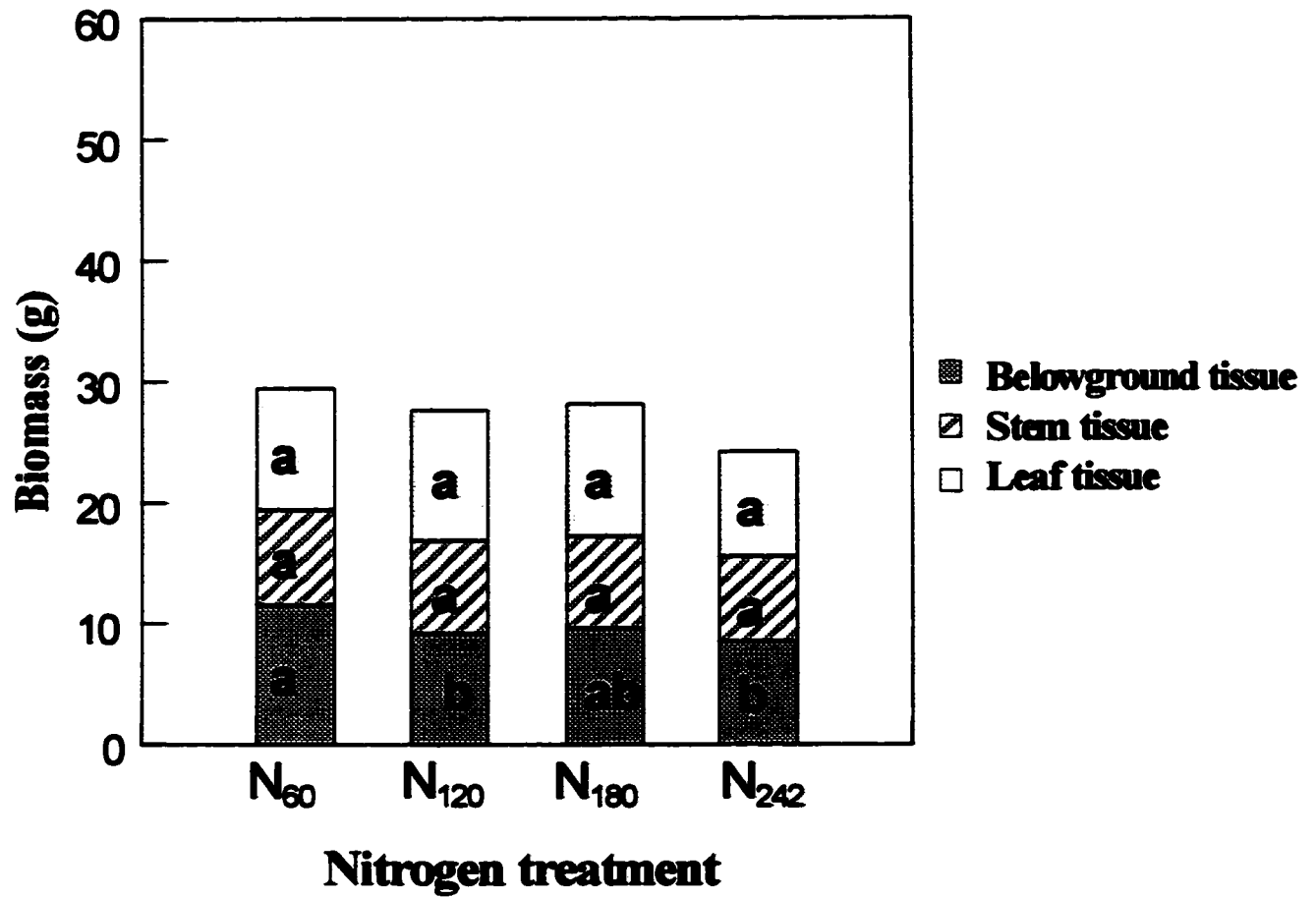


Figure 3.1. Effects of nitrogen treatment on the biomass (g) of leaf, stem, and belowground tissue in *Hypericum perforatum*. Between treatments, within the same tissue, bars with the same letter are not significantly different from each other. On x-axis N₆₀ = 60 mg N/L, N₁₂₀ = 120 mg N/L, N₁₈₀ = 180 mg N/L, N₂₄₂ = 242 mg N/L.

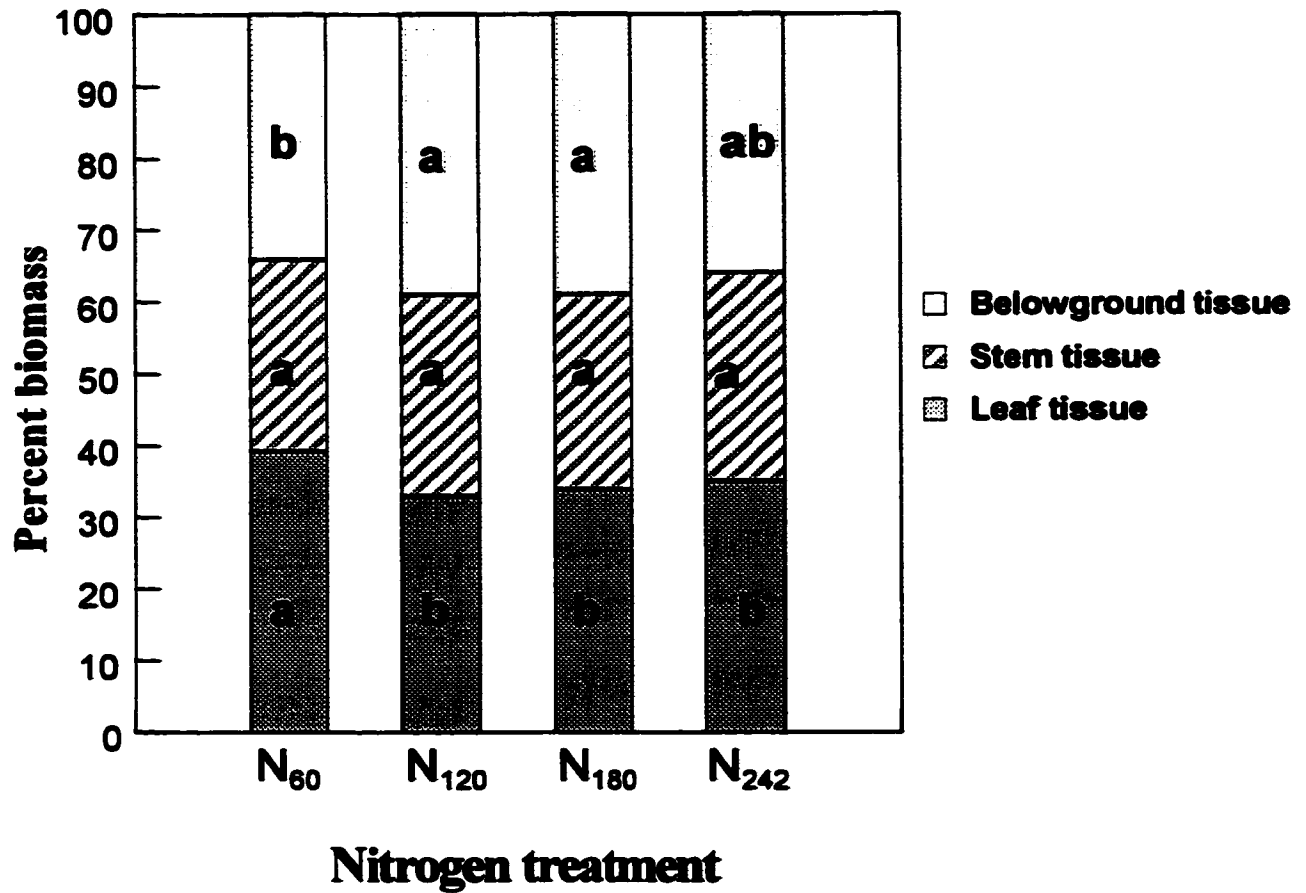


Figure 3.2. Proportional allocation of biomass to leaf, stem, and belowground tissue in *Hypericum perforatum*, for each of the nitrogen treatments. Between treatments, within the same tissue, bars with the same letter are not significantly different from each other. On x-axis N₆₀ = 60 mg N/L, N₁₂₀ = 120 mg N/L, N₁₈₀ = 180 mg N/L, N₂₄₂ = 242 mg N/L.

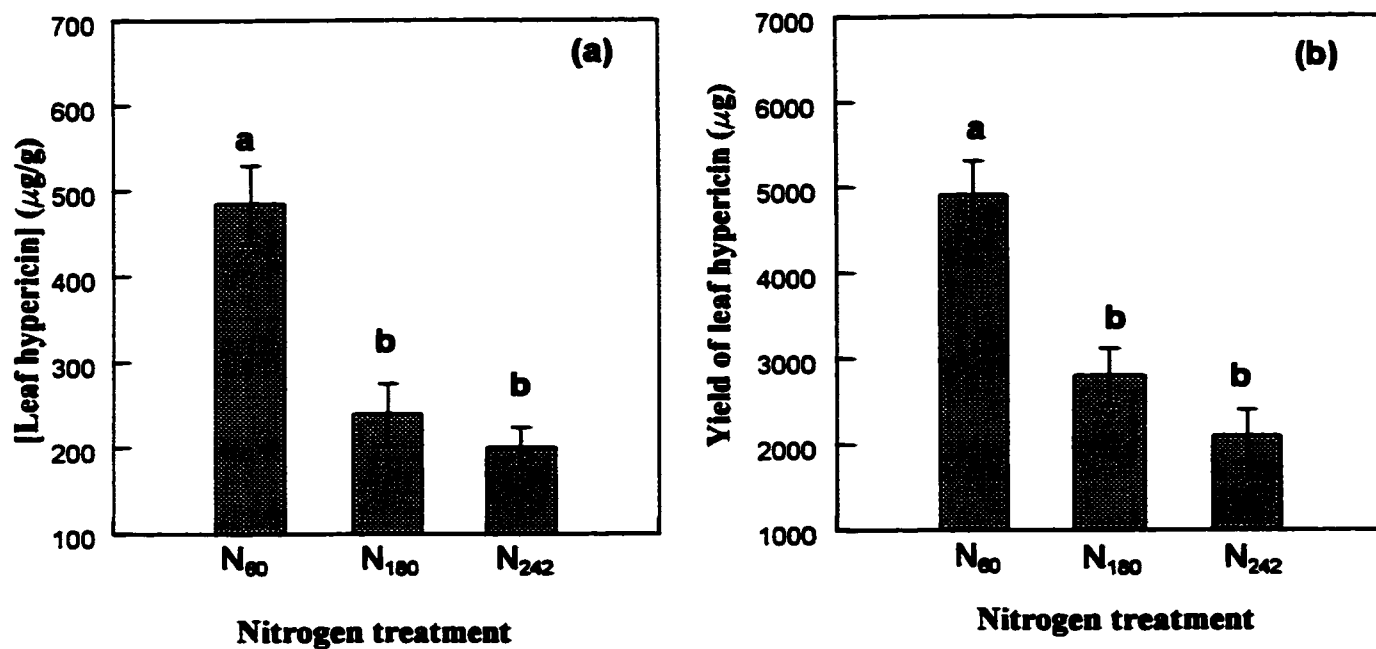


Figure 3.3 (a) and (b). Effects of nitrogen treatment on (a) leaf hypericin ($\mu\text{g/g}$) and (b) leaf hypericin yield (μg) in *Hypericum perforatum*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis N₆₀ = 60 mg N/L, N₁₈₀ = 180 mg N/L, N₂₄₂ = 242 mg N/L.

Tanacetum parthenium

Tables 3.3 and 3.4 summarize the results of the nested ANOVA for various growth measurements. Significant effects between treatments were observed for only leaf parthenolide concentration ($p \leq 0.01$), and leaf parthenolide yield ($p \leq 0.05$). Parthenolide concentrations were the highest for plants in the N₂₄₂ treatment, but only significantly greater than plants in the N₁₂₀ treatment. The yield of leaf parthenolide had a clear upward trend with increasing nitrogen level. However, according to the Tukey post-hoc test, these differences in parthenolide yield were not significant (Figure 3.4 a, b). Significant differences were observed between tubs within specific treatments for leaf parthenolide concentration, leaf parthenolide yield, and number of nodes on the primary stem. These differences within specific treatments may be due to tubs in the N₂₄₂ treatment containing plants with visible symptoms of nutrient deficiency (Table 3.5) and harbouring a large spider mite population. These plants had the highest parthenolide levels compared to plants in all treatments.

In addition, nested ANOVAs were also performed to compare the effects of herbivory on the proportion of biomass allocated to reproductive tissue, leaf tissue, stem tissue, and belowground (caudex + root) tissue (Figure 3.5). Plants in the N₆₀ treatment allocated a significantly greater proportion of biomass to belowground tissue compared to plants in the N₁₈₀ and N₂₄₂ treatments.

Table 3.3. Results of nested analysis of variance to determine the effects of nitrogen treatment on various *Tanacetum parthenium* leaf and reproductive growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Number of leaves	3	69600.612	1.494	0.223	20	34457.913	0.740	0.773
Leaf dry mass (g)	3	24.695	0.503	0.681	20	42.255	0.860	0.637
Dead leaf dry mass (g)	3	0.126	0.074	0.974	20	2.477	1.455	0.112
Total leaf dry mass (g)	3	31.939	0.551	0.648	20	48.839	0.843	0.657
Leaf area (cm ²)	3	511102.064	0.128	0.943	20	3310933.159	.830	0.671
Leaf [parthenolide] (µg/g)	3	2401525.785	4.829	0.008	18	1337688.625	2.690	0.011
Leaf parthenolide yield (µg)	3	2.045 x 10 ⁹	3.858	0.019	18	1.567 x 10 ⁹	2.955	0.005
Number of flowers	3	56531.215	1.038	0.957	20	27540.187	.507	.957
Reproductive tissue dry mass (g)	3	50.132	0.784	0.506	20	46.755	0.731	0.785

Table 3.4. Results of nested analysis of variance to determine the effects of nitrogen treatment on various *Taraxacum parthenium* stem, root, and plant growth parameters.

Growth parameter	Source of variation: nutrient solution				Source of variation: tub(nutrient solution)			
	Degrees of Freedom	Mean square	F-ratio	P-value	Degrees of Freedom	Mean square	F-ratio	P-value
Primary stem height (cm)	3	33.209	0.588	0.624	19	70.087	1.241	0.244
Overall plant height (cm)	3	52.134	0.732	0.535	20	84.906	1.192	0.274
Number of nodes	3	19.401	2.561	0.059	19	20.474	2.703	0.001
Number of secondary stems	3	10.384	0.548	0.651	20	29.037	1.532	0.084
Primary stem dry mass (g)	3	0.126	0.119	0.949	20	1.020	0.966	0.508
Higher order stem dry mass (g)	3	10.545	0.226	0.878	20	22.126	0.475	0.971
Total stem dry mass (g)	3	9.792	0.171	0.916	20	29.445	0.515	0.955
Caudex dry mass (g)	3	0.03	0.870	0.460	18	0.028	0.822	0.670
Root dry mass (g)	3	21.617	1.126	0.342	20	14.057	0.732	0.785
Total aboveground dry mass	3	40.853	0.197	0.818	20	150.302	0.726	0.792
Total belowground dry mass (g)	3	24.555	1.118	0.347	17	18.781	0.855	0.626
Total plant dry mass (g)	3	604.487	0.961	0.417	16	602.423	0.958	0.572

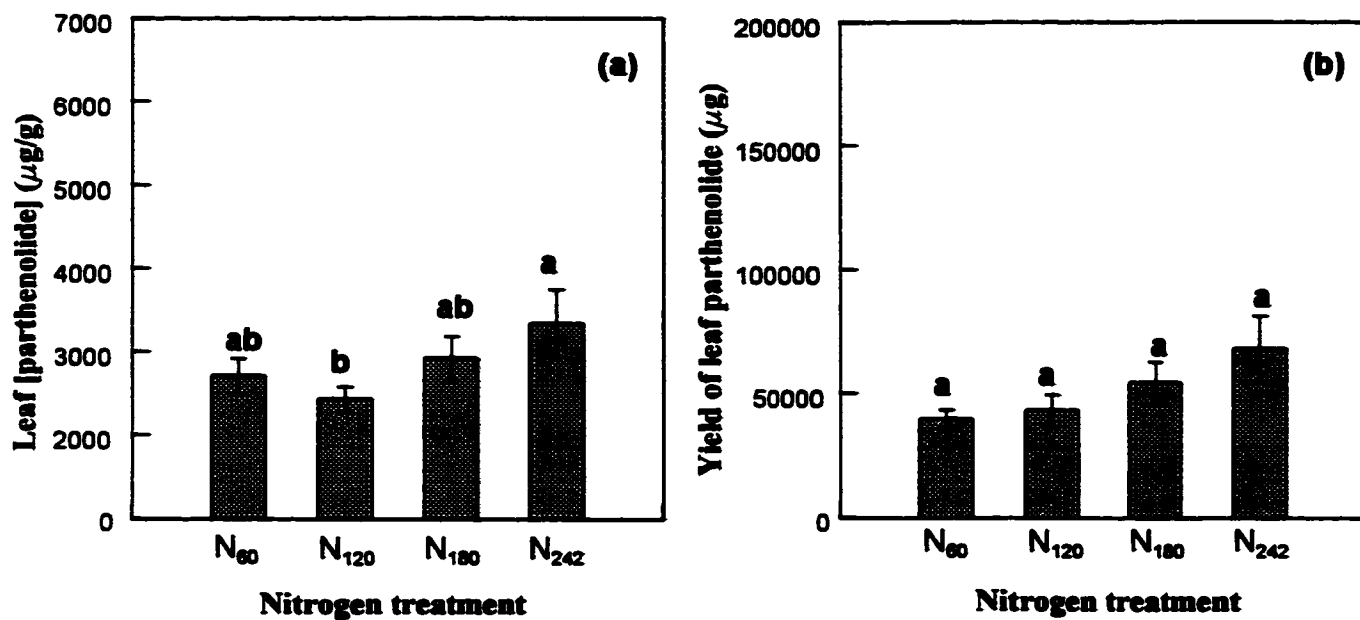


Figure 3.4 (a) and (b). Effects of nitrogen treatment on (a) leaf parthenolide concentration ($\mu\text{g/g}$) and leaf parthenolide yield (μg) in *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x - axis, N₆₀ = 60 mg N/L, N₁₂₀ = 120 mg N/L, N₁₈₀ = 180 mg N/L, N₂₄₂ = 242 mg N/L.

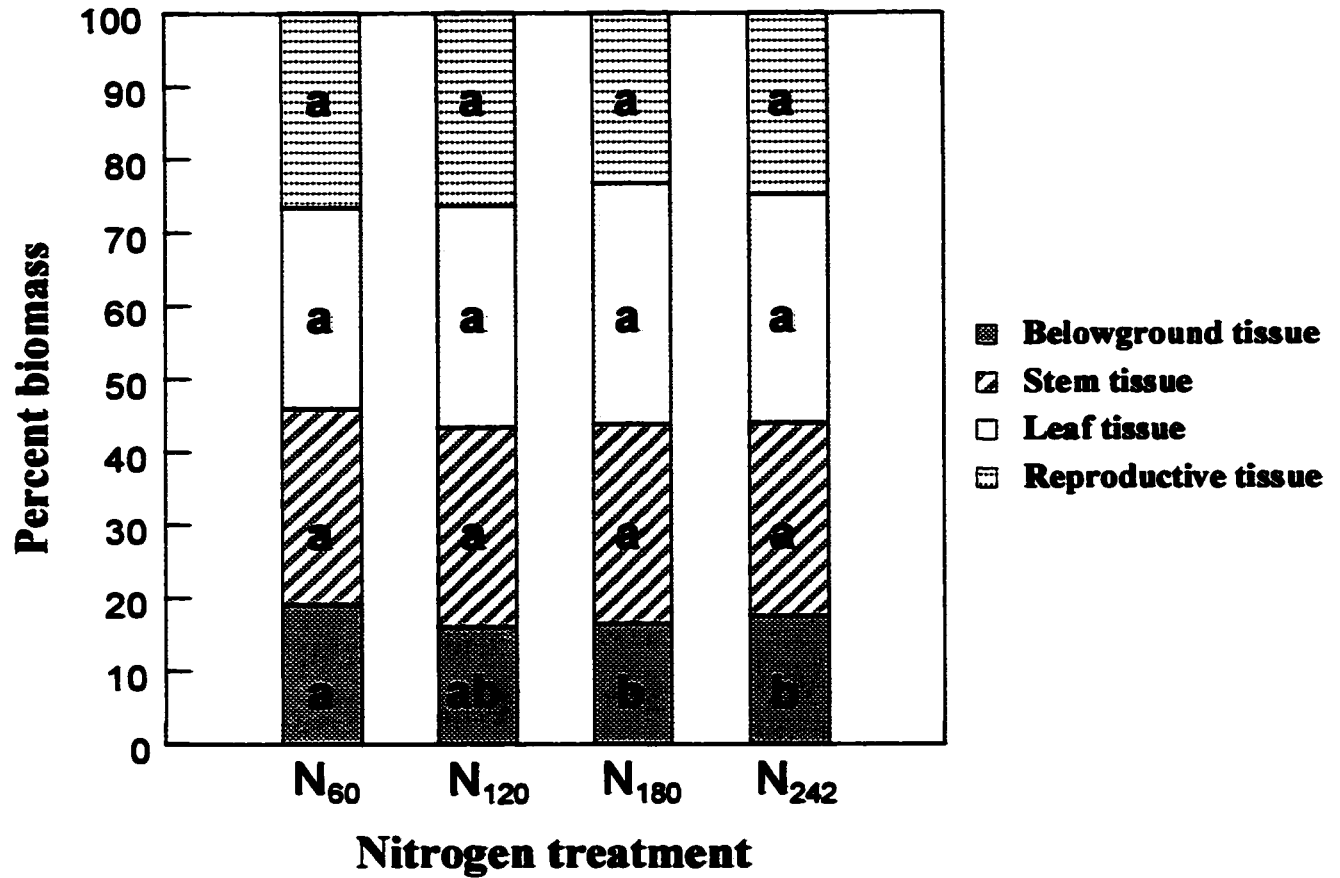


Figure 3.5. Proportional allocation of biomass to reproductive, leaf, stem, and belowground tissue in *Tanacetum parthenium* for each of the nitrogen treatments. Between treatments within the same tissue, bars with the same letter are not significantly different from each other. On x-axis, N₆₀ = 60 mg N/L, N₁₂₀ = 120 mg N/L, N₁₈₀ = 180 mg N/L, N₂₄₂ = 242 mg N/L.

Table 3.5. Leaf parthenolide concentrations ($\mu\text{g/g}$ dry mass), nitrogen deficiency symptoms and pest infestation on *Tanacetum parthenium* in the different nitrogen treatments. Leaf necrosis was graded as 0 = no leaf necrosis, 1 = low leaf necrosis, 2 = moderate leaf necrosis, and 3 = severe leaf necrosis. The pest population was rated as either 0 (no pests apparent), + (few pests observed), ++ (moderate pest infestation), or +++ (severe pest infestation).

Nitrogen treatment (mg/L)	Mean parthenolide concentration (\pm SE)	Leaf necrosis	Two-spotted spider mite
60	2715.03 (\pm 203.38)	0	0
120	2264.09 (\pm 95.74)	0	0
180	2926.62 (\pm 254.07)	0	0
242	3490.34 (\pm 393.80)	2	+++

Discussion

Hypericum perforatum

Since the leaves of *Hypericum perforatum* contain the naphodianthrone, hypericin, and the phloroglucinol, hyperforin, which contribute to the plant's medicinal activity, the leaf yield at harvest is important. Plant productivity has been shown to improve with increasing levels of nitrogen (Munsi 1992, Papadopoulos 1998). However, the different levels of nitrogen utilized in this study did not significantly influence leaf production or total plant yield. The low nitrogen levels did not cause any visible symptoms of mineral deficiency in the plants.

Nitrogen treatments had a significant effect on both root dry mass and belowground dry mass, with plants in the N₆₀ treatment having the greatest root dry mass, a common response to the low nitrogen levels (Chapin 1980). The mechanism by which root:shoot ratio increases in response to decreased nutrient availability is unknown, but it has been suggested that because root meristems are closer to the nutrient supply, they receive a disproportional share of nutrients and thus grow faster than shoot meristems (Chapin 1980).

The supply of nitrogen had a significant effect on the concentration and yield of hypericin in the leaves of *Hypericum perforatum*. Plants in the lowest nitrogen treatment (N₆₀) had a significantly higher concentration of leaf hypericin and yield of hypericin compared to plants in the higher nitrogen regimes (Table 3.2, Figure 3.3). These plants produced approximately 2.0 to 2.4 times more leaf hypericin than plants in the N₁₈₀ and N₂₄₂ treatments, respectively. These results are similar to those found by Brisikin *et al.* (2000), with *Hypericum perforatum* (cv. Topas), where plants grown in greenhouse sand

culture with decreasing levels of nitrogen, increased their production of hypericins and plants grown in low nitrogen-containing soil, experienced a decreased production of hypericins, with increasing nitrogen supplementation. Denke *et al.* (1999) also observed that, although nitrogen fertilization in the field yielded more plant material, hypericin content decreased. These results are in accordance with the Carbon/Nutrient hypothesis and the resource availability hypothesis (Bryant *et al.* 1983, Coley *et al.* 1985). As the plant is compromised by low nitrogen, more hypericin is produced. Although, Briskin *et al.* (2000) postulate that if increases in hypericin occur prior to any nitrogen deficiency symptoms, then increased production of hypericins may not depend on the metabolism of the carbon pool.

In this study, hypericin concentrations ranged from 641.77 to 155.57 $\mu\text{g/g}$ dry mass across treatments. These hypericin levels were comparable to those found by Jensen *et al.* (1995) in St. Johnswort collected in Canada.

Leaf hyperforin concentrations and yield appeared to be unaffected by the nitrogen levels used in this study. Hyperforin has also been shown to be unaffected by geographic location and accession (Buter *et al.* 1998).

In conclusion, there is no benefit in terms of raising nitrogen levels in this hydroponic system, since lower nitrogen levels produced similar yields of target tissue as did higher nitrogen levels, without producing any symptoms of mineral deficiency. Indeed there are measurable benefits to using low nitrogen levels, in terms of promoting elevated levels of leaf hypericin, and *Hypericum perforatum* tissue that may be pharmaceutically more active.

Tanacetum parthenium

The sesquiterpene, parthenolide, is believed to play an important role in the migraine prophylactic activity of *Tanacetum parthenium* (Heptinstall *et al.* 1992) so its level within a plant is an important factor affecting its medicinal effectiveness. In this study, the highest parthenolide concentrations were found in the highest nitrogen treatment, but were only significantly higher than plants in the N₁₂₀ treatment. Mihaliak and Lincoln (1985 and 1989), found leaf mono- and sesquiterpenes to have increased in *Heterotheca subaxillaris* with decreasing nitrogen availability, following the predictions of the Carbon-Nutrient Balance hypothesis in which carbon-based defenses should be in higher concentrations when nutrients are limiting, than when nutrients are well supplied.

Three explanations can be proposed to account for *Tanacetum parthenium*'s response to nitrogen, which was contrary to the Resource Availability hypothesis and the Carbon/Nutrient Balance hypothesis. First, in this study, concentrations of leaf parthenolide did not increase with decreased levels of nitrogen, possibly because nitrogen levels of 60 mg/L may not have been low enough to raise secondary metabolite production, by increasing carbon availability for parthenolide synthesis.

A second explanation looks to the symptoms of nutrient deficiency in plants grown in the different nitrogen regimes. Plants grown in the highest nitrogen regime exhibited signs of nutrient stress through visual symptoms of mineral deficiency, such as necrotic lesions on leaf tissue, and to a lesser degree lower leaf chlorosis. The high NO₃ and NH₄ levels in this nutrient solution may have caused competition with anions such as phosphorous and cations such as potassium, respectively, inhibiting and decreasing the uptake of these other essential mineral ions by the plant (Papadopoulos 1994) and thus

decreasing their availability for plant growth, and possibly increasing the availability of nonstructural carbon for the production of parthenolide.

Third, plants grown in the highest nitrogen treatment experienced a high infestation of *Tetranychus urticae* (two-spotted spider mite). The high pest infestation may have induced parthenolide production as a defense response against herbivory.

The proportion of biomass allocated to root tissue was greatest in plants grown in the lowest nitrogen regime (N₆₀). Such an increase in allocation of reserves to root growth is a common response to low nutrient levels (see above) (Chapin 1980, Ruess *et al.* 1983)

In conclusion, parthenolide levels in *Tanacetum parthenium* were relatively stable across treatments ($2858.20 \pm 137.792 \mu\text{g/g}$), with mean levels that were higher than the minimum (2000 $\mu\text{g/g}$) proposed by health Canada for quality control. Thus, elevated levels of nitrogen were not needed in order to maximize plant growth or parthenolide levels.

CHAPTER 4

EFFECTS OF SIMULATED HERBIVORY ON THE VEGETATIVE GROWTH AND CONTENTS OF SECONDARY METABOLITES IN *HYPERICUM PERFORATUM* AND *TANACETUM PARTHENIUM*

Introduction

A substantial amount of evidence has demonstrated that plants may increase productivity, reproductive potential and secondary metabolites in response to herbivory or stress (Paige and Whitman 1987, Gianoli and Niemeyer 1997, Baldwin 1998, Lennartsson *et al.* 1998, Agrawal 2000). Such changes in plants occurring after damage are termed induced responses (Karban and Myers 1989). Other changes in response to herbivory include increases in physical defenses such as thorns or trichomes, emission of volatiles attractive to predators and parasites of herbivores, reversion to juvenile growth form, and/or a reduction in plant nutritional status for herbivores (Karban and Myers 1989, Agrawal 2000). If the preference and/or performance of subsequent herbivores is reduced, these changes are labeled as induced resistance. Moreover, an induced response is considered a defence if plant fitness is increased relative to an undamaged plant (Agrawal 2000).

A number of studies have shown that, when grazed or experimentally clipped, plants may sometimes overcompensate in growth (e.g., Paige and Whitman 1987, Lennartsson *et al.* 1998, Agrawal 2000). This is well illustrated by Paige and Whitman (1987) in which naturally-browsed *Ipomopsis aggregata* plants produced four times more flowering stalks than unbrowsed plants and produced a greater mass of leaves, stems, flowers, and fruits compared to ungrazed plants. Lennartsson *et al.* (1998) experimentally clipped *Gentianella campestris* plants, removing approximately half of

the aboveground dry mass and half of the potential meristems, which resulted in more flowering branches and increased fruit production compared to unclipped plants.

In addition, plants may respond to herbivory and or simulated damage by changes in the synthesis of secondary metabolites (Collantes *et al.* 1997, Gianoli and Niemeier, 1997, Baldwin 1988). Neimeyer *et al.* (1989) investigated the effects of aphid infestation on four wheat cultivars and found that hydroxamic acids, a class of secondary metabolites that play a role in resistance to herbivores, were induced by aphid infestation and that this induction was affected by genotype. Baldwin (1988) suggested that since induced defenses appear to be stimulated by the release of endogenous cues from cell wall fragments, that plants may have difficulty distinguishing between leaf damage caused by biotic sources and those resulting from abiotic sources. Baldwin (1988) demonstrated that although the alkaloidal levels in *Nicotiana sylvestris* significantly increased in response to larval feeding compared to undamaged plants, the alkaloidal levels induced by mechanical damage were even higher. On the other hand, Agrawal (1998) studied *Raphanus sativas* and found plants to be induced by caterpillar larvae to have increased concentrations of mustard oil glycosides, lower subsequent herbivory, and a higher female fitness than control plants, whereas experimentally induced *Raphanus sativas* plants had no associated induced response and lower fitness than control plants.

Hypericum perforatum (St. Johnswort) and *Tanacetum parthenium* (Feverfew) are among the most commonly used and best studied medicinal plants (O'Hara *et al.* 1998). However, very little is known about their management practices that would result in high yields of target tissues and bioactive chemicals, which is an impediment to their potential development as an alternative crop in greenhouses. Nitrogen fertilization has been shown

to have a profound effect on hypericin concentrations in leaf tissue of *Hypericum perforatum* with no influence on hyperforin leaf concentrations and minimal effects on above ground tissues in *Hypericum perforatum* (refer to Chapter three). The photodynamic pigment, hypericin, has been proposed to serve as a defense against herbivores (Fields *et al.* 1990).

It has been suggested that parthenolide, a sesquiterpene lactone, in *Tanacetum parthenium*, is also produced as a defensive compound (Knight 1995). Picman (1986) demonstrated sesquiterpene lactones, a family of secondary metabolites, to have anti-herbivore activity on insects and mammals. However, we know of no previous studies on the modulation of secondary metabolites in *Tanacetum parthenium* or *Hypericum perforatum*.

In the present study, *Hypericum perforatum* and *Tanacetum parthenium* were grown in a deep root hydroponic system under greenhouse conditions. Leaf herbivory was simulated to determine the effect on biomass production and leaf hypericin and leaf hyperforin yields in *Hypericum perforatum*. Moreover, treatments involving moderate, severe and repeated leaf removal and repeated flower bud removal were applied in order to determine the effect on leaf number and mass, parthenolide yield and other growth parameters in *Tanacetum parthenium*.

Materials and Methods

Hypericum perforatum

Experimental plants

In order to minimize variability in growth and active constituents between individual plants, *Hypericum perforatum* plants were micropropagated. From a single plant grown from seed (Richters Herbs, Goodwood, ON, Canada) under controlled conditions, shoot tips (5-6 cm) were collected. Expanded leaves were removed and the remainder of the shoot tips were washed for 10 minutes in a 1 % solution of Javex in a closed container. Explants were rinsed thoroughly with distilled water and then placed in a distilled water bath for 20 minutes. Shoot tips were isolated under aseptic conditions into culture tubes and Phytatrays (Sigma Chemical Company, St. Louis, MO, USA) containing growing media. The cloned cultures were refrigerated at 24°C and 16 hours of light. As new shoots developed from the cuttings, they were separated and put into new culture media. Plants (10-12 cm) were transferred from media into rockwool plugs covered with soil and placed in a misting chamber for 3 weeks.

Experimental setup

The experiment was set up on December 17, 2000 at the Greenhouse and Processing Crops Research Centre (Agriculture and Agri-Food Canada, Harrow, ON, Canada). Plants (about 25 cm in length with roots emerging from the bottom of the rockwool plug) were lodged into matching holes in styrofoam floats. Each float had six holes (2 cm diameter) large enough to fit each rockwool plug. The floats were placed in 53 L Rubbermaid™ tubs filled with nutrient solution, and equipped with air stones (30.5cm) connected to an airline (pressure was maintained at 4 PSI) by PVC tubing (4.8mm diameter and 70 cm long) to provide aeration and water movement. The roots of

the plants were completely immersed in nutrient solution. The nutrient solution had previously been found to be optimal for the support of healthy plant growth; it contained the following salts per L of solution: 500.0 mg $\text{Ca}(\text{NO}_3)_2$, 227.2 mg KNO_3 , 63.9 mg NaNO_3 , 88.9 mg CaCl_2 , 399.6 mg K_2SO_4 , 111.0 mg KCl , 174.85 mg KH_2PO_4 , 318.0 mg MgSO_4 , 1.5 mg Fe chelate and micronutrients (1.885 mg MnSO_4 , 0.20 mg ZnSO_4 , 3.265 mg H_3BO_4 , 0.24 mg CuSO_4 , 0.15 mg NaMoO_4). Water levels were maintained by adding tap water every four days. Herbivory simulation was initiated March 4, 2001. The three herbivory treatments were:

1. Low herbivory (a randomly distributed 100 leaves were cut in half at the tip of the leaf)
2. High herbivory (all leaves present on the adaxial side of every stem were removed)
3. Control (plant remained intact).

Any foliage removed was labeled, bagged and dried for later inclusion in total plant mass measurements. Plants were grown at 21 °C, and 83 % RH under natural photoperiods (without supplemental lighting). Each treatment was assigned at random to each of six plants in 12 tubs, giving a total of 24 plants in each treatment.

Data collection

A destructive harvest of the plants was conducted in early April 2001, one month after treatments began. Plant tissues were separated and placed in paper bags, labeled, and dried at 35-40 °C to constant mass. Two weeks later, the dry masses of leaf, stem, caudex, and root tissues were recorded.

Extraction and determination of hypericin and hyperforin

Hypericin

Dried plant samples from each treatment were ground to a fine powder using a Wiley-Mill grinder (Arthur H. Thomas CO., Philadelphia). A portion of the powder (0.2 g) was extracted four times with 10 mL 70 % acetone, vortexed, then placed for 5 minutes at 60 °C in a Branson model 5210 ultrasonic bath (Branson, Danbury, CT). After that, the sample was centrifuged for 5 minutes and the supernatant of each aliquot was collected. For each sample, four successive aliquots were pooled, and the extract volume was adjusted to 40 mL with 70 % acetone. Samples (1 mL) were filtered through a 0.2 µm PTFE membrane (Chromatographic Specialties, Brockville, Canada), then 5 µL of sample was injected into the HPLC system. To ensure reliable results, this procedure was replicated two times for each powdered sample.

Hyperforin

The extraction procedure for hyperforin was the same as above, except that 100 % ethanol was used for the extraction.

HPLC apparatus and chromatographic conditions

The Beckman HPLC system was equipped with an autosampler (Module 502, with a 5 µL loop), solvent delivery system (Module 126), and a photo diode array detector (Module 168). Separation was accomplished on a 4 µm LiChrospher 100 RP-18, 75 x 4.6 mm analytical cartridge with a 5 µm LiChrospher RP-18, 4 x 4.6 mm guard cartridge (E. Merck/BDH Inc, Toronto, Canada). Chromatographic data were processed using System Gold Software (Beckman System Gold, version 8.10, Fullerton, CA). The mobile phases consisted of solvent A: 50 mM Na₂HPO₄ pH 7.1 with H₃PO₄, B: acetonitrile. All solvents were degassed using a pump (KNF Neuberger model UN820.3 FTP, Fisher Scientific, Ottawa, Canada). Gradient elution was run at a flow rate of 1.1

mL/min: 10-25 % B in 5 min; 25-90 % B in 5 min; 90 % B held for 2.5 min; 90-10 % B in 2.5 min. The detection wavelength was set at 587 nm for hypericin, and 290 nm for hyperforin.

Method Validation

Hypericin and hyperforin elute at about 10 and 10.5 minutes, respectively. The peak identities of both compounds were confirmed by relative retention time and spectral analyses of the experimental samples compared to injections of purified commercial standards (Sigma-Aldrich Canada Ltd., Oakville, ON). Hypericin and hyperforin quantification in samples were based on the response factor (slope of standard curve) calculated from known amounts of pure standard for each chemical. Results are reported in $\mu\text{g/g}$.

Calibration, precision, and accuracy

Hypericin

Standard solutions used for calibration were prepared by dissolving 15 mg hypericin in a volumetric flask and bringing the volume up to 100 mL with 70 % acetone. Solutions with concentrations of 150, 75, 37.5, 18.75, 9.38, 4.69 and 0.000 $\mu\text{g/mL}$ were generated from the stock solution by serial dilution with acetonitrile.

A standard solution for determination of accuracy and precision was prepared by placing 2 mg hypericin in a volumetric flask and bringing the volume up to 100 mL with 70 % acetone. This standard was injected at the beginning and end of each run, and after every 7 injections of samples. The standard solutions used for calibration were also injected at the beginning of each run.

Hyperforin

A quality control solution containing 62 µg/mL hyperforin was injected at the beginning and end of each run, and after every 7 injections of samples. Hypericin standard solutions were also injected at the beginning of every run.

Statistical analyses

Statistical analyses were conducted using the SYSTAT 9.0 statistical package (SPSS Inc., 1999). One-way Analyses of variance were conducted to determine whether there were significant differences among herbivory treatments with respect to various growth parameters. Tukey post hoc multi-comparison tests were then used to examine the detail of the differences among herbivory treatments for each growth parameter. Two sample t-tests were used to compare the leaf hypericin concentration and leaf hypericin yield between plants in the control and high herbivory treatment. Leaf and root parameters were natural log-transformed to normalize their distribution.

Tanacetum parthenium

Experimental plants

For this experiment, seeds from a single selfed *Tanacetum parthenium* plant were used (Richters Herbs, Goodwood, Ontario, Canada). The parent plant had been raised under highly controlled conditions in the greenhouse. On December 28, 2000, seeds from this individual plant were sprinkled on top of 240 moist rockwool plugs (2.5 cm x 2.0 cm); the seeds were then covered with a thin layer of peat moss and placed in a Conviron CMP 3244 growth chamber under the following conditions: 16 hours of light at 20 °C, and 8 hours of darkness at 18 °C. Germination began December 31, 2000. All

germination was complete within 7 days. All plugs were thinned on January 8, 2001 leaving one seedling per plug.

Experimental setup

The experiment was set up on January 24, 2001 at the Harrow Research Station (Agriculture and Agri-Food Canada, Harrow, Ontario, Canada). Small plants (about 4 cm in height with roots emerging from the bottom of the rockwool plug) were lodged into matching holes in styrofoam floats. Each float had six holes (2 cm diameter) large enough to fit each rockwool plug. The floats were placed in 53 L Rubbermaid™ tubs filled with nutrient solution, and equipped with air stones (30.5cm) connected to an airline (pressure was maintained at 4 PSI) by PVC tubing (4.8mm diameter and 70 cm long) to provide aeration and water movement. The roots of the plants were completely immersed in nutrient solution. The nutrient solution had previously been found to be optimal for the support of healthy plant growth; it contained the following salts per litre of solution: 500.0 mg $\text{Ca}(\text{NO}_3)_2$, 227.2 mg KNO_3 , 63.9 mg NaNO_3 , 88.9 mg CaCl_2 , 399.6 mg K_2SO_4 , 111.0 mg KCl , 174.85 mg KH_2PO_4 , 318.0 mg MgSO_4 , 1.5 mg Fe chelate and micronutrients (1.885 mg MnSO_4 , 0.20 mg ZnSO_4 , 3.265 mg H_3BO_4 , 0.24 mg CuSO_4 , 0.15 mg NaMoO_4). Water levels were maintained by adding tap water every four days. Herbivory simulation was initiated March 10, 2001, when plants were approximately 11 weeks old, and flower buds were developing. The five herbivory treatments included:

4. Low herbivory (a random 25% of leaves present were cut in half)
5. High herbivory (a random 50% of leaves present were cut in half)
6. Sustained leaf herbivory (every three days, 50% of the existing leaf area of a random 15 leaves was removed)

7. Complete flower bud removal (every 3 days all flower buds were excised) and
8. Control (leaves, flowers and buds remained intact).

Any leaves or flower buds removed were labeled, bagged and dried for later inclusion in total plant mass measurements. Plants were grown at 21 °C, and 83% RH under natural photoperiods (without supplemental lighting). Each treatment was assigned at random to each of six plants in 18 tubs), giving a total of 20 plants in the control treatment and 22 plants in each of the other treatments.

Data collection

A destructive harvest of the plants was conducted on April 9th, one month after treatments began, before flower bud development. Plant measurements taken at harvest were: number of flowers and leaves; total leaf area; number of secondary stems; number of nodes on the primary stem; plant height; and primary stem height. Plant tissues were separated and placed in paper bags, labeled, and dried at 35-40 °C to constant mass. Two weeks later, the dry masses of leaf, stem, caudex, and root tissues were recorded.

Extraction and determination of parthenolide

Dried plant samples from each treatment were ground to a fine powder using a Wiley-Mill grinder (Arthur H. Thomas Co., Philadelphia). A subsample of the powder (0.5g) was extracted three times with 10 mL methanol, vortexed, then placed for 15 minutes at 60 °C in a Branson model 5210 ultrasonic bath (Branson, Danbury, CT). Then, the sample was centrifuged for 5 minutes and the supernatant of each aliquot was collected. For each sample, three successive aliquots were pooled, and the extract volume was adjusted to 30 mL with methanol. Samples (1.5 mL) were filtered through a 0.2 µm PTFE membrane (Chromatographic Specialties, Brockville, Canada), then 5 µL

of sample was injected into the HPLC system. To ensure reliable results, this procedure was replicated three times for each powdered sample.

HPLC apparatus and chromatographic conditions

The Beckman HPLC system was equipped with an autosampler (Module 502, with a 5 μ L loop), solvent delivery system (Module 126), and a photo diode array detector (Module 168). Separation was accomplished on a 4 μ m LiChrospher 100 RP-18, 75 x 4.6 mm analytical cartridge with a 5 μ m LiChrospher RP-18, 4 x 4.6 mm guard cartridge (E. Merck/BDH Inc, Toronto, Canada). Chromatographic data were processed using System Gold Software (Beckman System Gold, version 8.10, Fullerton, CA). The mobile phases consisted of solvent A, which was water and solvent B, which was acetonitrile. Both solvents were degassed using a pump (KNF Neuberger model UN820.3 FTP, Fisher Scientific, Ottawa, Canada). Isocratic elution occurred at a flow rate of 1.1 mL/min using 45% B over 15 minutes, with UV detection at 210 nm.

Method validation

Parthenolide elutes at about 3 minutes. The peak identity of parthenolide was confirmed by relative retention time and spectral analyses of the experimental samples compared to injections of purified commercial standards (Sigma-Aldrich Canada Ltd, ON). Parthenolide quantification in samples was based on the response factor (slope of standard curve) calculated from known amounts of pure standard. Results are reported in μ g/g.

Stock solutions

Standard solutions used for calibration were prepared by dissolving 40 mg parthenolide in a volumetric flask and bringing the volume up to 100 mL with

acetonitrile. Solutions with concentrations of 0.400, 0.200, 0.100, 0.050, 0.025 and 0.000 mg/mL were generated from the stock solution by serial dilution with acetonitrile.

A standard solution for determination of accuracy and precision was prepared by placing 5 mg parthenolide in a volumetric flask and bringing the volume up to 100 mL with acetonitrile. This standard was injected at the beginning and end of each run, and after every 7 injections of samples. The standard solutions used for calibration were also injected at the beginning of each run.

Statistical analyses

Statistical analyses were conducted using the SYSTAT 9.0 statistical package (SPSS Inc., 1999). ANOVAs were conducted to determine whether there were significant differences among herbivory treatments with respect to various growth parameters. Tukey post-hoc multi-comparison tests were then used to examine the detail of the differences among herbivory treatments for each aspect of growth. Two-sample t-tests were used to compare the leaf area and leaf size between plants in the bud removal and control treatments.

Results

Hypericum perforatum

Table 4.1 summarizes results for the following plant growth analyses: leaf dry mass, leaf hyperforin concentration, leaf hyperforin yield, aboveground dry mass, belowground dry mass (caudex + roots), plant length, stem dry mass, caudex dry mass, root dry mass, and total plant dry mass. Herbivory treatments had significant effects on

Table 4.1. Results of analysis of variance to determine the effects of herbivory treatment on various *Hypericum perforatum* growth parameters.

Growth Parameter	Degrees of freedom	Mean-square	F-Ratio	P-value
Leaf dry mass (g)	2	0.442	5.135	0.008
Leaf [hyperforin] (ug/g)	2	51223039.642	0.385	0.683
Leaf hyperforin yield (ug)	2	1.246 x 10 ¹⁰	3.181	0.055
Aboveground dry mass (g)	2	218.018	2.840	0.065
Belowground dry mass (g)	2	76.609	3.256	0.045
Plant length (cm)	2	178.395	1.360	0.264
Stem dry mass (g)	2	20.449	1.556	0.219
Caudex dry mass (g)	2	0.026	0.715	0.493
Root dry mass (g)	2	0.269	2.360	0.102
Total plant dry mass (g)	2	520.409	3.064	0.053

leaf dry mass at harvest ($p \leq 0.01$). Plants grown in the high herbivory treatment had a significantly lower dry mass than plants in both the control and low herbivory treatments (Table 4.1, Figure 4.1). ANOVA results also revealed a significant difference among treatments in belowground dry mass ($p \leq 0.05$). However, the differences were not statistically significant from each other according to a Tukey post-hoc test. Results of a one-way ANOVA on the proportion of biomass allocated to leaf tissue, stem tissue, and root tissue showed no significant differences between treatments.

The mean leaf hyperforin concentration and mean leaf hyperforin yield were lowest with the high herbivory treatment, although, the effects were not statistically significant.

No statistically significant differences were found for hypericin concentrations between control plants and plants in the high herbivory treatment (Table 4.2, Figure 4.2). However, two-sample t-test results showed that the mean leaf hypericin yield ($p \leq 0.001$) was greater in the control plants compared to plants in the high herbivory treatment (Table 4.2, Figure 4.2).

Tanacetum parthenium

Table 4.3 summarizes the results of the ANOVA comparing various stem and root growth measures. Results of the ANOVA illustrate that the different herbivory treatments had significant effects on primary stem height, number of primary stem nodes, number of secondary stems, primary stem dry mass, higher order stem dry mass, total stem dry mass (primary stem + higher order stems) (all $p \leq 0.001$). In addition, overall plant height, caudex dry mass, and root dry mass differences were significant at $p \leq 0.05$.

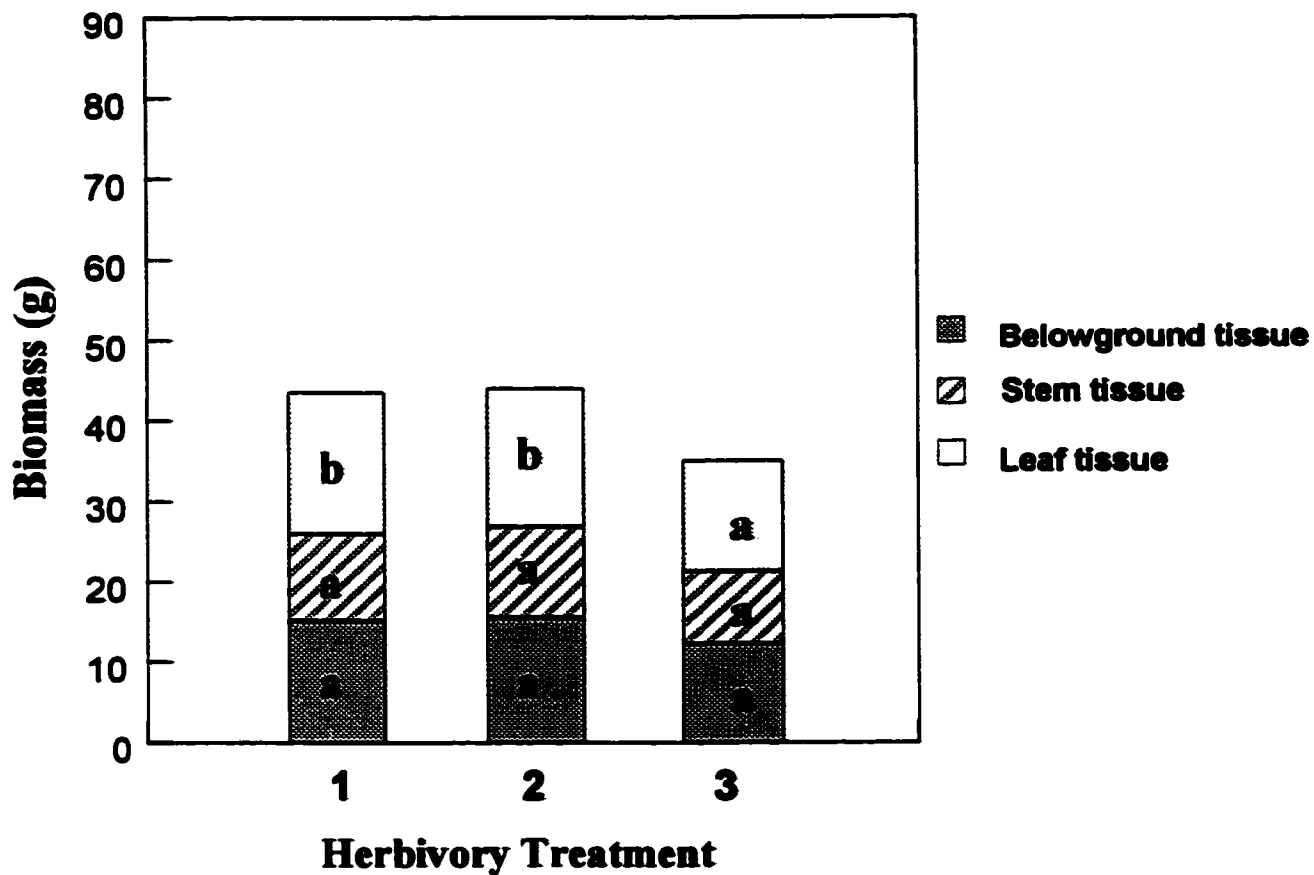


Figure 4.1. Effects of herbivory treatment on the biomass (g) of leaf, stem, and belowground tissue in *Hypericum perforatum*. Between treatments, within the same tissue, bars with the same letter are not significantly different from each other. On x axis 1 = control, 2 = low herbivory (100 leaves present were cut in half at the tip of the leaf), 3 = high herbivory (all leaves on adaxial side of every stem were removed).

Table 4.2. Comparison of leaf hypericin concentration and leaf hypericin yield of *Hypericum perforatum* in the control and high herbivory treatments. For all comparisons, N=7.

Growth Parameter	Mean (\pmSE) for control	Mean (\pmSE) for high herbivory	Probability
Leaf [hypericin] (ug/g)	442.032 (18.512)	400.350 (22.089)	0.174
Leaf hypericin yield (ug)	7701.154 (1281.036)	5563.442 (412.598)	0.001

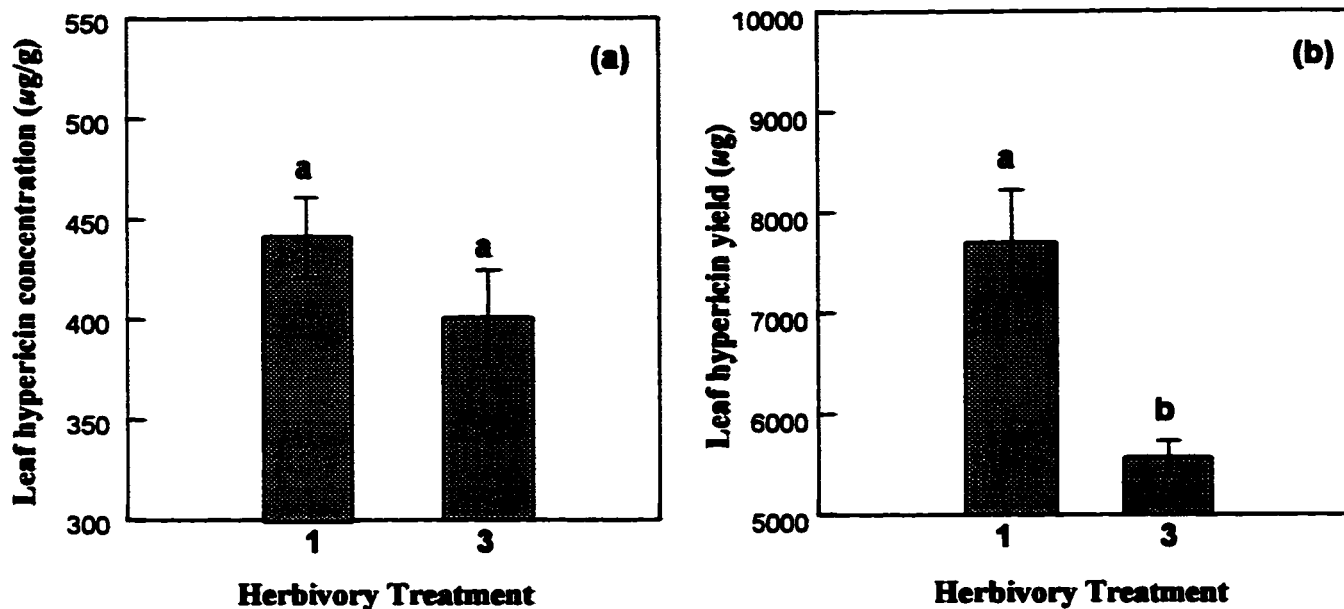


Figure 4.2 (a) and (b). Effects of herbivory treatment on (a) leaf hypericin concentration (\pm SE) and (b) leaf hypericin yield (\pm SE) of *Hypericum perforatum*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, and 3 = high herbivory (all leaves on adaxial side of every stem were removed).

Table 4.3. Results of analysis of variance to determine the effects of herbivory treatment on various *Tanacetum parthenium* stem and root parameters.

Growth Parameter	Degrees of freedom	Mean-square	F-Ratio	P-Value
Primary stem height (cm)	4	1257.737	22.653	0.000
Overall plant height (cm)	4	160.543	2.474	0.049
Number of nodes on primary stem	4	118.453	20.563	0.000
Number of secondary stems	4	44.406	7.479	0.000
Primary stem dry mass (g)	4	4.865	5.204	0.001
Higher order stem dry mass (g)	4	255.518	9.317	0.000
Total stem dry mass (g)	4	328.494	7.756	0.000
Caudex dry mass (g)	4	0.048	2.669	0.046
Root dry mass (g)	4	327.764	4.289	0.003

Plants grown in the bud removal treatment produced a significantly shorter primary stem than plants in all other treatments (Figure 4.3 a); despite the fact that the primary stem was shorter, plants in the bud removal treatment nevertheless produced a greater primary stem mass than plants in both the low herbivory treatment and high herbivory treatment (Figure 4.3 b).

ANOVA results revealed a significant difference in overall plant height among herbivory treatments ($p \leq 0.05$), but post-hoc comparisons of means indicated the differences were not statistically significant.

Plants grown in the bud removal treatment produced a significantly lower number of nodes and secondary stems on the primary stem than plants from all other herbivory treatments. (Figure 4.4 a, b). However, the total dry mass of higher order stems was significantly greater in these plants from the bud removal treatment than in plants from all other treatments (Figure 4.5).

Plants from the bud removal treatment also had a significantly greater caudex dry mass than plants in the high herbivory treatment. The root dry mass in plants grown in the bud removal treatment was significantly higher than plants from the low herbivory treatment, high herbivory treatment, and control treatment.

The results of a one way ANOVA between four herbivory treatments (low herbivory, high herbivory, sustained leaf herbivory, and control treatments) showed no significant differences in terms of number of flowers, bud dry mass, flower and peduncle dry mass and total reproductive dry mass (buds + flowers + peduncles). The bud removal treatment could not be included in the comparison of reproductive biomass because flower development was deliberately prevented in this treatment.

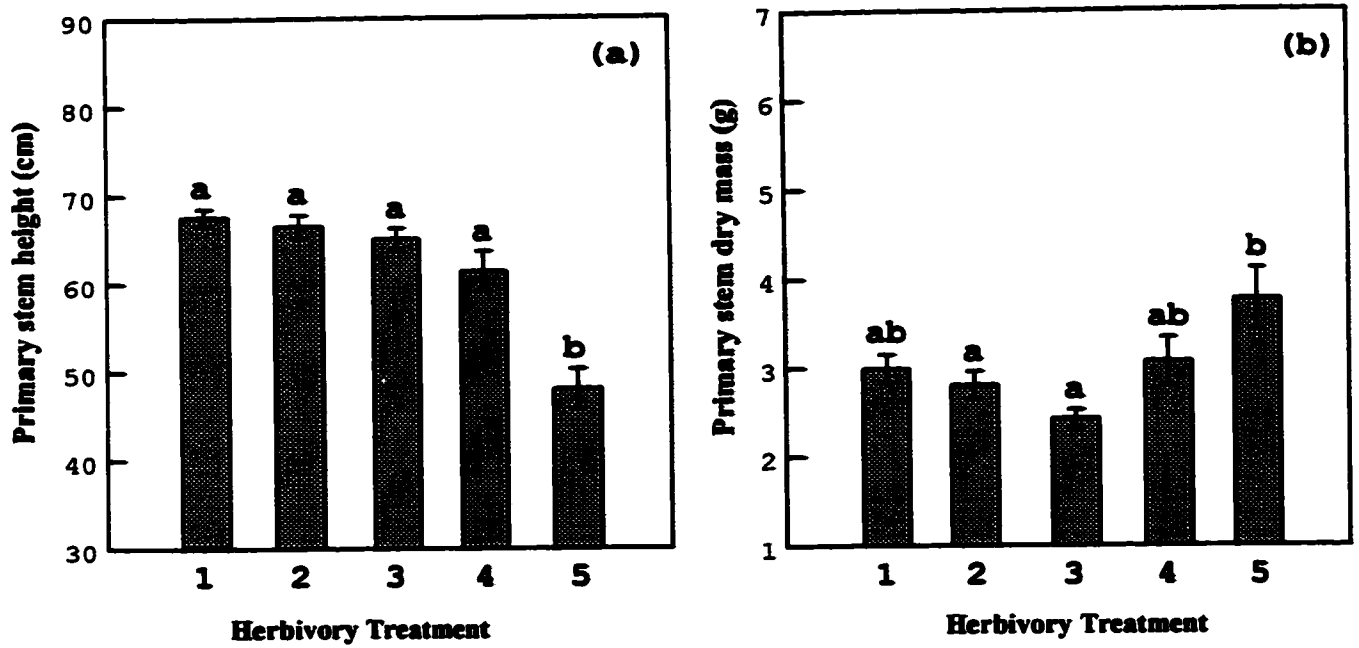


Figure 4.3 (a) and (b). Effects of herbivory treatment on (a) primary stem height (\pm SE) and primary stem dry mass (\pm SE) of *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

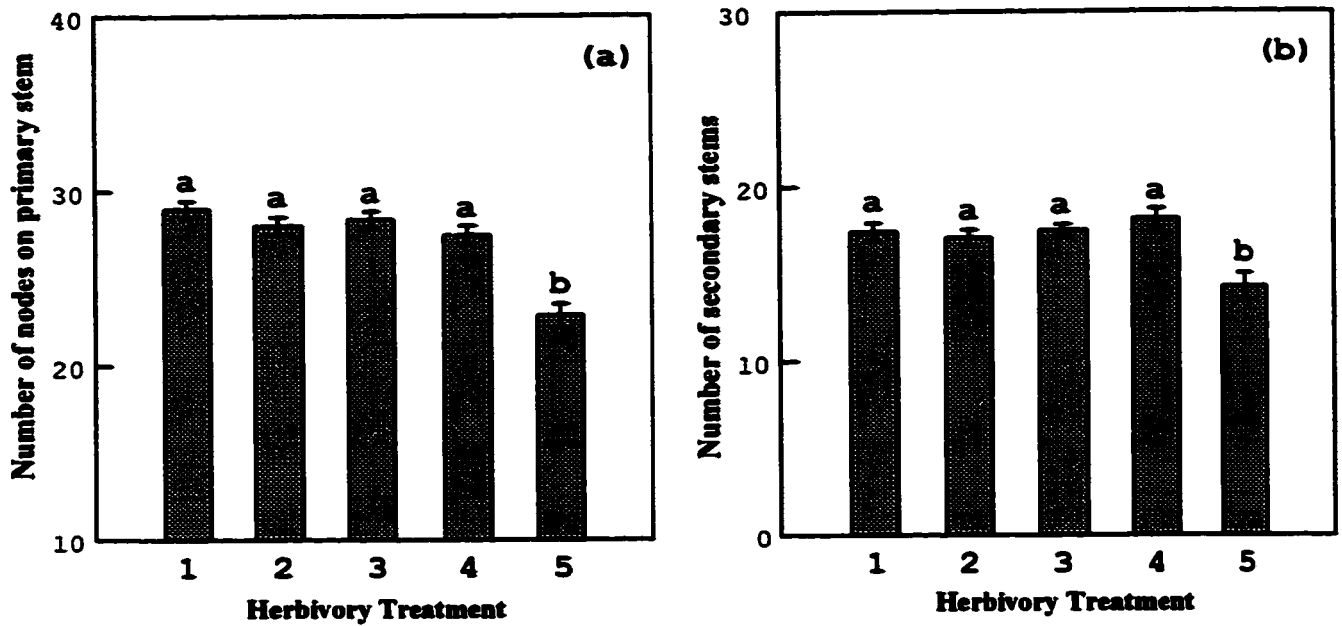


Figure 4.4 (a) and (b). Effects of herbivory treatment on (a) number (\pm SE) of nodes on the primary stem and (b) number (\pm SE) of secondary stems of *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

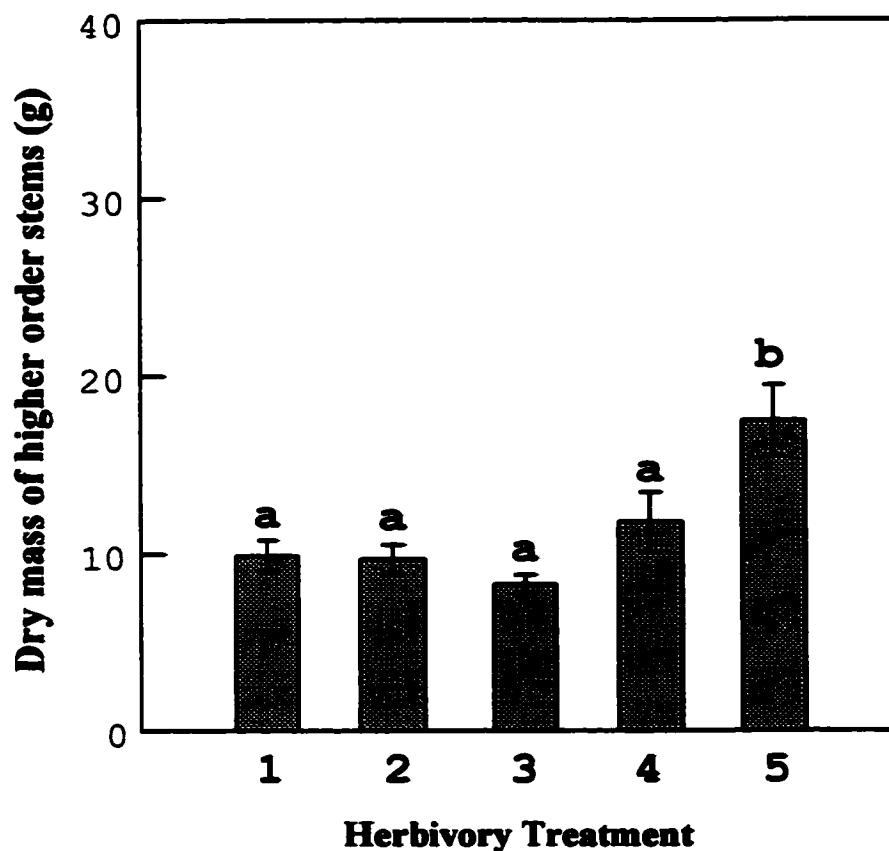


Figure 4.5. Effects of herbivory treatment on dry mass (\pm SE) of higher order stems of *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

Table 4.4 summarizes the ANOVA results of various leaf growth parameters. Herbivory treatments had statistically significant effects on the number of green leaves, green leaf dry mass, total leaf dry mass (green leaves + dead leaves), leaf parthenolide concentration, and leaf parthenolide yield (all $p \leq 0.001$). In the case of dead leaf dry mass, differences were significant at $p \leq 0.01$.

The number of green leaves and green leaf dry mass produced from plants grown in the bud removal treatment were significantly higher than in all other herbivory treatments (Figure 4.6 a, b). Plants in the bud removal treatment also had a significantly greater total leaf (green + dead leaves) dry mass than plants from all other treatments. The control plants had significantly more dead leaf dry mass than either plants grown in the sustained leaf herbivory treatment or plants grown in the bud removal treatment (Figure 4.7).

Figure 4.8 illustrates the absolute biomass allocated to reproductive tissues, leaf tissue, stem tissue, and belowground (caudex + roots) tissue in plants in each treatment. There was significantly more leaf tissue, and stem tissue in the bud removal treatment than all other treatments, and the mass of belowground tissue in the bud removal treatment was significantly greater than in the low herbivory treatment, high herbivory treatment, and control treatment.

A one-way ANOVA was also performed to compare the effects of herbivory treatments on the proportion of biomass allocated to reproductive tissue, leaf tissue, stem tissue, and belowground tissue (Figure 4.9). Plants in the bud removal treatment allocated a significantly greater proportion of biomass to leaf tissue ($p \leq 0.001$) and a significantly greater proportion of biomass to stem tissue ($p \leq 0.001$) than plants in all

Table 4.4. Results of analysis of variance to determine the effects of herbivory treatment on various *Tanacetum parthenium* leaf parameters.

Growth Parameter	Degrees of freedom	Mean-square	F-ratio	P-value
Number of green leaves	4	384145.990	15.653	0.000
Green leaf dry mass (g)	4	301.527	17.093	0.000
Dead leaf dry mass (g)	4	0.534	4.098	0.004
Total leaf dry mass (g)	4	299.888	16.394	0.000
Leaf [parthenolide] ($\mu\text{g/g}$)	4	1222070.466	5.161	0.001
Leaf parthenolide yield (μg)	4	5.186×10^9	42.615	0.000

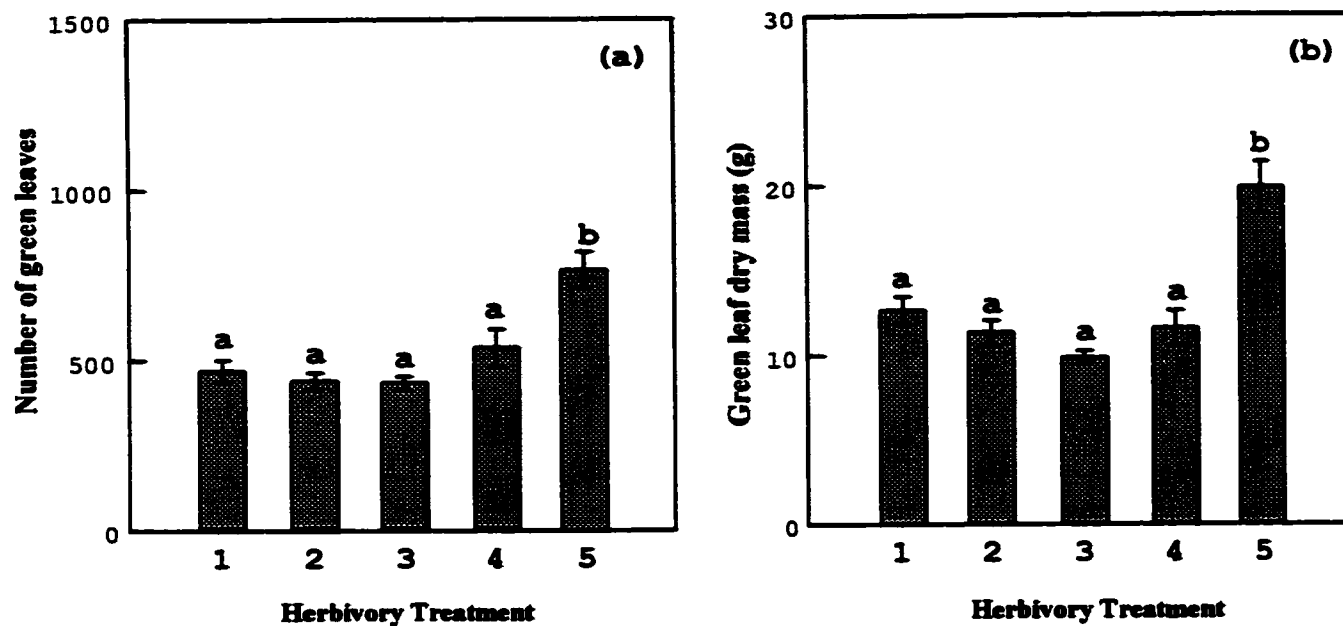


Figure 4.6 (a) and (b). Effects of herbivory treatment on (a) number (\pm SE) of green leaves and (b) green leaf dry mass (\pm SE) of *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

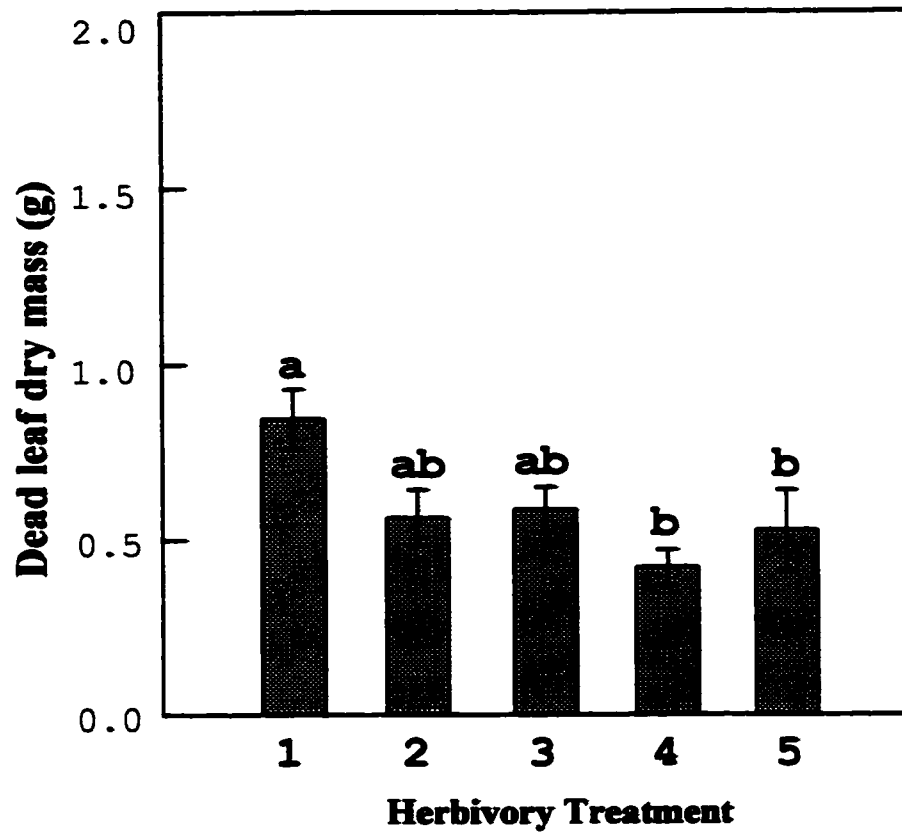


Figure 4.7. Effects of herbivory treatment on the dry mass (\pm SE) of dead leaves of *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

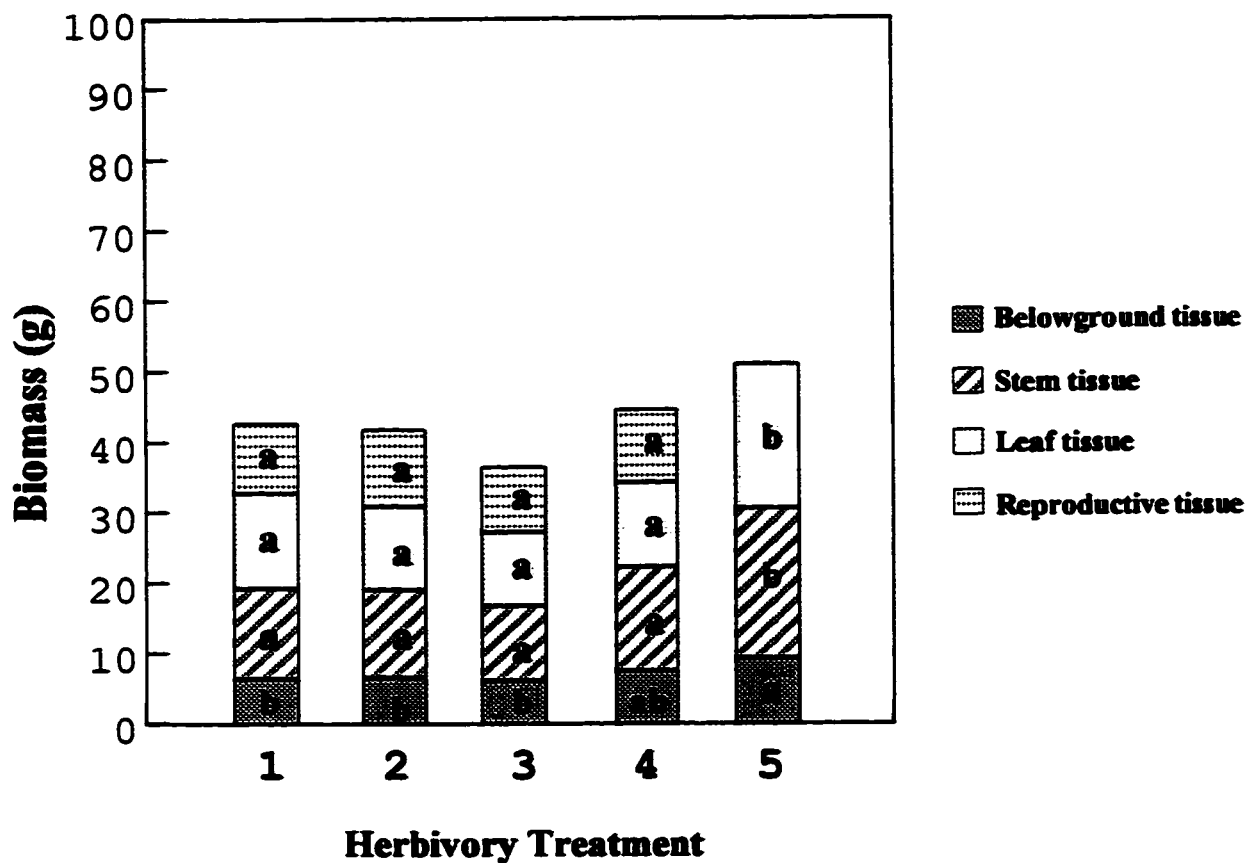


Figure 4.8. Effects of herbivory treatment on the biomass (g) of reproductive, leaf, stem, and belowground tissue of *Tanacetum parthenium*. Between treatments, within the same tissue, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

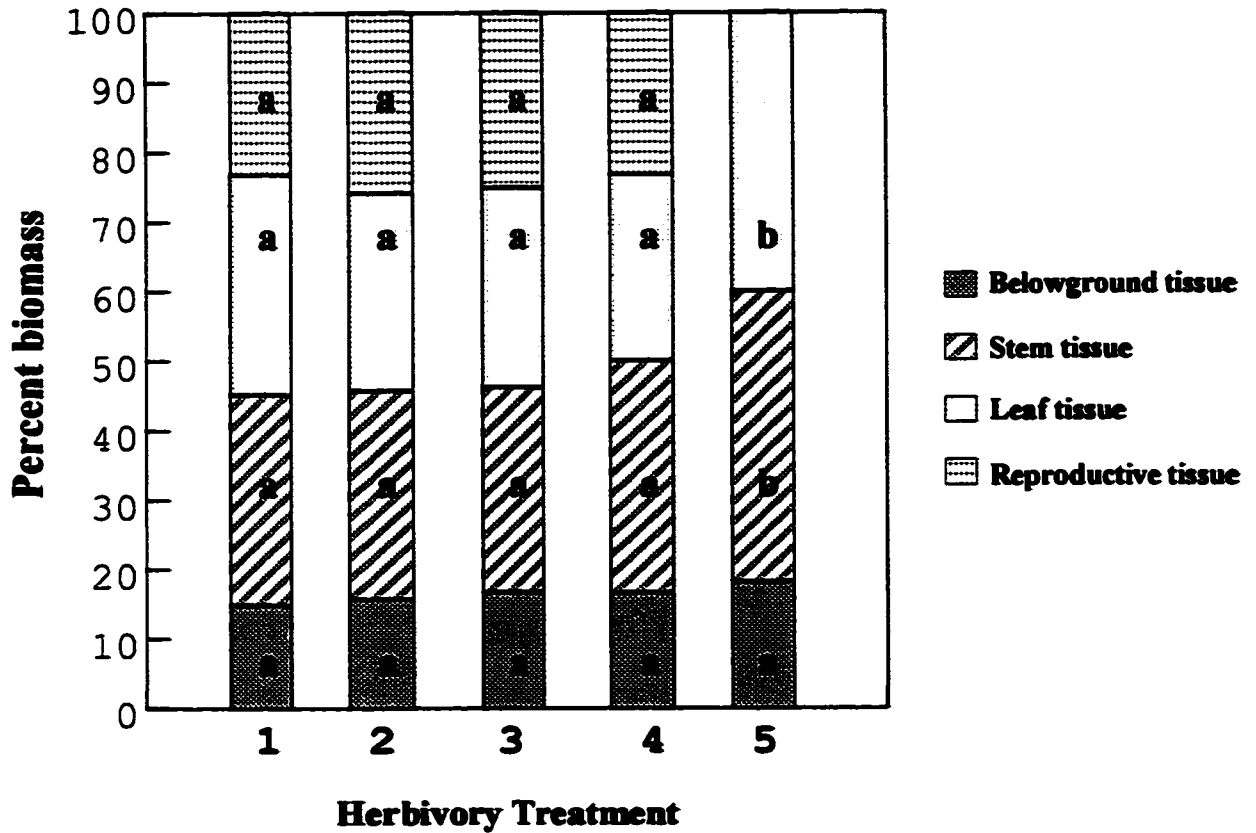


Figure 4.9. Proportional allocation of biomass to reproductive, leaf, stem and belowground tissue in *Tanacetum parthenium*, for each of the herbivory treatments. Between treatments, within the same tissue, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

other treatments; again it should be noted that these plants, by virtue of the bud removal treatment, had been prevented from allocating biomass to reproductive tissues.

The highest leaf parthenolide concentration was found in plants grown in the sustained herbivory treatment; that concentration was significantly higher than in either plants from the high herbivory treatment or plants from the control treatment (Figure 4.10).

Total yield of leaf parthenolide per plant was significantly greater in plants grown in the bud removal treatment ($84132.72 \pm 7490 \mu\text{g}$) compared to all other treatments (Figure 4.11). In order to investigate whether the greatest yield was due to larger, or more smaller leaves, two-sample t-tests were used to compare the mean leaf area between plants in the control treatment and plants in the bud removal treatment, and to compare the mean leaf size (leaf area divided by number of leaves) between plants in the control and in the bud removal treatment (Table 4.5). The results show that the significantly greater leaf area in the bud removal treatment ($p \leq 0.05$) was produced by having more, significantly smaller, leaves in the bud removal treatment ($p \leq 0.01$).

Discussion

Hypericum perforatum

We had anticipated that plants in the herbivory treatment would have a higher leaf dry mass and aboveground dry mass compared to controls, since overcompensation has been seen in a number of studies (Paige and Whitman 1987, Lennartsson *et al.* 1998, Agrawal 2000). However, under our experimental conditions this was not the case. Plants did not overcompensate in response to damage by increasing the growth of

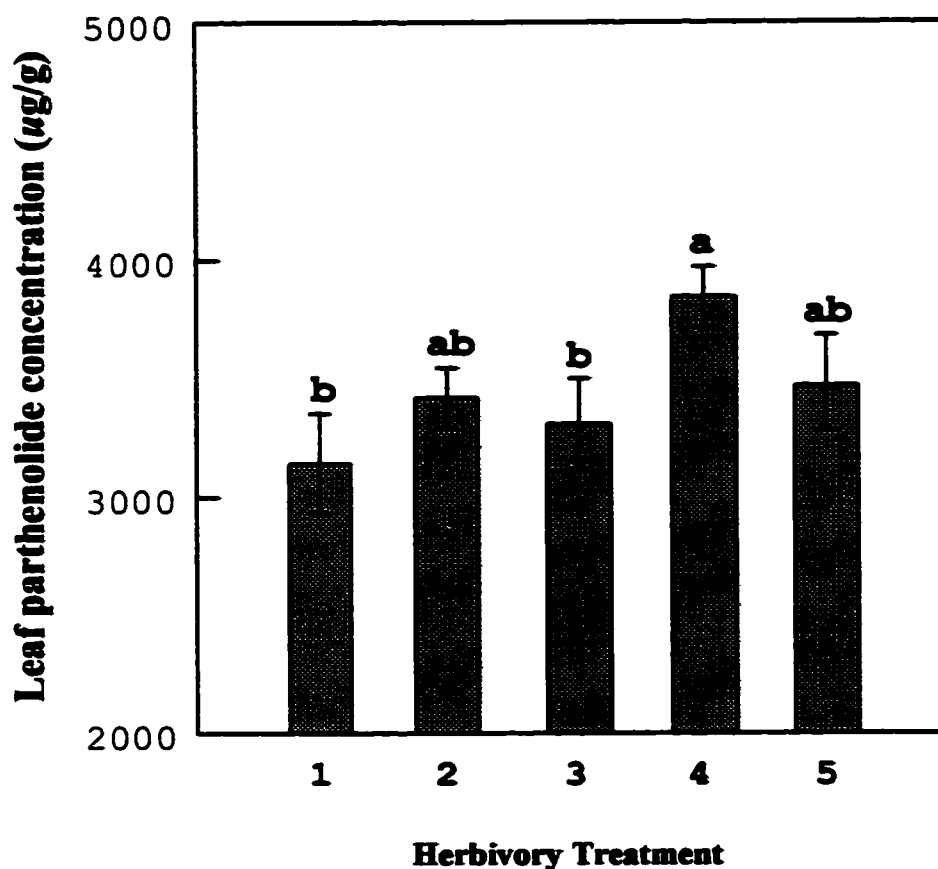


Figure 4.10. Effects of herbivory treatment on the concentration (\pm SE) of leaf parthenolide ($\mu\text{g/g}$) in *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % of leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

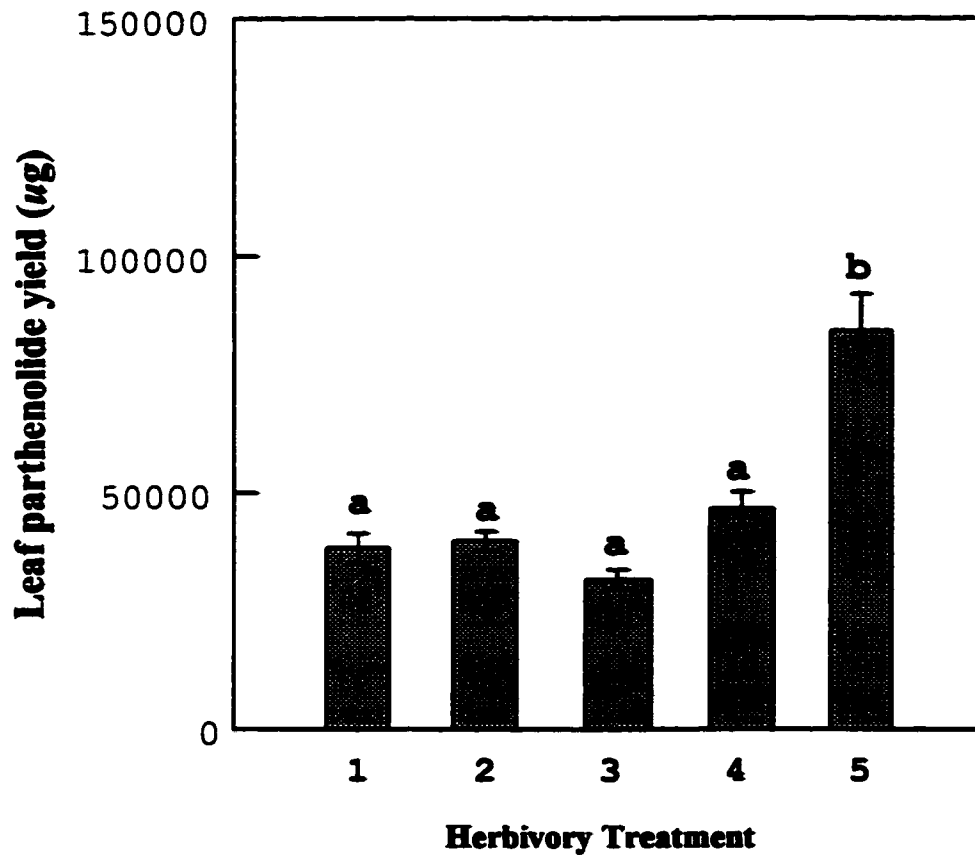


Figure 4.11. Effects of herbivory treatment on the yield (\pm SE) of leaf parthenolide (μg) in *Tanacetum parthenium*. Between treatments, bars with the same letter are not significantly different from each other. On x-axis 1 = control, 2 = low herbivory (25 % leaves present removed), 3 = high herbivory (50 % of leaves present removed), 4 = sustained herbivory (50 % of 15 leaves removed every 3 days), 5 = bud removal (repeated every 3 days).

Table 4.5. Comparison of leaf area and average leaf size for *Tanacetum parthenium* in the control and bud removal treatments. For all comparisons, N=6.

Growth Parameter	Mean (YSE) for control	Mean (YSE) for bud removal	Probability
Leaf area (cm ²)	4770.323 (542.477)	7891.778 (929.825)	p≤0.05
Leaf size (cm ²)	10.537 (0.270)	8.290 (0.447)	p≤0.01

remaining tissues. Instead, aboveground dry mass in plants experiencing both low and high herbivory did not differ significantly from control plants. Although Paige and Whitman (1987) provided evidence that the relative fitness of grazed *Ipomopsis aggregata* plants was greater than that of ungrazed individuals, a later study by Bergelson and Crawley (1992) found that clipped *Ipomopsis aggregata* did not differ in final shoot dry weights from control plants. Other studies have also shown aboveground parts of defoliated plants to be undistinguishable from control plants. Prins *et al.* (1989) studied the effects of various levels of artificial defoliation on *Cynoglossum officinale* and *Senecio jacobaea* grown in sand under controlled conditions and observed both species to experience regrowth but no overcompensation. Similarly, Oosterheld (1992) found *Briza subaristata* and *Stipa batioensis*, subjected to various levels of defoliation ranging from 0% to 100%, to fully compensate in aboveground biomass compared to controls, but no overcompensation was evident. *Briza subaristata* and *Stipa batioensis* increased their relative growth rates exponentially with defoliation intensity (Oosterheld 1992).

In this study, plants in the high herbivory treatment had a significantly lower leaf mass than all other plants. This suggests that the “high” herbivory treatment may have been too severe to allow the plants to fully recover, relative to the controls. Full leaf compensation was seen in plants experiencing low herbivory. The mechanism used to injure leaves may also have been a factor. Sometimes the method is as important in determining a plant’s response to damage, as is the degree of damage to the leaf itself (Baldwin 1988). Plants in the low herbivory treatment experienced more damage because the leaves were torn in half at the tip, compared to plants in the high herbivory treatment where leaves were pulled from the stem at their petiolar attachments.

It is also possible that the post-treatment recovery time (one month) may have not been sufficient enough to allow for full leaf compensation in plants in Treatment III (high herbivory). Prins *et al.* (1989) working with *Senecio jacobaea* and *Cynoglossum officinale* reported that the recovery time after manual leaf removal depended on the degree of defoliation. The results of our herbivory simulations on *Hypericum perforatum* may have been different if the plants had been harvested at a later date, following flowering and seed set.

Numerous studies have shown that the production of secondary compounds may increase in response to herbivory or simulated herbivory (Collantes *et al.* 1997, Gianoli and Niemeyer 1997, Baldwin 1988). Hypericin has been suggested to play a role in herbivore defence and has been shown to vary with factors such as site characteristics, nutrients, and source or accession (Buter *et al.* 1998, Denke *et al.* 1999, Briskin *et al.* 1999). Hyperforin, on the other hand, seems to vary little according to location and accession (Buter *et al.* 1988).

In this experiment, hypericin and hyperforin concentrations in plants subjected to low and high herbivory did not differ significantly from from each other, nor did they differ significantly from control plants. In herbivory simulations, the lack of induced responses has been suspected to result from low levels of leaf tissue damage, or the absence of herbivore saliva (Agrawal 2000). The allocation of energy to defence in damaged tissue could be delayed until the degree of herbivory threatens a plant's survival and fitness. Such a threshold requirement for damage might save resources for growth and reproduction in plants with a high level of tolerance (Zangerl and Bazzaz 1992, cited

by Gianoli and Niemeyer 1997). The amount of leaf surface area damaged, and mechanism of damage may also play a role in chemical induction (Baldwin 1988).

The induction of secondary metabolites may have also be affected by the duration of the damage, which in this experiment was very short. Gianoli and Niemeyer (1997) found that hydroxamic acid induction in *Triticum aestivum* was dependent on the duration as well as intensity of aphid feeding. In a later study, Gianoli and Niemeyer (1998) found that induction of hydroxamic acid in *Triticum uniaristatum* lasted only 48 hours. Thus, if induction of hypericin and hyperforin occurs in *Hypericum perforatum*, and lasts only for a short period, then these plants may have had their initial concentrations of hypericin and/or hyperforin rise after initial damage (which occurred one month prior to harvest) but return to normal levels with time such that they no longer differed significantly by the end of the experiment.

In conclusion, *Hypericum perforatum* plants experiencing herbivory did not overcompensate with respect to leaf production or aboveground biomass production. Neither the concentration nor the yield of hypericin and hyperforin increased in response to herbivory. In fact, the yield of hypericin was significantly higher in control plants than in plants in the high herbivory treatment. Although herbivory did not increase the concentrations of secondary metabolites, the hydroponic cultivation of *Hypericum perforatum* did yield concentrations of leaf hypericin (311 – 507 µg/g) and leaf hyperforin (5225 – 19418 µg/g) that were comparable to concentrations observed in *Hypericum perforatum* collected from Nova Scotia, Ontario, British Columbia, Oregon, and Australia (121 – 804 µg/g hypericin) by Jensen *et al.* (1995) and higher than those

observed in the foliage of *Hypericum perforatum* in Slovenia (550 – 11330 µg/g hyperforin) by Umek *et al.* (1999).

Tanacetum parthenium

Flower bud removal had a significant effect on the number of leaves, dry mass of leaves, and dry mass of higher order stems compared to plants in which flowering was allowed to occur. These plants also had significantly shorter and heavier primary stems than plants in all other treatments. The smaller primary stem height was probably a result of the removal of the main stem's apical meristem, promoting lateral growth. However, overall plant height was similar across treatments because of the growth of higher order stems. Due to the shortness of the main stem, the number of primary nodes and secondary stems it bore were significantly fewer in the bud removal treatment than in all the other treatments.

Overcompensatory leaf production by *Tanacetum parthenium* was induced only in plants experiencing bud removal; these plants produced 1.6 to 2 times as much dry leaf mass as plants in all the other treatments. These plants also allocated a greater percentage of their biomass to leaf tissue compared to plants from the other herbivory regimes. Hurd *et al.* (1979) demonstrated in tomatoes (*Lycopersicon esculentum*) that, during anthesis and fruiting, vegetative growth was reduced as resources became diverted to developing fruit. In an experiment by Daugaard (1999), flowers were removed from *Fragaria X ananassa*; the result was an increase in the numbers of leaves and fresh mass of runners per plant compared to controls. In the present experiment, the pattern of overcompensation in leaf and stem tissue in *Tanacetum parthenium* in the bud removal

treatment may have been a result of more energy being available for vegetative growth since flower development is being prevented. Literature reviewed by Bazzaz *et al.* (1987) suggests that since reproduction and vegetation compete for resources within the plant, the prevention of flowering may extend a plant's vegetative growth. Also, when apical meristems are removed, the auxin source which inhibits lateral growth is also removed, and branching is stimulated (Berg 1997). Overcompensation has also been suggested to be a result of redirection of assimilates from the roots (McNaughton 1983; Paige and Whitman 1987). Reuss *et al.* (1983) showed that foliage removal increased energy allocated to vegetation at the expense of roots. If this were true in this case, a reduction in root biomass would have been evident in our bud removal treatment. Instead, these plants had an equal or higher root mass than all other treatments, suggesting that leaf overcompensation was not at the expense of the root system. The absence of flowers may have promoted vegetative growth with increased root production being a consequence of the plant's increased demand for water and minerals.

Plants in the bud removal treatment had a significantly greater total leaf area, but significantly smaller individual leaves than control plants. The prevention of flowering may have prolonged the growth period so that the plants produced more juvenile leaves (Karban and Myers 1989).

The biomass of dead leaves (mainly found at the base of the plant) was greatest in the control treatment. The difference was significant only for the comparison between plants from the sustained leaf removal and the bud removal treatments. In these plants, the persistent removal of buds or leaves may have prolonged the active lifetime of the remaining leaves by reducing shading of the lower leaves, which would ordinarily result

in a decline in the photosynthetic capacity of the lower leaves followed by senescence and death (Woledge 1978, McNaughton 1983).

Numerous studies have shown that the production of secondary compounds may increase in response to herbivory or simulated herbivory (Baldwin 1988, Cronin and Hay 1996, Gianoli and Niemeyer 1997). Plants in which leaf removal was sustained throughout the duration of the experiment produced the highest concentration of parthenolide (this value was significantly higher than for plants in the control and high herbivory treatments). The other levels of herbivory simulation may not have been high enough to induce elevated concentrations of defense chemicals. These results indicate that persistent, sustained leaf herbivory may induce a rise in the secondary metabolite, and implicate a facultative response. The induction of certain secondary metabolites may be either systemic or localized to the damaged leaf (Baldwin 1988, Gianoli and Niemeyer 1998). Gianoli and Niemeyer (1998) found that induced hydroxamic acid levels in *Triticum uniaristatum* were restricted to the aphid-infested leaf. If parthenolide induction were confined to the damaged leaves, then the higher observed concentrations in plants in the sustained herbivory treatment could simply be due to the fact that more leaves were damaged. There may also be a refractory period for the facultative process and if induction of a secondary metabolite lasts for only a short time after damage (Gianoli and Niemeyer 1998), then plants damaged once (a month prior to harvest) may have had their parthenolide concentration rise initially, but then return to normal levels by harvest time. In contrast, plants experiencing continuous leaf removal had been damaged repeatedly, until two days prior to harvest.

The highest yield of parthenolide was in plants experiencing bud removal, although the absolute concentration of parthenolide was no higher in these plants than in the other treatments, they had more, small, young leaves that, taken together provided a greater total yield of the target substance.

In conclusion, plants prevented from flowering produced a greater number and dry mass of leaves, the tissue of greatest importance in the medicinal applications of *Tanacetum parthenium*. This indicates that the hydroponic cultivation of *Tanacetum parthenium* is a very viable approach, yielding concentrations of parthenolide that are relatively stable (with an average of $3433.87 \mu\text{g/g} \pm 78.41$ over all treatments). These levels are superior to the minimum of $2000 \mu\text{g/g}$ proposed by Health Canada for quality control (<http://www.hc-sc.gc.ca>), and can be maximized in terms of total yield per plant by removing flower buds throughout the growth period. This would be a labour-intensive practice, taking about 3 minutes per plant, but the total yield of parthenolide is 1.8 to 2.6 times more that of the other treatments. A cost/benefit analysis would be necessary to evaluate the cost-effectiveness of this treatment regime in a commercial setting.

APPENDIX

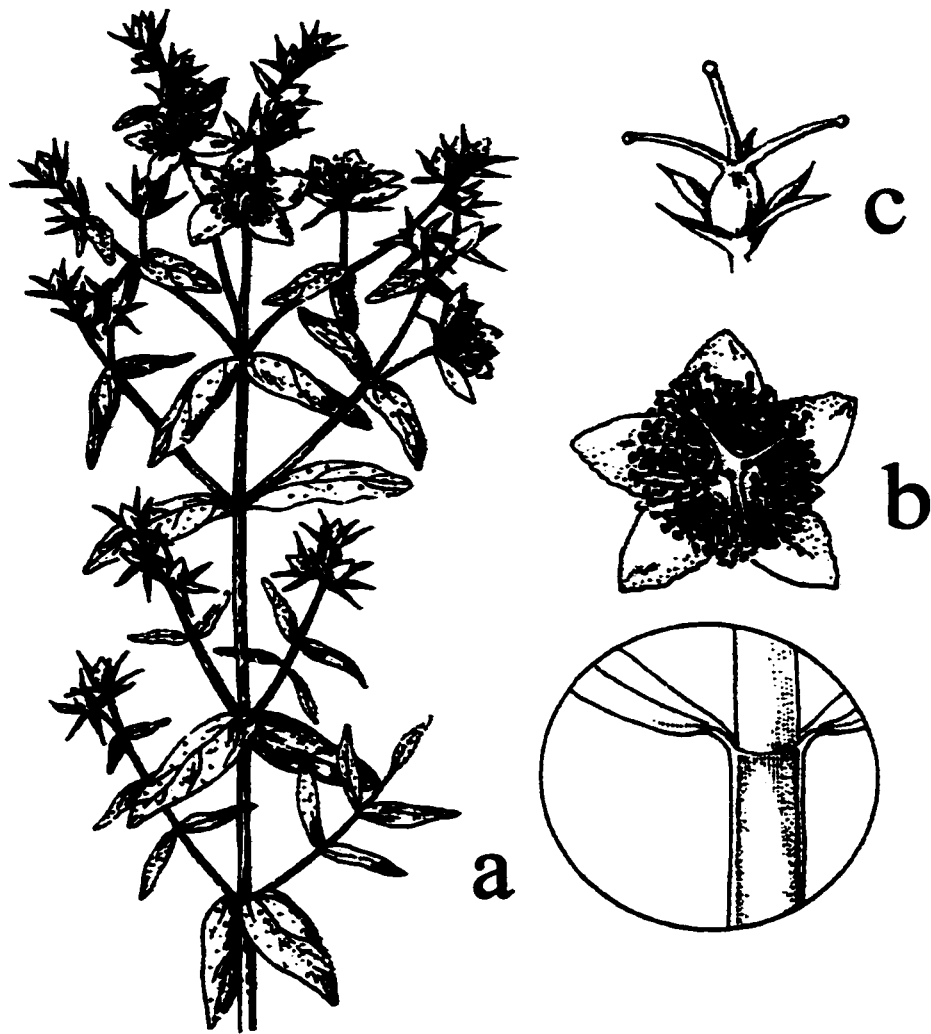


Figure A.1. Morphological features of *Hypericum perforatum*, a member of the Hypericaceae family (adapted from Holmgren 1998). A herbaceous perennial (a) multibranched, with many opposite, pinnately netted, oval leaves with numerous pelucid glands on the surface and black glandular dots along the margins. The stamens (b) are numerous, the styles (c) are three, and the ovary is ovoid (Bombardelli and Morazzoni 1995, Crompton 1988).



Figure A.2. Morphological features of *Tanacetum parthenium*, a member of the Asteraceae family (adapted from Holmgren 1998). A herbaceous perennial with round, leafy, branching stems bearing alternative bipinnate leaves with ovate green leaflets, coarsely toothed. The flowers are numerous, daisy-like, with a yellow center consisting of many yellow disks and from 10-20 white toothed ray florets (Hobbs 1989, Murray 1995).

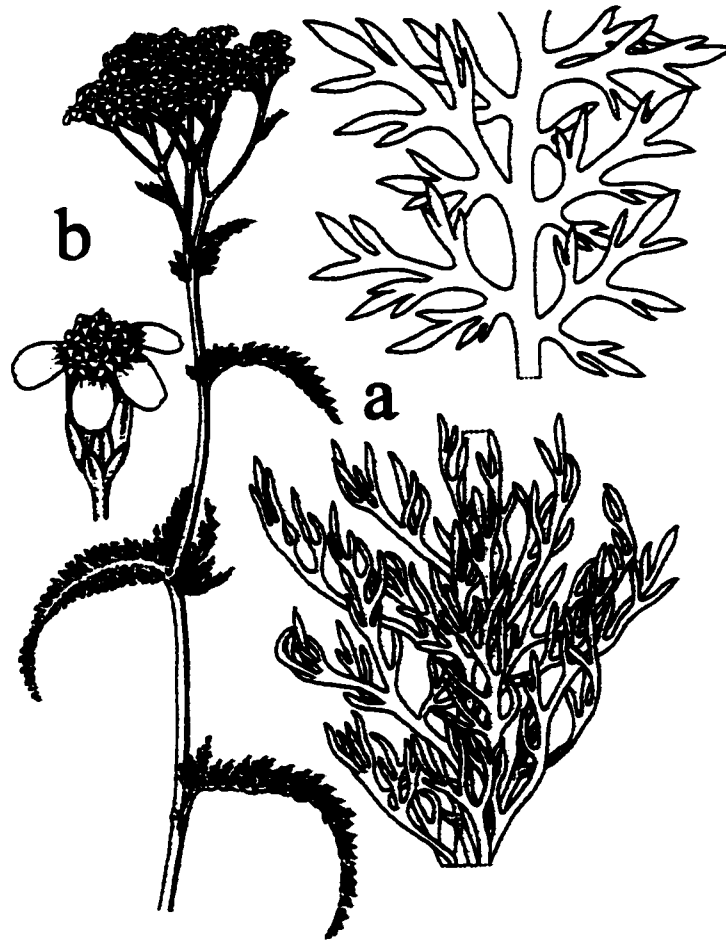


Figure A.3. Morphological features of *Achillea millefolium*, a member of the Asteracea family (adapted from Holmgren 1998). A perennial herb, 30-60 cm tall, with a single sometimes forked stem. Leaves (a) are lanceolate, 2-4 times pinnately dissected and finely toothed. The leaves are clustered at the base of the stem with smaller feather-like leaves alternating upwards along the stem. Flowers (b) are numerous, in a flat topped corymb, ray flowers mostly five, three toothed, white or occasionally pink (Chandler *et al.* 1982, Warwick and Black 1982, Mitich 1990).



Figure A.4. Morphological features of *Leonurus sibiricus*, a member of the Labiatae family (adapted from Holmgren 1998). A perennial herb with 3-lobed leaves; lobes toothed. Flowers are pinkish in whorls in axils.

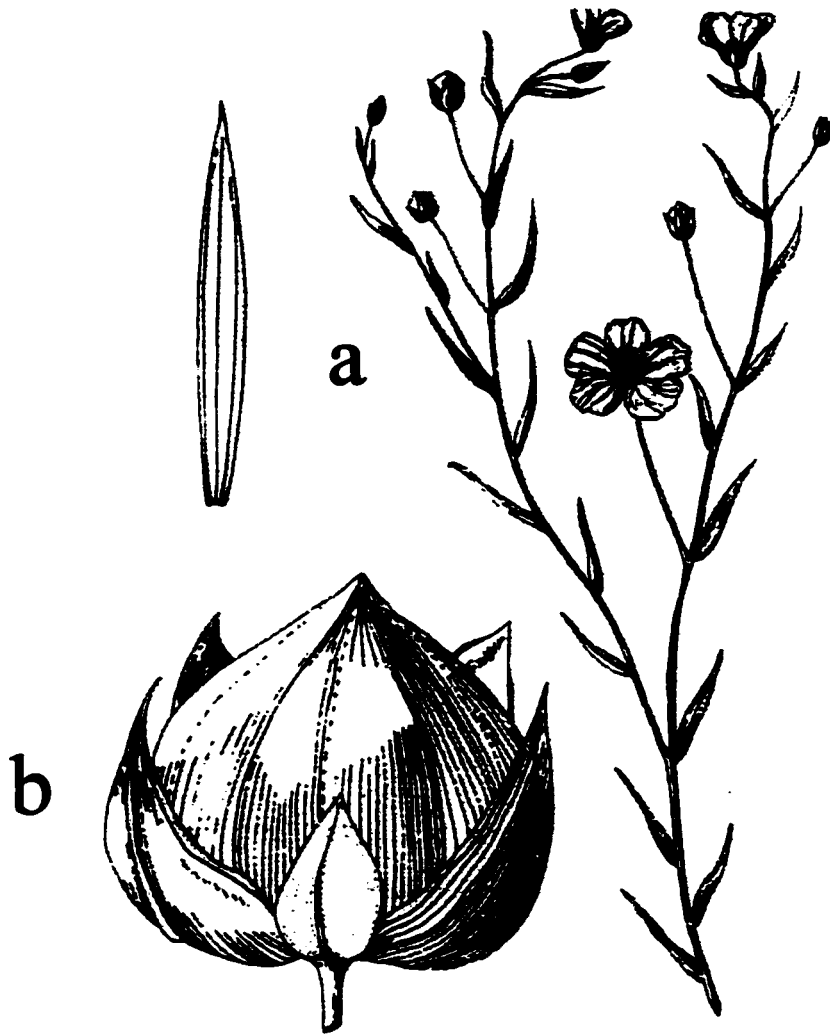


Figure A.5. Morphological features of *Linum usitatissimum*, a member of the Linaceae family (adapted from Holmgren 1998). An annual plant (a), with many small, three-veined, linear, alternate leaves. Flowers are borne on branch terminals, are many, five-petaled, may vary from hues of blue, pink, lavender or white. The mature fruit (b) is a dry capsule divided into five segments, with each segment producing two seeds (<http://agric.gov.ab.ca>, <http://gears.tucson.ars.ag.gov>).

Table A.1. Macronutrients, micronutrients, and electrical conductivity of the four nitrogen treatments.

Salts	Nitrogen treatment			
	N ₆₀ mg/L	N ₁₂₀ mg/L	N ₁₈₀ mg/L	N ₂₄₂ mg/L
Macronutrients				
Ca(NO ₃) ₂	156.60	500.00	500.00	626.30
KNO ₃	100.95	227.20	672.65	583.50
NH ₄ NO ₃	20.20	0	0	4.35
Mg(NO ₃) ₂	0	0	0	334.75
NaNO ₃	85.20	63.9	63.05	164.1
CaCl ₂	330.55	88.90	88.90	0
NaCl	0	0	0	0
K ₂ SO ₄	648.95	399.60	98.40	205.05
KCl	0	111.00	20.5	0
KH ₂ PO ₄	174.85	174.85	174.85	174.85
MgSO ₄	318.00	318.00	318.00	0
Micronutrients				
MnSO ₄	1.885	1.885	1.885	1.885
ZnSO ₄	0.200	0.200	0.200	0.200
H ₃ BO ₄	3.265	3.265	3.265	3.265
CuSO ₄	0.240	0.240	0.240	0.240
NaMoO ₄	0.150	0.150	0.150	0.150
EC (Electrical conductivity) mS/cm	2.4	2.4	2.4	2.6

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VITA AUCTORIS

NAME: Dana Simeunovic

PLACE OF BIRTH: Windsor, Ontario

YEAR OF BIRTH: 1974

EDUCATION: St. Anne highschool, Tecumseh, Ontario

1989-1994

University of Windsor, Windsor, Ontario

1994-1999 B.Sc.

University of Windsor, Windsor, Ontario

1999-2002 M.Sc.