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The Effects of Nitrogen, Harvest Method and Substrate on the Growth and the Medicinal Compound Concentration of Hydroponically-Grown Sundew (*Drosera adelae* F. Muell.)

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August 2000

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of Master of Science

Suggested Short Title:

Effects of Agronomic Factors on Sundew Growth and Quality

Abstract

Some species of sundew (*Drosera* spp.), harvested from the wild for use in homeopathic cold remedies, are now endangered. This research consisted of two experiments in hydroponic production of sundew. The first investigated the effects of four nitrogen (N) fertilization levels (0.0, 5.1, 25.5 or 51.0 mg/L added N) and three harvest methods (after two months, four months, and sequential harvest at two and four months) on the growth of *Drosera adelae* (F. Muell.). In addition, the effect of N on the production of a medicinal active ingredient in *D. adelae* was examined. The second experiment investigated effects of the same nitrogen treatments in combination with three substrates (long fiber sphagnum, peat or rockwool) on growth. The growth rate of *D. adelae* was greatest at low levels of nitrogen fertilization, and slowed after two months of growth. Plants that were sequentially harvested had the lowest growth rate among all the harvest method treatments. Young plants and plants that received little or no N fertilizer had a greater water concentration than the other plants. Substrate and N level interacted, but generally peat and rockwool produced greater yields than did sphagnum. The medicinal compound plumbagin and a similar but unidentified compound were detected in the leaf extracts of *D. adelae*. However, whether N fertilization influenced the concentration of these compounds remains inconclusive.

Résumé

Plusieurs espèces de drosera (*Drosera* spp.), qui servent d'ingrédients pour des remèdes homéopathiques, sont en voie de disparition. Ce projet de recherche a compris deux études en production de drosera dans un système hydroponique. Une de ces études a exploré les effets de quatre taux d'azote (N) (0.0, 5.1, 25.5, 51.1 mg/L N) et trois méthodes de récolte (après deux mois, après quatre mois ou, récolte séquentielle à deux et à quatre mois) sur la croissance de *Drosera adelae* (F. Muell.). La deuxième étude a examiné les effets de ces mêmes taux de N en combinaison avec trois substrats (mousse de sphaigne, tourbe et laine à roche) sur la croissance de *D. adelae*. Le taux de croissance des plantes était plus élevé lorsque le niveau de la fertilisation azotée était bas, et diminuait après deux mois. Les plantes ayant subis la méthode de récolte séquentielle se sont caractérisées par un rendement faible. Les jeunes plantes, ainsi que celles non- ou peu fertilisées, contenaient plus d'eau que les autres plantes. Des interactions significatives entre les substrats et la fertilisation azotée ont affecté la croissance d'une manière variable, néanmoins la tourbe et la laine à roche ont produit en général des rendements plus favorables que la sphaigne. Le produit médicinal plumbagine, et un deuxième produit apparenté, ont été identifié chez les feuilles du *D. adelae* mais l'effet de la fertilisation azotée sur les concentrations de ces produits n'est pas encore certain.

Acknowledgements

Perhaps the most enjoyable part of writing a thesis is coming to the end and, with a feeling of accomplishment, reflecting on the long hours of work, and those who made it possible, endurable and at times even fun. I wish to begin by thanking Agriculture Canada for funding I received through the Horizons Programme, and Dr. Katrine Stewart who supported me with funding through much of my Masters degree, and who funded the agronomic portion of the research project. Dr. André Bélanger of Agriculture and Agri-Food Canada funded the extraction and mass spectrometry work for this project and I thank him, and his technician France Boudreau for the many hours they put into the analyses, answering my many questions, and showing me how it all worked. I wish to thank Dr. Katrine Stewart again for her guidance through the very early stages of my experiments. I give my thanks to Dr. Diane Mather, Dr. Timothy Paulitz and Dr. Timothy Johns who helped me to put the whole thing together. My thesis, with their help, has undergone a complete metamorphosis - from a disorganized, confusing blob, to a beast that ate up all my time, to the form it has today. I am grateful to Richard Smith for the wonders he worked in the greenhouse whenever it all went awry, and for his sense of humour that made the repetitive tasks bearable. Carolyn Bowes was especially helpful when paperwork threatened to take over my life and future. Huge thanks go to Mahtab Afaghi and Erica Fava who sweated it out with me in the greenhouse at 30°C, and for a hundred other things. My family was very supportive through the entire, lengthy Masters process, weathering the ups and downs with me, and sometimes I think, feeling them more. Without my father Richard's advice, and my mother Tanya's cups of tea, the whole process might have aborted. Finally, I wish to thank Dr. Ed Schneider. Ed was the one who showed me science could be enjoyable, rewarding and even exciting, instead of stodgy and boring. Without him, I would have given up on a career in science in 1993.

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1. Introduction

Sundews (*Drosera* spp.) are insectivorous plants that are adapted to bog habitats and other areas of low fertility. Three species of sundew are native to Québec, and over 90 species occur world-wide (Erickson, 1978). Sundews have a long history of use in traditional, homeopathic and allopathic medicine (Finnie and van Staden, 1993). With a recent return to the homeopathic style of medical treatment that was popular and effective at the end of the 19th century in Europe and North America, demand for sundew plants for cough and cold remedies has increased, and numerous sundew cold remedies are now widely available at pharmacies. Some *Drosera* species are listed as endangered in some countries, are protected in many countries, and have been targeted for conservation practices throughout Europe (World Wildlife Fund, 1998). This alarming decline has been largely due to habitat destruction and to wild-harvest for medicine. Habitat destruction and wild harvesting must be stopped if these plants are to survive. The popularity of sundew-based cough and cold remedies suggests that a halt to wild-harvest will occur only if an agricultural production method is found and used.

Caniato et al. (1989) noted that many of the European *Drosera* species are small and can be difficult to cultivate. In addition, they claimed that European species, such as *D. rotundifolia* L., contain low concentrations of medicinal compounds compared with many Australian species. They recommended that further study should focus on the medicinal compound levels and methods of cultivating the larger Australian species. In spite of this recommendation, almost a decade later the World Wildlife Fund (1998) reported that all material trade of sundew was still of plants collected from wild stocks.

The goal of this research was to investigate the effects of some agronomic factors on the growth in hydroponic culture of *Drosera adelae* (F. Muell.), one of the largest Australian species of sundew. The factors investigated were the type of substrate, method of harvest and nitrogen fertilizer level.

In addition, because the quality of plant material produced is clearly of interest to consumers, producers and processors of sundew, as well as to homeopathic practitioners, I hoped to ascertain whether *D. adelae* contains either or both of the compounds that modern science has deemed to be the source of sundew's therapeutic effects, namely plumbagin and ramentaceone. In the case that either was found, a final goal was to determine whether nitrogen fertilization affects the concentration of the product. This final goal was not meant to determine a specific mechanism or pathway between nitrogen fertilizer level and medicinal compound concentration. Rather, it was meant to provide preliminary guidance for continued research on production methods for this plant.

2. Literature Review

2.1. Description of Sundew Plants

Sundews are insectivorous plants of the genus *Drosera* in the botanical family Droseraceae. Over 90 species of sundew are distributed throughout the world, except in arctic regions (Erickson, 1978). Australia is home to 54 species of *Drosera* and is the region of the greatest concentration of species. In Québec, only three species are found; *D. anglica* Hudson, *D. intermedia* Hayne, and *D. rotundifolia* L. (Schnell, 1976).

Sundew plants vary in size from less than 1 cm in diameter, as in the case of the pygmy sundew *D. pygmaea* D.C., to the relatively giant proportions of *D. regia* Stephens. A native of South Africa, *D. regia* has leaves 35 cm long, and 2 cm wide (Lloyd, 1942).

Almost all of the sundew species exhibit one of two growth habits. The most common habit is a basal rosette, with flowering scapes arising from the rosette centre, as seen in *D. rotundifolia*. *D. capensis* L., the cape sundew, typifies the second growth form, which is an upright stem from which the leaves grow. Racemes are borne in leaf axils (Lloyd, 1942).

The leaves of sundew plants vary in shape from round to filiform, may be sessile or have petioles, and may or may not be ligulate (Lloyd, 1942). Cotyledons are simple and spatulate, with few glands. Leaves of *D. binata* Labill. and *D. dichotoma* Smith are once and twice forked, respectively. Some species have peltate leaves (as in *D. peltata* Thunb.), although this character manifests itself only in the adult plant. *D. auriculata* Planch. exhibits another possible variation; leaves with two basal lobes. One species of sundew, *D. gigantea* Lindl., has some modified leaves that help it climb. These leaves twist and form discs, which cement the leaves to plant or twig supports. The adaxial surface of the leaf is covered with glands borne on stalks. Each gland produces a drop of sticky mucilage from its tip. The colour of the mucilage varies with the species. *D. rotundifolia*, for example, produces red mucilage. Denoël (1949) determined that red pigments in sundew plants are attributable to an anthocyanin. The mucilage of *D.*

filiformis Raf. is often clear or whitish. It is the dew-like appearance of sun-bathed leaves that gave rise to the common name of these plants: sundew. A second set of glands found on the leaves is usually sessile and appears embedded in the epidermis at the centre of the adaxial leaf surfaces. When a small insect lands on the adaxial surface of the leaf, it becomes stuck to the leaf by the mucilage. Both sets of glands are implicated in the digestion of the insect, the nutritional components of which are then absorbed by the plant.

The young leaves of round-leafed sundew plants are folded so that the upper face is adjacent to the petiole (Lloyd, 1942). They are held this way by hyponasty of a narrow zone at the base of the petiole. In sundews with elongate leaves, true circination occurs, the leaves being tightly spiralled before opening. In both cases, the adaxial surface of the leaves is protected. As the leaves grow, they unfold.

The inflorescences of sundews are coiled racemes, although sometimes the flowers may be solitary (Heywood, 1993). Sundew flowers are regular, actinomorphic and perfect. They are characterised by 5 sepals, 5 petals, up to 20 stamens, and a superior ovary containing 3 or more ovules. The sepals are green and typically connate at the base. The petal colour varies with species, although this is not always a dependable taxonomic characteristic. Petals may be red, orange, pink, purple or white. The ovary comprises 2 to 5 fused carpels forming a single locule with basal placentation. Flowers open for a single day. If cross-pollination does not occur, the flowers will self-pollinate as they close in the evening. The resulting fruits are loculicidal capsules with 2 to 5 valves. The seeds are most often black, with basal embryos surrounded by endosperm.

The roots of sundew plants may take different forms. The germinating seed develops a fugacious taproot (Nitschke, 1860). This taproot ceases to elongate soon after germination, and then swells into a round structure covered with root hairs (Heinricher, 1902, as cited by Lloyd, 1942).

Numerous species develop tubers after the seedling stage (Lloyd, 1942). These tubers can germinate and send up shoots to form new rosettes or upright plants next to the parent plant. Conversely, other species grow axillary shoots that are positively geotropic, and grow into the ground, forming tubers at their ends. Species native to northern climates over-winter by forming a hibernaculum; a scaly bud from which new leaves grow in spring.

D. erythrorhiza Lindl. has rhizoids instead of roots (Lloyd, 1942). One to three root-like organs, complete with root hairs, grow from the base of each scale leaf. These organs differ from roots not only in their placement, but also in lacking a root cap. At the apex of each rhizoid is an apical meristem, which is covered by three layers of epidermal cells containing starch grains. These cells are never sloughed off as with a root cap. In the axil of each rhizoid-bearing scale leaf, very tiny tubers can grow, as another means of vegetative reproduction.

2.2. Description of Species Used

Drosera adelae F. Muell. has an upright stem, and leaves measuring up to 25 cm in length (Kondo et al., 1976). When grown in shady conditions, the leaves are dark green. High light intensity results in paler, reddish leaves, which are shorter in length. The plant reproduces sexually by forming a flowering scape with a raceme of fifteen to thirty orange or red flowers. The flowers are insect- or self-pollinated. Asexual reproduction occurs as new shoots form from adventitious buds on the roots, and on the mid-vein and margins of senescent leaves. No literature could be found stating whether *D. adelae* contains either plumbagin or ramentaceone, two compounds of medical interest, that have been reported in other *Drosera* species.

2.3. Habitat

Sundew plants grow on rotting logs, on acidic sandy soils, stream edges, by the sides of roads and on abandoned pastureland. Most frequently, however, they are associated with highly acidic peat bogs, which are characterized by sphagnum mosses

and other acidophilic plants, a large accumulation of organic matter, and large, open spaces un-shaded by trees or shrubs (Schnell, 1976). A few sundew species are found in highly alkaline marl bogs. However, even in these conditions, the sundew plants find an acidic niche, for they grow from acidic hummocks within the marl bogs. Sundew habitats are poor in available soil nutrients, and the plants are adapted to low nutrient availability (Schnell, 1976). In regions of high fertility, sundews fail to thrive.

2.4. Medicinal Importance

The historical basis for using sundew as medicine goes back several centuries, and perhaps earlier in undocumented cultures. In 1633, *Gerard's New Herbal* reported that sundew extract should be used against consumption of the lungs, although the same source also reported fatalities linked with the medicine (as cited by Slack, 1988). Sundew has been recommended for its aphrodisiac properties, based on results obtained when cattle and sheep were given small doses (Slack, 1988). Sundew was also used for removal of corns, warts, and freckles and was used to treat other dermatological conditions. Culpeper (1995) recorded sundew in his definitive herbal. Finnie and van Staden (1993) included tuberculosis, arteriosclerosis, inflammations, intestinal illness and syphilis among the list of illnesses sundew may be effective against. Lloyd and Middlebrook (1944) wrote of inhibitory effects of medicinal compounds found in *Drosera* spp. on the tubercle bacillus. Homeopathic and allopathic use of *Drosera* spp. in the treatment of tuberculosis was reported by Denoël (1949). Caniato et al. (1989) pointed out that a tincture made from the dried plants has been used in the treatment of asthma and pertussis in Europe since the 16th century, and that *Drosera* spp. are not only included in old herbals, but also in modern pharmacopoeias. According to Caniato et al. (1989), the herb is used widely in homeopathic medicine in tinctures made from both fresh and dried plants.

Paris and Quevauviller (1947) investigated the ability of sundew extract to prevent histaminic and acetylcholinic bronchospasms using a 1:1000 dilution of raw sundew extract. They discovered that the extract does not affect the onset of histaminic

bronchospasm, but does interfere with acetylcholinic bronchospasm, although it does not entirely prevent it. The therapeutic properties of sundew extract were attributed to the naphthoquinone compounds by Denoël (1949), who reported that *Bacillus subtilis* and *Staphylococcus aureus* were inhibited by *Drosera* spp. naphthoquinones, but *Escherichia coli* was not. The presence of the naphthoquinone compounds, plumbagin or ramentaceone, occurring singly or in combination in numerous species of *Drosera* was later confirmed (Zenk et al., 1969; Durand and Zenk, 1974b; Caniato et al., 1989).

Plumbagin has been studied in greater depth, due to its presence in the root of the Southern African medicinal plant, *Plumbago* sp.. Ray and Majumdar (1976) tested ethyl alcohol extracts from numerous plants, including *Plumbago zeylanica*, against a range of bacteria and fungi responsible for illnesses in humans. *P. zeylanica* root extracts had anti-microbial activity against *Staphylococcus aureus*, *Bacillis subtilis*, *Escherichia coli*, *Salmonella typhi*, *Vibrio cholera*, *Saccharomyces cerevisiae*, *Candida albicans*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Aspergillus niger*.

According to Watt and Breyer-Brandwijk (1962), plumbagin stimulates the central nervous system of animals including rabbit, mouse and frog, and large doses of plumbagin cause convulsions and paralysis. Studies conducted on rabbits also showed that plumbagin dilated peripheral blood vessels thus lowering blood pressure and depressing respiration.

In a study performed by Sharma et al. (1991), plumbagin reduced the serum cholesterol and LDL-cholesterol of rabbits by 53 to 86% and 61 to 91% respectively, and lowered the cholesterol/phospholipid ratio by 45.8%, while raising HDL-cholesterol levels in the blood. Plumbagin also prevented accumulation of cholesterol and triglycerides in the liver and aorta, and caused the regression of atheromatous plaques in the aorta.

Resistance to the antibiotic streptomycin developed in *Escherichia coli* and *Staphylococcus aureus* (Durga et al., 1990), resulting in colonies of cells unaffected by the antibiotic. In plumbagin-amended growth media, death of all cells occurred, perhaps due to the inhibition of the development of resistance. Fetterer and Fleming (1991) found that plumbagin was inhibitory to the development of the parasitic nematodes *Haemonchus contortus* at the first larval stage, and *Ascaris suum* at the fourth larval stage, although the development of the latter was enhanced at very low doses of plumbagin.

Melo et al. (1974) reported that plumbagin treatment caused a total cure of some cases of cellular carcinoma and Kaposi's carcinoma. Plumbagin enhanced phagocytosis of human granulocytes *in vitro*. This indicated immune system stimulation, and thus potential for the treatment of cancer (Kreher et al., 1990). Parimala and Sachdanandam (1993) reported regression of tumours of 3-methyl-4-dimethyl-aminoazobenzene-induced hepatoma in rats that were treated with plumbagin. In rats with the hepatoma, the glycolytic enzymes hexokinase, phosphoglucoisomerase and aldolase were elevated. Treatment with plumbagin decreased these levels to near normal. The gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-diphosphatase were low in rats with hepatoma, but plumbagin treatment increased these levels to normal.

Didry et al. (1994) recommended plumbagin for treatment of oral infectious disease, based on observations that its super-oxidative abilities give it a wide spectrum of activity. Plumbagin may be the active ingredient in folk remedies that use the plant *Pera benensis* to cure cutaneous leishmaniasis (Fournet et al., 1992). Plumbagin inhibited five strains of the protozoan *Leishmania* sp. in various stages of their life cycle.

Plumbagin can also have negative health effects. Bhuyan et al. (1991) found that plumbagin, paraquat and diquat, all free-radical enhancers, induced early cataracts when injected into the eyes of rabbits. An injection of 300 ηM plumbagin in 30 µl of isotonic saline induced cataract in 24-72 hrs.

Bhargava (1986) studied the effects of plumbagin on fertility and found that plumbagin decreased the total number of spermatids, decreased the numbers of resting and pachytene spermatocytes and reduced the diameter of Leydig cells and seminiferous tubules in immature rats fed 1 mg/100 g of body mass per day. It is not known whether these anti-fertility symptoms are a direct effect of plumbagin on the testes, or whether plumbagin acts on the pituitary gland. Watt and Breyer-Brandwijk (1962) examined the effects of plumbagin on isolated rabbit uterus tissue. Concentrations of 10×10^{-8} % stimulated the tissue, while 10×10^{-7} % plumbagin inhibited it. In rats, intraperitoneal injection of plumbagin caused secondary ovary dysfunction and abortion.

2.4. Medicinal Compounds in Sundew

2.4.1. Plumbagin and Ramentaceone in Sundew

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) and ramentaceone (5-hydroxy-7-methyl-1,4-naphthoquinone), or simply 7-methyljuglone, are the major quinones in the genus *Drosera*.

Durand and Zenk (1974b) investigated 17 species of sundew, and discovered that in 15 species, either plumbagin or ramentaceone, but not both, was present. The other two species, *D. capensis* and *D. cistiflora* L. contained both naphthoquinones. Zenk et al. (1969) reported that *D. longifolia* L. (now recognized by the name *D. anglica*) contained plumbagin, but not ramentaceone. In *D. capensis*, ramentaceone accounted for 0.7% of the plant dry weight.

Caniato et al. (1989) conducted a study on *D. binata*, *D. binata* var. *dichotoma*, *D. capensis* and *D. rotundifolia* and confirmed that both forms of *D. binata* contain only plumbagin, while *D. capensis* also contains ramentaceone. They reported that naphthoquinone concentrations vary seasonally with propagation method (division vs. root cuttings) and with organ sampled (leaves vs. roots). In the case of both forms of *D. binata*, plumbagin concentrations of plants propagated by division were approximately double those of plants propagated by root cutting, in every month of the year.

Concentrations of plumbagin in leaves of divided plants were about 300 mg/100 g fresh weight for *D. binata* var. *dichotoma* at the lowest level, after only one month of growth. This increased monthly, until concentration peaked at about 800 mg/100 g fresh weight six months later, and then declined. The root concentration of plumbagin was more variable. In the first months of growth, it was similar to leaf concentrations, but it declined as the concentrations of aerial plant parts peaked. Interestingly, the fresh weight of both roots and aerial parts of the plants continued to increase for about one month after the peak concentration in above ground parts was reached. Peak plumbagin concentration in roots was about 500 mg/100 g fresh weight. The plants propagated by root cutting had a peak plumbagin concentration of about 600 mg/100 g fresh weight, and were considerably smaller than those propagated by division. The same trends occurred for *D. binata*. However, the plumbagin concentration of aerial plant parts was slightly lower, peaking at 700 mg/100 g fresh weight. In the case of *D. capensis*, propagation method had no significant effect on plumbagin concentration, and in general, ramentaceone levels remained relatively constant. At its peak, ramentaceone concentration in aerial parts was 160-200 mg/100 g fresh weight, similar to the peak root concentration. Caniato et al. (1989) compared these results with data taken using *D. rotundifolia* grown under the same conditions. They found that naphthoquinone concentration in this species was about 130 mg/100 g fresh weight in both roots and shoots, much lower than the plumbagin concentrations of *D. binata*.

Plumbagin and ramentaceone are not the only phenolic compounds found in sundews. *D. intermedia* and *D. spathulata* Labill., grown *in vitro*, produced the following phenolic metabolites: the flavonoids myricetin 3-0-rhamnoside, quercetin 3-0-rhamnoside, quercetin 3-0-galactoside, quercetin 3-0-glucoside, as well as 5,6-dimethoxy-1, 2,4,7-tetrahydroxsynaphthelene 1-0-beta-glucopyranoside (Budzianowski et al., 1993).

2.4.2. Plumbagin and Ramentaceone Chemistry

Plumbagin is 5-hydroxy-2-methyl-1,4-naphthoquinone, which has the chemical

formula $C_{11}H_8O_3$. The substance exists in pure form as orange-yellow coloured, long, smooth, needle-like crystals. It has a melting point of 78°C, and is crimson or pink in solution (Watt and Breyer-Brandwijk, 1962). Ramentaceone, also called 7-methyljuglone is 5-hydroxy-7-methyl-1, 4-naphthoquinone, which has the chemical formula $C_{11}H_8O_4$ (Kreher et al., 1990). In its pure form it exists as scaly orange crystals, and has a melting point of 168°C.

Durand and Zenk (1974a, 1974b) fed tyrosine to *Drosophyllum lusitanicum* (a close relative of the sundews), and demonstrated that this amino acid is degraded to acetate, via the homogentisate pathway, which is then converted to plumbagin via the acetate-polymalonate pathway. Twenty percent of beta-^{14}C-labelled tyrosine was incorporated into the plumbagin (Durand and Zenk, 1974b). They noted that animals and microorganisms are able to metabolize acetogenic amino acids using the same homogentisate pathway. They suggested that carnivorous plants evolved the homogentisate pathway in order to use insects for nitrogen. Acetogenic amino acids, such as tyrosine, leucine, and isoleucine, are stripped of their nitrogen, and the hydrocarbon skeleton is then recycled for use in plumbagin, which may function in reducing competition by microorganisms for nitrogen. Plumbagin has bactericidal properties, and therefore may be able to prevent microorganisms from scavenging nitrogen from its prey. Durand and Zenk (1976) found that *Plumbago europaea*, a known source of plumbagin, was unable to degrade tyrosine, demonstrating that the sundew and *Plumbago* have different mechanisms for the biosynthesis of the naphthoquinone carbon skeleton. Ramentaceone (5-hydroxy-7-methyl-1, 4-naphthoquinone) is synthesized in sundew, using the same pathways that are involved in plumbagin biosynthesis. Identical pathways are involved in the formation of the naphthoquinone mollisin in fungi (Durand and Zenk, 1976).

2.4.3. Detection of Plumbagin and Ramentaceone

Denoël (1949) used a colorimetric approach to quantify naphthoquinone concentrations of several *Drosera* species. In this method, chloroform extraction and

alkaline fractionation led to a sample in which naphthoquinone concentration was proportional to the intensity of a yellow colour. However, this method does not accommodate the determination of the concentration of each naphthoquinone present.

Another approach, used by Zenk et al. (1969), was to separate the naphthoquinones, using thin layer chromatography (TLC), followed by spectrophotometric assay and identification using mass spectrometry. TLC was the method of choice for Heble et al. (1974), Caniato et al. (1989), and Budziankowski et al. (1993). Bonnet et al. (1984) used TLC in order to recover naphthoquinones for identification using mass spectrometry.

Bonnet et al. (1984) used high-performance gas liquid chromatography (GLC) to separate and quantify naphthoquinones in a single step. Mass spectrometry was used to identify the compounds. Crouch et al. (1990) used high-performance liquid chromatography (HPLC) to identify plumbagin in extracts from several *Drosera* species, and spectrophotometry to quantify the plumbagin in the extracts by comparison of values to a standard curve.

Electron spin resonance was used by Pedersen (1978) to identify and quantify quinones in crude plant extracts. The technique involves converting the quinone to its corresponding semi-quinone radical, and then observing the pattern of electron spin resonance. Semi-quinone radicals lacking ortho- or para-dihydroxy groups have lifetimes too brief to allow observation. Non-phenolic compounds are not observed because they do not form para-magnetic species. Only the semi-quinone nucleus is identified. In the case of methyl-juglones, this is sufficient to give absolute identification. In order to quantify the compound, a standard curve must be determined for the intensity of the spectrum, using electron spin resonance at various known concentrations. The intensity observed may then be found using the standard curve. Pedersen (1978) used this method successfully to determine the identity and concentrations of juglone, plumbagin, and ramentaceone.

2.5. Propagation and Culture

Caniato et al., (1989) investigated division and root cutting as ways of multiplying stocks of greenhouse-raised sundews. They claimed that leaf cuttings are a common way of propagating sundew. Leaves or partial leaves should be laid, adaxial surface upward, atop wetted sphagnum moss, and kept at high humidity and high temperature for several weeks. Schnell (1976) suggested a variation of the same technique, involving pinning the edges of the leaf to the sphagnum moss with toothpicks to ensure good contact of the leaf surface with the moist moss. When this method is successful, plantlets appear at the leaf margins, and on the cut edge. This method is very effective for *D. rotundifolia* and *D. capensis*.

Sundews are readily propagated by seed. In the case of species from northern climates, stratification of seeds in a refrigerator at 4 °C is required (Schnell, 1976). Germination of seeds from North American sundew species should take 2 to 4 weeks after stratification. Kukulczanka and Czastka (1988) reported that *D. rotundifolia* seeds germinated between 16 and 24 days after being placed *in vitro* conditions. Burger (1961) collected *D. intermedia* seeds from the wild in September in New York State, and then stored them at room conditions for 8 months. Out of 3 200 seeds subsequently plated on slant medium only 2 germinated over a period of 3 months. Burger (1961) then tested the effects of indole-3-acetic acid (IAA), gibberellic acid (GA), dilute sulfuric acid and high temperature on seed germination, and found no increased germination due to any of these treatments. Using alternate temperatures of 38°C and 15°C for 12 hours each for all photoperiod treatments, Burger (1961) obtained 44% germination of seeds under 24 hr daylight, 31% germination of seeds with 12 hr daylight, and only 2% germination of seeds kept in the dark.

Bonnet et al. (1984) micropropagated *D. intermedia* and *D. rotundifolia* on Murashige/Skoog (MS) (Murashige and Skoog, 1962) medium fortified with benzylaminopurine (BAP) and naphthaleneacetic acid (NAA), and compared naphthoquinone levels in these plants with those of plants collected from the wild. BAP

concentration in the medium was 10^{-7} M and NAA concentration was 10^{-5} M for *D. rotundifolia*. Concentrations were reversed for *D. intermedia*. Results indicated that the micropropagated plants had about 5 times the concentration of naphthoquinones of their wild counterparts. *D. rotundifolia* biosynthesized ramentaceone exclusively when cultivated *in vitro*, with a concentration of 6.01% of the dry weight. In contrast, wild plants produced only 1.72%, but also produced 0.06% plumbagin. *D. intermedia* produced 9.86% and 0.69% plumbagin and ramentaceone, respectively, when grown *in vitro*, and produced 1.99% and 0.13% plumbagin and ramentaceone, respectively, in the wild (Bonnet et al., 1984). Interestingly, Crouch et al. (1990) found that sundew plants grown *in vitro* contained plumbagin in the concentration 0.001% of fresh weight, while *in vivo* sundew plants contained a concentration of plumbagin of 0.0048% of fresh weight, although it is not clear how *in vivo* conditions differed from *in vitro* conditions. Crouch et al. (1990) concluded that in either case, concentrations of plumbagin were too low in sundew plants to enable any type of commercial production of the plant for the exploitation of this compound.

Bonnet et al. (1984) cultured *D. intermedia in vitro* on modified MS medium. Kukulczanka and Czastka (1988) also used MS medium, but reported greater success using Reinert/Mohr medium (Reinert and Mohr, 1967), when growing *D. rotundifolia*. Anthony (1992) tested various concentrations of MS medium. Sixty percent of *D. rotundifolia* and *D. binata* leaf explants produced plantlets on the 1/8 concentration of Murashige/Skoog medium, whereas only 38% produced plantlets on the full strength medium. All but one leaf explant grew plantlets on 1/4 and 1/2 concentrations of medium.

The effects of NAA and BA alone and in combination on the growth of micropropagated *D. rotundifolia* plants was summarized by Kukulczanka and Czastka (1988); plants given 5 x 10^{-7} M BA produced the most rosette formation (110-167 per explant), and the least root formation (0 per explant). Root production was maximal when

neither BA nor NAA were added. When 5.3 x 10^{-7} M NAA alone was added, all shoots and roots died.

Anthony (1992) also investigated the effects of growth hormones, in conjunction with MS medium on the growth of sundew plants. The hormones NAA and BA were tested in the concentrations 5.3 x 10^{-8} M and 10^{-7} M respectively. *D. rotundifolia* and *D. capensis* produced flowers without hormones on 1/2 strength medium. *D. binata* required NAA and BA with the 1/2 strength medium in order to flower.

Bobak et al. (1995) screened 49 MS medium variants on sundews and discovered that the best direct shoot organogenesis was obtained on liquid medium containing no BA and no NAA. No intermediate callus formation was found, and it was determined that the leaf epidural cells showed the highest potential for regeneration leading to bud formation. In the same study, it was noted that media supplemented with 10^{-6} to 2 x 10^{-5} M NAA and up to 2 x 10^{-5} M BA caused intensive red pigmentation in the cultured tissue.

Blehova et al. (1995) investigated the influence of several supplements to ½ MS medium on the concentration of ramenteceone in *D. spathulata*. The best growth of calli occurred on ½ MS supplemented with coconut milk (5% v/v) and 0.05 mg/L 2,4-D with 16 hours of daylight. Calli grown in the dark were friable, light green and developed red pigment spots. In the light, the calli that formed were dark green and very hard. Concentration of 7-methyljuglone was highest in complete plants compared to callus, higher in shoots compared to roots, and higher in red-pigmented calli than in calli without red pigmentation. Complete plants from seed had higher concentrations of 7-methyljuglone than complete plants from callus. After 23 passages over two years, plants showed a decrease in 7-methyljuglone.

A complete method for culturing *D. natalensis* Diels. was given by Crouch and van Staden (1988). When surface sterilized for seven minutes in 1.5% sodium hypochlorite, 35% of mature leaves survived uncontaminated, whereas 90% of flower

buds died when sterilized for only 3.25 minutes. Production of buds occurred after placing explant material on 1/5 MS supplemented with 1.3 x 10^{-7} M NAA and 5 x 10^{-7} M BA. Shoots formed on the same medium, but root induction required 5.3 x 10-7 M NAA and 6.3 x 10^{-8} M BA.

2.6. Insectivory

Sundews are insectivorous plants. They are able to derive nutrients from insects, which become entrapped on their leaves in sticky mucilage excreted from stalked glands. According to Givinish et al. (1984) a plant is considered insectivorous if it has two characteristics:

1. It must be able to absorb nutrients from dead animals juxtaposed to its surfaces, and thereby gain some increment of fitness in terms of increased growth, chance of survival, pollen production, or seed set; 2. The plant must have some unequivocal adaptation or resource allocation whose primary result is the active attraction, capture and (or) digestion of prey.

Stewart and Nilsen (1992) interpreted this definition to mean that a truly insectivorous plant must benefit from insectivory, and therefore must gain a significant nutritional contribution from insect prey.

Various nutritional experiments have been conducted, involving feeding insects to sundew plants. Under controlled conditions, Dixon et al. (1980) determined that the average nitrogen content of a *Drosophila melanogaster* fly was 26.8 μg. They radio-labelled this nitrogen and determined that 76.1% of the *Drosophila* nitrogen was absorbed by *D. erythrorhiza*, corresponding to 20.39 μg per fly. Stewart and Nilsen (1992) found that the nitrogen content of mature *D. rotundifolia* at highest biomass was 14.78 mg/plant. Using data from Dixon et al. (1980), and Nilsen (1992) calculated that for a *D. rotundifolia* to satisfy its entire nitrogen requirement through

insectivory, it would have to devour at least 200 fruit-fly sized insects per growing season.

Thum (1988) investigated the average arthropod catch by *D. rotundifolia* in a growing season in natural bog conditions, and found that it was 0.46 mg of arthropod/plant. In addition, Thum (1988) found the mean biomass of a *Drosophila melanogaster* to be 0.179 mg. This indicates that *D. rotundifolia* generally captures 2 to 3 fruit-fly-sized insects per growing season. Thum (1989) pointed out that the actual capture may be considerably greater than what is observed being digested by the leaves of sundews, for in one of his studies, ants were observed to walk on the leaves without becoming trapped in the mucilage, and to remove trapped insects for their own uses.

In spite of the low catch of insects by *D. rotundifolia* plants in the wild, several studies into the role of carnivory in sundew fitness involved feeding large numbers of flies to plants in the wild. Thum (1988) fed up to 104 fruit flies to each *D. rotundifolia* in one growing season. Krafft and Handel (1991) fed each plant up to 160 fruit flies, again in just one growing season.

Stewart and Nilsen (1992) conducted field studies of *D. rotundifolia*. Plants were fertilized with 113 g nitrogen (40-0-0) added to the substrate, while others were fertilized with 130 g phosphorus (0-46-0). A third group were fertilized with both additions, and a fourth were fed one *Drosophila* per month for 3 months. A final group received no feeding or fertilizers. Each of these treatments was carried out with and without the exclusion of naturally available insects. Stewart and Nilsen (1992) used Kjeldahl digestions to determine nitrogen from the leaves harvested at the end of the season, and determined phosphorus colorimetrically. Nitrogen levels available to plants increased when phosphorus was also added, probably because phosphorus was limiting to mineralization by microbes in the substrate. The addition of phosphorus alone decreased plant biomass, rosette diameter and flower scape density, whereas the addition of nitrogen alone decreased flowering. Plants with naturally available insect food had

significantly fewer leaves, smaller rosette diameter and lower biomass than plants in treatments that excluded naturally occurring insects. Flowering was apparently not influenced by the digestion of naturally occurring insects.

Stewart and Nilsen (1992) also investigated the nutrient use efficiency (NUE) of *D. rotundifolia*. NUE has been defined by Chapin and Van Cleve (1989) as the quantity of nutrient used per gain in carbon. Because sundews are adapted to soils of very low fertility, these plants may have a high NUE, indicating an ability to use nutrients very efficiently, and to retain them for future use. The NUE of *D. rotundifolia* for nitrogen and phosphorus were 0.35 and 1.42, respectively. Chapin and Van Cleve (1989) list the NUE of most crops and wild plants at 0.58 for nitrogen, and at 0.40 for phosphorus. Stewart and Nilsen (1992) thought that the NUE of phosphorus may be critical to survival of *D. rotundifolia*. They calculated the nutrient retention index (NRI) for phosphorus, and found that plants (regardless of whether naturally occurring insects were excluded) with no nutrient additions of phosphorus, nitrogen, or insects, had almost twice the NRI of plants with one or more of these additions. Phosphorus is very limiting to sundew growth, so sundew plants have adapted to become very efficient at absorbing and storing phosphorus for later use. However, even small fluxes of phosphorus (such as those incurred in the feeding of one insect per plant per month) may hamper phosphorus re-absorption. Stewart and Nilsen (1992) concluded that, in this way, insectivory could be deleterious to phosphorus nutrition. The NRI for nitrogen showed that plants (regardless of whether naturally occurring insects were excluded or not) fed nitrogen had about 50% lower NRI than all other treatments. The low levels of nitrogen re-absorption were effectively counteracted by phosphorus additions, since the NRI of the plants in the treatment with nitrogen and phosphorus additions were more than double the NRI of N alone.

Although Stewart and Nilsen (1992) did not find any evidence to support the hypothesis that *D. rotundifolia* gains any additional increment of fitness due to insectivory, Wilson (1985) demonstrated earlier an improved ability of *D. intermedia* to

compete interspecifically with the vigorous plant *Lysimachia terrestris*. Wilson and Keddy (1985) reported that *D. intermedia* was found growing along lake shores in Ontario, not only on sandy, acidic soil, but also on densely vegetated, organic sediment with high nutrient levels.

2.7. Nitrogen Fertilizers

Simola (1978) investigated the use of dipeptides as sole nitrogen sources for *D. rotundifolia* growing in aseptic culture. Seventeen dipeptides at 2 mM in the medium were compared with 2 mM ammonium nitrate (NH_4NO_3). In some treatments, no growth occurred, and plant leaves turned reddish in colour. Generally, the pH of the medium in treatments where growth did not occur was between 5.0 and 5.5. On the other hand, generally the treatments with pH between 2.5 and 3.6 gave the greatest yield. Exceptions to this trend were leucyl-tyrosine and arginyl-aspartic acid. In the former case, growth did not occur, although the pH was 2.6. The arginyl-aspartic-acid-treated plants grew, although the pH was greater than 6. The arginyl-aspartic acid treatment produced the greatest plant growth and was the only dipeptide treatment for which the biomass production exceeded that of the NH_4NO_3 treatment, giving an additional 31% dry matter. Arginine can also constitute the sole nitrogen source for *Sphagnum nemorum* and *Scapania nemorosa* (Basile, 1967; Simola, 1975).

Simola (1978) also found that glycine was better used by *D. rotundifolia* in the form of dipeptides, rather than in pure form. Glycyl-aspartic acid gave 70% of the growth recorded in the NH_4NO_3 treatment, and glycyl-glycine gave 77%. A synergism between glycine and alanine may occur since glycyl-alanine gave 31% more growth than the NH_4NO_3 treatment, much greater than either the glycyl-glycine or the alanyl-alanine treatments alone.

Simola (1978) reported that the dipeptides prolyl-methionine, prolyl-valine and methionyl-proline are toxic to *D. rotundifolia*. The same study found that *D. rotundifolia* would not use the constituent amino acids of these compounds when applied solely.

Leucyl-tyrosine was also toxic to *D. rotundifolia* (Simola, 1978). Analyses of the growing media after the experiment indicated that the amino acid components of the dipeptides that had been used as the nitrogen source, since they were absent or detected only in minute quantities. From this, Simola (1978) concluded that an active dipeptide transport system may be present in *D. rotundifolia*.

Small et al. (1977) investigated the effects of NH_4NO_3 (4 mM), NH_4Cl (8 mM), $NaNO_3$ (8 mM) and cheese as nitrogen sources on the growth of *D. aliciae* Hamet at different pHs. $NaNO_3$ as the N source resulted generally in the lowest biomass in the plants. It was, however the best-used N source at pH 4.5. Cheese, applied to the leaves, resulted in the next highest biomass, and the optimum pH was 5.5. *D. aliciae* varied in its efficiency at using NH_4Cl. At pH 4.0, plants had lower yield than plants using any other nitrogen source, but had higher yield than plants using any other nitrogen source at pH 5.0. The peak biomass yield occurred at pH 6.0. NH_4NO_3 was best used at pH 6.0 and the biomass of the plants at this pH exceeded all others. NH_4NO_3 was the best-used N source at low pH (4.0-4.5).

D. aliciae plants in all of the treatments of the experiments by Small et al. (1977) flowered and set seed, and the seeds from all treatments were viable. They were able to grow three generations of *D. aliciae* on aseptic agar medium using $NaNO_3$ as the sole nitrogen source. This showed that *D. aliciae* is able to complete its entire life cycle successfully, using only inorganic N. This is in contrast to studies carried out with another carnivorous plant *Utricularia exoleta*, which only flowered when fed beef extract (Pringsheim and Pringsheim, 1962).

Small et al. (1977) investigated, the enzymatic activities of *D. aliciae* plants fed ammonium, nitrate or organic nitrogen in the form of cheese. Nitrate reductase, nitrite reductase, glutamate dehydrogenase, glutamate synthase, glutamine synthetase and a peroxide able to reduce nitrate, were assayed in the cell-free extracts of the roots and leaves of *D. aliciae* grown on medium of pH 4.5, containing $NaNO_3$ (8 mM), NH_4Cl (8

mM) or cheese. Results showed that nitrate and nitrite reductases were absent from the extracts of ammonium- and cheese-fed plants. Nitrate-grown plants gave extracts with higher levels of glutamine synthetase and glutamate synthase, as well as a lower level of the peroxidase able to reduce nitrate, than plants in the cheese or ammonium treatments. Root extracts of plants that were grown on ammonium medium showed higher levels of glutamate dehydrogenase than root extracts of plants grown using other nitrogen sources. Because the plants were grown in aseptic conditions, these enzymes were not due to contamination with bacteria or other organisms. These results led the researchers to conclude that ammonia from nitrate assimilation is incorporated into the plant by the glutamine synthetase/glutamate synthase pathway. However, when ammonia levels are high, incorporation occurs using glutamate dehydrogenase. They concluded that the carnivorous abilities of *D. aliciae* have not modified the ability of the plant to use inorganic nitrogen in the same way that non-carnivorous plants do.

3. Objectives

Two experiments were designed with the following objectives:

1. Establish *D. adelae* in hydroponic culture.
2. Determine the effect of nitrogen rate (0 to 51.5 mg N/L) on the growth of *D. adelae* in order to establish an appropriate fertilisation regime for the species.
3. Evaluate two harvest dates and a sequential harvest method to establish the best method for *D. adelae* biomass production.
4. Evaluate the effects of three substrates (rockwool, peat and long fibre sphagnum) on the growth of *D. adelae* in order to learn which of the three represents the best choice for a sundew production medium.
5. Determine whether *D. adelae* contains plumbagin and ramentaceone.
6. Determine the effect of nitrogen fertilisation rate on the concentration of plumbagin in *D. adelae*.

4. Materials and Methods

4.1. Transplants

Transplants of D. adelae for the two experiments were handled in the same manner prior to transplantation. Plants were obtained from Agristarts II Inc. in Apopka, Florida. The plants had been produced in tissue culture from a single explant and had subsequently been transplanted into 72-cell packs (cells 32 mm wide by 32 mm long by 44 mm deep) filled with Canadian peat moss containing no additives. Each 32-mm-square plug contained from 2 to 20 plantlets. Plants were held in cell packs for about one week at a temperature of 35°C, and at a relative humidity of 60%.

The plants were placed in the greenhouse at 27 ± 1.6°C during the daytime, and 24± 1.6°C at night with a relative humidity of 95± 5%. The photoperiod was fixed at 12 hours by supplementing natural light with light from high-pressure sodium lamps when necessary. After one week, relative humidity was reduced to 80± 5%.

Each cell pack was placed in a plastic tray (53 by 27 cm) with no drainage holes. To water the plants, each plastic tray was filled with warm tap water. After two hours of soaking in the warm water, excess water was decanted from each plastic tray to prevent waterlogging of the roots. Plants were watered every second day.

4.2. Experiment 1

4.2.1. Experimental Design

The experiment was conducted as a randomized complete block design (RCBD) with two blocks arranged perpendicular to the light gradient in the greenhouse room. Each block contained 13 replications of 12 treatments arranged in a 4 x 3 factorial design. Each experimental unit consisted of one rockwool cube containing one *D. adelae* plant.

4.2.2. Treatments

The twelve treatments consisted of a complete factorial arrangement of two factors: fertilizer regime with four levels, and harvest method with three levels.

Three levels of nitrogen fertilizer were each applied in a solution of "complete-minus-nitrogen" fertilizer. These nitrogen concentrations were 51.1 mg/L (51.1 ppm) (N1), 25.5 mg/L (25.5 ppm) (N2) and 5.1 mg/L (5.1 ppm) (N3), in the form of ammonium nitrate. The composition of the stock solutions used to supply these solutions is presented in Table 1. The fourth level (N4) consisted of watering plants with tap water adjusted to pH 5.8 without any fertilizer additions. The composition of the tap water was determined by analysis at the Quebec Ministry of Agriculture and Fisheries Laboratory in Rock Forest, Quebec, and appears in Table 2.

Three harvest methods were used. The first method (H1) involved harvesting complete plants from designated sampling units after two months of growth. The second method (H2) involved sequential harvest of plants from designated sampling units. The shoot was harvested after two months of growth, and the remaining plant and any re-growth was harvested two months later, coinciding with the harvest of plants undergoing the third method. The third method (H3) involved the harvest of complete plants from designated sampling units after four months of growth.

Table 1: The concentration of components in fertigation stock solutions, the stock solution injection rates, and the final nitrogen (N) concentrations of the fertigation treatment solutions used in both Experiment 1 and 2.

Component	Chemical formula	Stock (mg/L)			
		X	Y	Z	W
Ammonium nitrate	NH_4NO_3	15	7.5	1.5	0
Potassium phosphate, dibasic	K_2HPO_4	0	0	0	6.53
Magnesium sulphate	$MgSO_4*7H_2O$	0	0	0	3.07
Calcium carbonate	$CaCO_3$	0	0	0	0.07
Chelated micronutrients	**	0	0	0	0.72
Seplex (ml/L)	***	0	0	0	8.34
Injection rate (ml/L)		10	10	10	20
Final N concentration (mg/L)		51.0	25.5	5.1	0.0

** Chelated Micronutrient Mix containing in mg/g: B, 13; Mo, 0.6; Fe, 70; Mn, 20; Cu, 1.0; Zn, 4.0 (Plant Products, Laval, Quebec)

*** An organic acid containing only C, N, O (Greencare Fertilizers, Chicago, IL)

Table 2: Analysis of greenhouse tap water, conducted prior to Experiments 1 and 2.

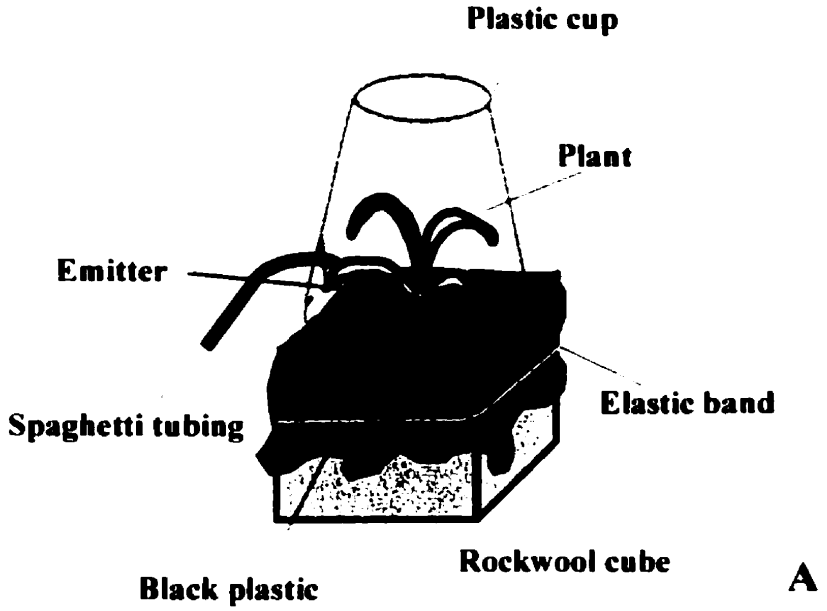
Quality analysed	Result
pH	7.6
Conductivity (mmhos)	0.14
NO_3 (mg/L)	0.2
P (mg/L)	0.2
K (mg/L)	1.4
Mg (mg/L)	2.3
Ca (mg/L)	9.6
Na (mg/L)	15.8
Mn (mg/L)	0.08
Cu (mg/L)	0.24
Fe (mg/L)	0.44
Zn (mg/L)	1.32
Sulfate (mg/L)	21.7
Cl (mg/L)	6.0
Mo (mg/L)	0.00

4.2.3. Construction of the Experimental Site

Cladmate polystyrene sheeting (Dow Chemical Canada Inc.) was cut and glued using foam bonder to form one sheet about 5 x 155 x 288 cm. All seams in each sheet were sealed with outdoor grade silicone sealer, and a continuous bead of silicone sealer was applied on the upper face, along the outer long edges of the sheets to prevent water from dripping off the sides of the sheets. One end of the sheet was raised to a height of about 4 cm with scrap pieces of wood to create a slope of 0.8°. This sloped sheet served to channel fertigation run-off toward the drain.

Grodan X-TRA individually wrapped rockwool seeding cubes (Canadian Hydrogardens, Niagara, ON), measuring 7.5 x 7.5 x 6 cm were placed in rows, supported along two opposite edges by pieces of spruce strapping, and finally formed five long, adjacent, continuous rows of cubes in each block. This system allowed clear space between the bottoms of the rockwool cubes and the Styrofoam sheet to allow proper drainage. The blocks were arranged parallel to the exterior-facing greenhouse windows and perpendicular to the light gradient. Three main irrigation lines encircled this arrangement, each designated to deliver only one fertilizer solution by way of spaghetti tubing and emitters attached to the main line. Each of these main lines fed 39 emitters for each block.

Each block contained 156 rockwool cubes as sampling units. Additional cubes were placed at the ends of the rows to make an even arrangement. While these were hand watered, they were not planted. The top of each cube was covered with a piece of black plastic mulch, which was held in place by an elastic band. A hole was cut in each cover, just large enough for transplantation of a sundew plant, and for the placement of an emitter. In each block, the 39 emitters attached by spaghetti tubing to each main line were placed in randomly selected rockwool cubes. The emitters were tucked underneath the black plastic covering and were held in place by the support sticks. The sampling unit used in Experiment 1 is illustrated in Figure 1.



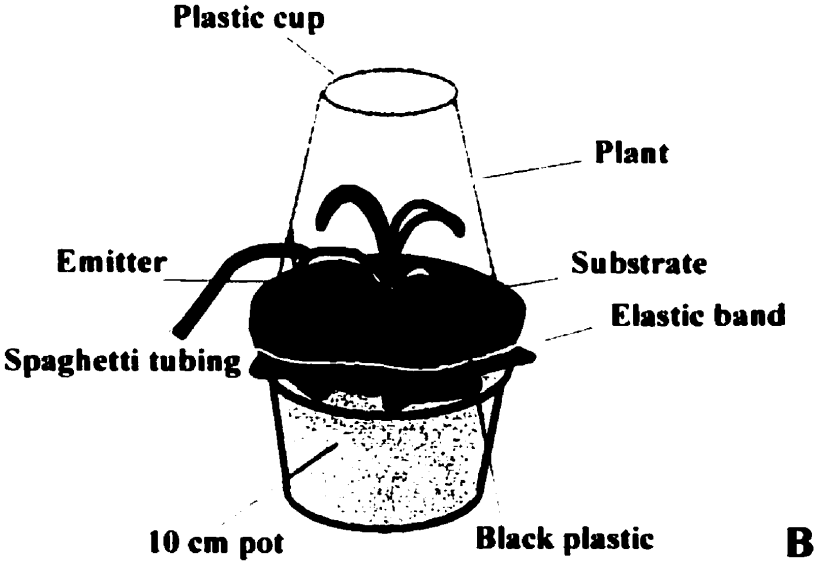


Figure 1: Sampling units used in Experiment 1 (A) and Experiment 2 (B).

A diagram of the fertilizer delivery system is shown in Figure 2. Water from a holding tank in the basement of the greenhouse flowed up through a pipe to a set of valves in the hallway outside the culture room. When the water was turned on, it flowed through hose H of inside diameter (i.d.) 3.8 cm. This hose connected with a pipe equipped with a Dosatron injector (M) (Dosatron International Inc., Clearwater, FL), which injected 20 mL of nutrient solution W per litre of irrigation water. This irrigation water continued through the pipe, and a second dosatron (N) injector injected fertilizer X, Y or Z at a rate of 10 mL/L of irrigation water, depending on the position of the fertilizer intake tube. The fertilizers X, Y and Z were each contained in separate tubs, into which the intake tube could be manually inserted. The irrigation water, with its full complement of fertilizer continued through the pipe, past a flush valve, and finally into a hose (I) of 3.5 cm i.d. Hose I connected with a valve which when opened allowed the fertigation water to pass through pipes in the floor, connecting with valves C, D and E inside the culture room. When open, these valves allowed the flow of fertigation water into 2.6 cm i.d. hoses (Kuritec Kuriyama, Canada), which in turn were connected to 3 main pvc irrigation lines of 1.0 cm i.d. and 1.3 cm outside diameter (o.d.). To each main irrigation line were attached 84 spaghetti tubes of 0.16 i.d. and 0.32 cm o.d., obtained from *Les Industries Harnois* (Saint Thomas-de-Joliette, Québec), and tipped by emitters with a drip rate of 2 L/hour. Support sticks 13 cm long were fused to the emitters. Fertilizer solutions containing X, Y or Z were delivered to the appropriate plants when the corresponding valve C, D or E was opened.

Prior to transplanting, all rockwool cubes were thoroughly soaked with the highest level of N fertilizer solution (51.1 mg/L N), and left overnight. Improperly wetted rockwool cubes were replaced and the emitters checked and replaced if necessary.

4.2.4. Transplanting

D. adelae plantlets were transplanted on 20 October, 1997. Plantlets were separated and the roots were gently washed free from peat under warm water. The roots were patted dry with a paper towel. Each plantlet was weighed, and its fresh weight

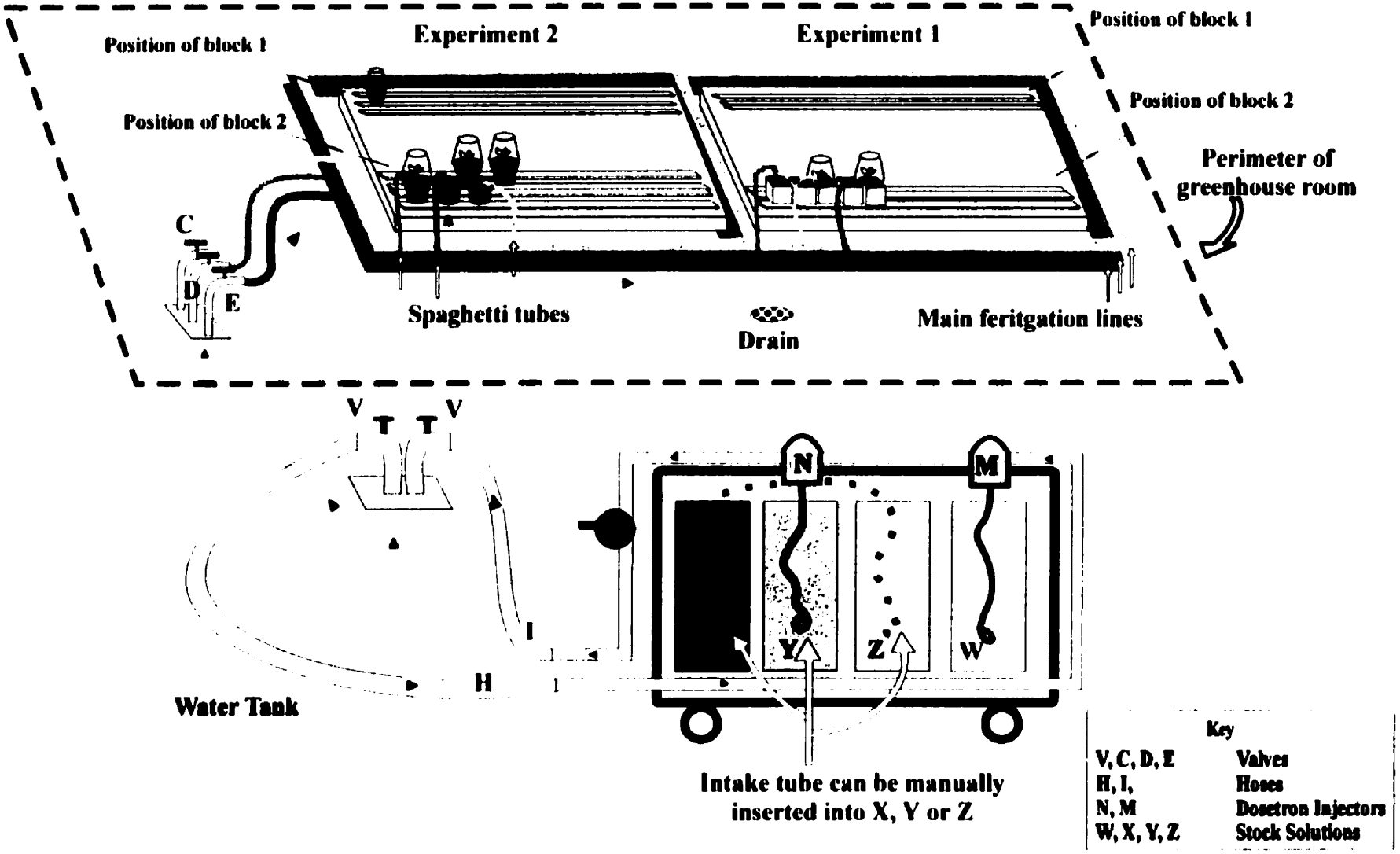


Figure 2: Fertilizer delivery system.

recorded. Plantlets between 0.2 and 1.8 g were used. The position of each plant was also recorded, so that later data from each plant could be linked with its transplant weight. Loose rockwool, wetted with the warm water, was immediately wrapped around the roots to prevent them from drying. The bundled roots were inserted into the pre-existing hole in the rockwool cube; one plant was transplanted to each cube. The emitter was arranged so that it dripped next to, but not on top of the plant. In order to maintain high relative humidity around the plants, each plant was covered by a clear plastic drinking cup (250 mL) which was affixed to the top of each cube or pot by sliding the spaghetti tubing near the emitter into a small cut made in the side of each cup. This cut also allowed for air exchange. The relative humidity of the greenhouse room was lowered to 65$\forall$5% after all plants had been transplanted and covered. This was necessary to prevent condensation,

and accumulated moisture from the mist system (used to maintain relative humidity) from dripping onto the experiments from overhead pipes.

4.2.5. Acclimation and Maintenance

For one month after transplanting, all the plants were all fertilized once daily for one hour with the highest N level fertigation solution. During this acclimation period, even the plants in treatments destined to have no fertilizer additions received this treatment by hand watering (800 mL, determined to be sufficient to fully exchange the fertigation solution in the sampling unit) with the highest N level fertigation solution. This served as an establishment period before beginning the treatments. During this time, transplants that suffered transplant shock and died were replaced.

After 30 days of acclimation, the experimental period began. Plants in the N-fertilized treatments were fertigated for one hour twice per week with their respective fertigation solutions. Plants undergoing the treatments with no N fertilizer added were each hand-watered with 800 mL of tap water adjusted to pH 5.8. Harvesting dates were calculated from the beginning of the experimental period commencing after 30 days of acclimatization. Changes were made to the original fertigation solution for the last

watering of the acclimatization period and the following experiments. Hydrochloric acid was replaced by Seplex (Greencare Fertilizers, Chicago, IL) as the acidifying agent, and potassium chloride was replaced by potassium phosphate (dibasic) to avoid chlorine toxicity and occupational hazards. The nutrient stock solution was made fresh each week to avoid bacterial contamination.

4.2.6. Harvest

In each block, 13 of the plants from each N level were randomly selected and were completely harvested after two months (roots and all aerial parts) (H1). Another 13 plants per N level in each block, selected in the same manner, were also harvested on that same date (H2). In this case only the main shoot of each of these plants was harvested while the roots and any side shoots were left intact. These roots and any re-growth that occurred were harvested after a further two months. Finally, the remaining 13 plants per N level in each block were harvested completely (roots and all aerial parts) after four months of growth. Due to irregularities in the labeling of sample envelopes, some numbers of plants to be harvested were changed. In treatment 0 mg/L N, H1, 14 plants were harvested in block 1, and therefore in block 1, only 12 plants undergoing treatment 0 mg/L N, H3 were harvested. Only 12 plants were harvested from the treatment 5.1 mg/L N, H2 in block 2, so 14 plants were harvested from treatment N 5.1 mg/L, H3 in block 2.

When harvested, fresh shoot and root weights of each plant were recorded separately and each portion was put into a separate paper envelope. Plants harvested completely at either two or four months were divided into two envelopes (roots and aerial parts) while plants harvested sequentially had three envelopes each (two for aerial parts, one for roots). Care was taken to identify all envelopes so that data from any single plant could be combined if required. Harvested plants were air-dried at 23°C for 48 hours. The dry weight was determined and all samples were subsequently kept frozen at –4°C.

4.2.7. Agronomic Data

In addition to fresh and dry weights, transplant weight and sampling period, the following characteristics of each plant were measured at final harvest: presence or absence of flowering scape, number of leaves on the main stem, number of new side shoots, and number of leaves on each side shoot. Shoot dry and fresh weights included leaves, stems, flowers and seed capsules, new shoots and the scape bearing the raceme. Total plant fresh and dry weights were values obtained from the addition of root and plant weights.

4.2.8. Statistical Analysis

From the data collected on individual plants, seven variables were tested for statistical differences. These were root dry weight, root fresh weight, shoot dry weight, shoot fresh weight, total plant dry weight, total plant fresh weight and growth rate (total plant fresh weight - transplant weight divided by the number of days of treatment).

The experimental design (randomized complete block design with up to 13 replications per treatment per block) permitted powerful tests of the significance of the block*treatment interactions. These were conducted using PROC GLM of SAS System 6.12 software. No significant block*treatment interactions were detected ($P>0.05$). Therefore, to provide more powerful tests of treatment effects, further analysis was conducted considering the experiment as a completely randomized design (i.e., ignoring the blocking). Each of the variables was analyzed using PROC GLM, with the transplant weight included as a covariate, except in the case of growth rate. No significant interactions occurred between the factors nitrogen and harvesting method, so the main effects of each factor were considered separately. Since the data set contained some missing values due to unbalanced design, least square means were computed for each level of each factor. Differences between the levels of each factor were tested using a t-test, interpreting only the linearly independent comparisons between levels of each factor.

4.3. Experiment 2

4.3.1. Experimental Design

The experiment was conducted as a randomised complete block design (RCBD) with two blocks arranged perpendicular to the light gradient in the greenhouse room. Each block contained 12 replications of 12 treatments arranged in a 4 x 3 factorial design. Each experimental unit consisted of one 4-inch (10.2 cm) pot containing one *D. adelae* plant (Figure 1).

4.3.2. Treatments

Twelve treatments were used in a complete factorial design by combining all levels of factor one with all levels of factor two. Factor one was the same four fertilizer regimes described in Experiment 1. Factor two was three types of substrate rockwool (grower grade Grodan, Canadian Hydrogardens), long fibre sphagnum moss (ornamental grade) or peat moss (greenhouse grower grade, un-amended Canadian peat moss).

4.3.3. Construction of the Experimental Site

The experimental site was constructed in much the same manner as the site for Experiment 1. Some differences were made. Each block in Experiment 2 contained 120 4-inch (10.2 cm) pots, each containing one of the types of substrate. The first substrate type was rockwool. Each of 80 pots (40 for each block) was filled with 450 g of thoroughly wetted loose Grodan rockwool. The second substrate type was peat, which was soaked over night to achieve a uniform moisture level. Each of 80 pots (40 for each block) was filled with 450 g of the wet peat. Similarly, 450 g of thoroughly water soaked sphagnum was put into 80 pots (40 for each block). The top of each pot was covered with a piece of black-plastic mulch as in Experiment 1. In each block, 30 emitters attached by spaghetti tubing to each main line were placed so that 10 pots containing each substrate were fertilized by any given fertigation solution. The remaining 10 pots containing each substrate constituted the sampling units receiving the treatments with no fertilizer added.

4.3.4. Transplanting

Few differences from the methods described in Experiment 1 occurred in the transplanting of *D. adelae* plantlets in Experiment 2. Each plantlet was weighed, but only transplants between 0.25 and 0.50 g were used in the experiment. These transplant weights were not recorded. Loose rockwool, long fibre sphagnum or peat moss, corresponding to the destined substrate of the individual plant, wetted with warm water, was immediately wrapped or squeezed around the roots to prevent them from drying. The bundled roots of a given plant were inserted into a hole made in the substrate of the pot for which it was destined.

4.3.5. Acclimation and Maintenance

The acclimatization and maintenance of Experiment 2 was identical to that described in Experiment 1.

4.3.6. Harvest

All plants were harvested in their entirety after two months of growth, beginning from the start of the experimental period. Fresh shoot and root weights of each plant were recorded separately and each portion was put into a separate paper envelope. All other harvest methods used are detailed in Experiment 1.

4.3.7. Agronomic Data

The same agronomic data was collected in Experiment 2 as in Experiment 1.

4.3.8. Statistical Analysis

The statistical analysis conducted in Experiment 2 was similar to that in Experiment 1. Block*treatment interactions were not significant at the 0.05 level, so the blocks were not considered and the experiment was analysed as a factorial CRD. No covariates were included in the analysis. Inspection of the analysis of variance tables from the factorial CRD showed that most variables exhibited significant ($P \leq 0.05$) nitrogen*substrate interactions. Those variables for which the interaction was not

significant were interpreted as in Experiment 1. Those for which the interaction was significant were analysed for simple effects using SAS PROC GLM, using a modified F-statistic of the simple effects mean square divided by the overall within-groups mean square. When this F statistic was significant ($P \leq 0.05$), least square means (to accommodate missing data of unbalanced design) were computed. Linearly independent comparisons between substrates of each level of nitrogen fertilization were conducted using a t-test.

4.4. Analysis of Medicinal Compounds

4.4.1. Selection of Samples for Extraction

Due to limited resources, only a sub-sample of the harvested *D. adelae* material was extracted for the analysis of medicinal compounds. The aerial portion of five plants from each of the four nitrogen treatments, all undergoing harvest treatment H3 were used from each block in Experiment 1.

Samples were sent to Dr. André Bélanger of Agriculture and Agri-Food Canada at the Horticulture Research and Development Centre in Saint Jean-sur-Richelieu, Québec for quantification of medicinal constituents. The following methods were used.

4.4.2. Extraction from Aerial Plant Parts

Each plant sample was weighed, transferred in its entirety to a 50-mL test tube and subsequently homogenized with three successive 5-mL portions of acetone using a Brinkman generator and grinding attachment. Each 5-mL portion was filtered (Buchner vacuum) using three layers of filtration material. The top layer consisted of Whatman No. 1 filter paper, the second of Whatman GF/C 1.2 µm filter and the third of Whatman GF/C 0.045 µm filter. The three portions were combined, evaporated and reconstituted with 1 mL of acetone (GPC grade). Plumbagin and a second related compound were detected and measured directly in the non-purified extracts by Gas Phase Chromatography (GPC).

A Varian gas chromatograph, model No. 3800, was used with a Varian injector (model No. 1079) in septum programmable injector mode, a Supelco SPB1 column (Supelco, Fisher Scientific) of 30 metres with an inside diameter of 0.25 mm, and a flame ionization detector. An automatic injector (Varian, model No. 8200) was used on some samples.

The injector was initially adjusted to 40°C and increased to a final temperature of 230°C at a rate of 180°C per minute. This final temperature was maintained for 36 minutes. The oven starting temperature of 60°C was increased at a rate of 5°C per minute to a final temperature of 230°C and maintained for two minutes. The detector temperature

was fixed at 250°C. The carrier gas was helium, with a flow rate of 1.6 mL/min and a pressure of 20 p.s.i (133.3 kPa).

A volume of 1 µl of extract diluted in acetone was injected. The retention time of plumbagin was 21.58 minutes and that of the second compound was 21.85 minutes. Plumbagin was quantified using a calibration curve based on an external standard of plumbagin (99.99% pure, Fisher Scientific). The concentration of the second compound was determined by comparison with the same plumbagin external standard. The linearity of the plumbagin curve ranged from 2 to 100 ηg/µl.

Because of contaminants in the extracts, the injector insert was frequently changed and the end of the column was clipped on a daily basis, which resulted in a long-term impact of shortened retention times. In addition, the needle of the syringe often became blocked, requiring cleaning and frequent replacement in order to ensure exact volume of injected samples. Certain non-purified samples, generally those from larger plant samples, could not be quantified without purification because they blocked the column and the syringe.

4.4.3. Purification

No reports of purification methods for sundew extracts could be found in the literature. Therefore, the following procedures were developed on a trial and error basis for purification of samples that could not be analyzed directly.

4.4.3.1. Final Procedure for Purification of Extracts

The acetone-diluted extract was evaporated and reconstituted with 5 mL H_20 (distilled and filtered with a Barnstead Nanopure II PCS system) at 50°C, ultra-sonicated for 3 minutes and applied by pipette to a 60-mL Chem-Elute™ cartridge. The tube that had contained the extract was rinsed with 5 mL of 50°C H_20 (distilled and filtered), which was also applied to the Chem-Elute™ cartridge. The solution was left in the cartridge for five minutes before eluting the plumbagin with a volume of 70 mL of a 10:90 acetone:chloroform solution. The eluant was evaporated under vacuum,

reconstituted with acetone, decanted into a test-tube, and subsequently evaporated under nitrogen. The purified extract was reconstituted to a desired volume (dependent on whether dilution was required to bring the plumbagin peak within the range of linearity of the standard) using acetone and was injected into the gas phase chromatograph for quantification. It should be noted that much of the second compound was lost during this purification procedure.

This purification procedure gave comparable results with respect to plumbagin recovery to the direct method using non-purified extracts. Table 3 illustrates the percentage recovery of plumbagin and the second compound from two portions of the same sample extract: non-purified (raw) and purified before injection. The eluant from the Chem-Elute™ cartridge was collected in different fractions in order to determine the amount of acetone:chloroform solution to apply to fully elute all of the plumbagin. Since all of the quantifiable plumbagin was recovered in the first 60 mL, 70 mL of solution were used for the purification procedure. Once the comparability of methods was established, the problematic samples were purified according to this procedure with one exception. Because samples were all initially injected without purification, only those that blocked the injector were subsequently purified. In these cases only a small quantity of sample remained. Therefore, instead of 1 mL of extract, 200 µl of extract was applied to the Chem-Elute™ cartridge.

Figures 3 and 4 show the gas phase chromatograms of the non-purified and purified extracts, respectively. Figure 4 demonstrates that the purification process removed many of the components of the non-purified extract, and notably diminished the peak corresponding to the second compound.

4.4.3.2. Recovery Rate of Plumbagin at Low Concentration

The recovery rate of plumbagin using the Chem-Elute™ purification was tested at both at a high concentration (compared with an evaporated and reconstituted standard of 50 ηg/µl plumbagin) and at a low concentration (compared with an evaporated and

Table 3: Comparison of recovery rates of plumbagin and the second compound from purified extract versus non-purified extract of *D. adelae*.

	Purified by Chem-Elute™ method		**Non-purified**	
Volume of eluant	**Plumbagin recovered (µg)**	**Second compound recovered (µg)**	**Plumbagin recovered (µg)**	**Second compound recovered (µg)**
0-50 ml	26.46	3.09	28.98	54.51
10 ml	3.18	-		
10 ml	*	-		
10 ml	-	-		
10 ml	-	-		
10 ml	-	-		
Total	29.64	3.09	28.98	54.51

* too low to be quantified
- none recovered

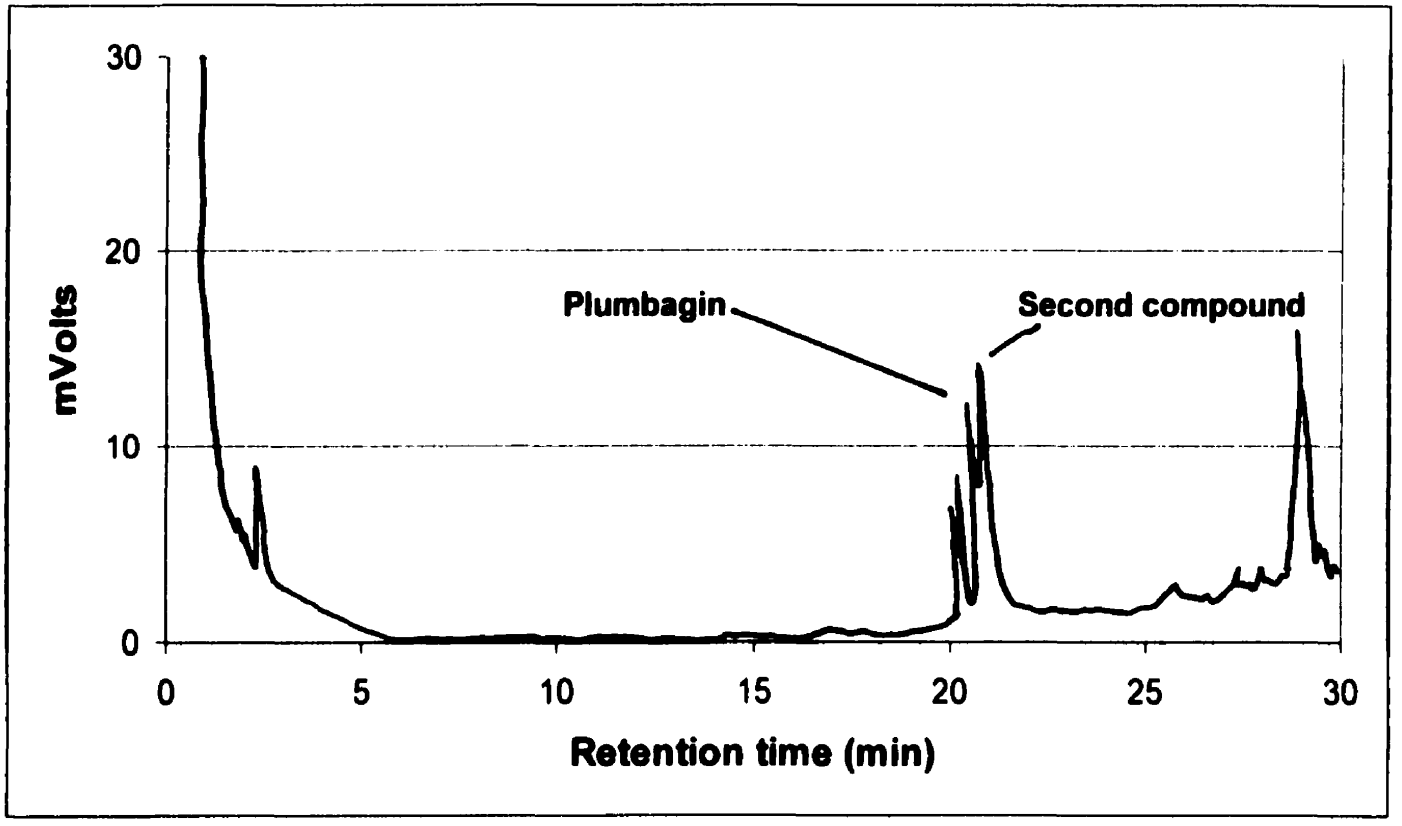


Figure 3: Gas phase chromatogram of non-purified *D. adelae* extract.

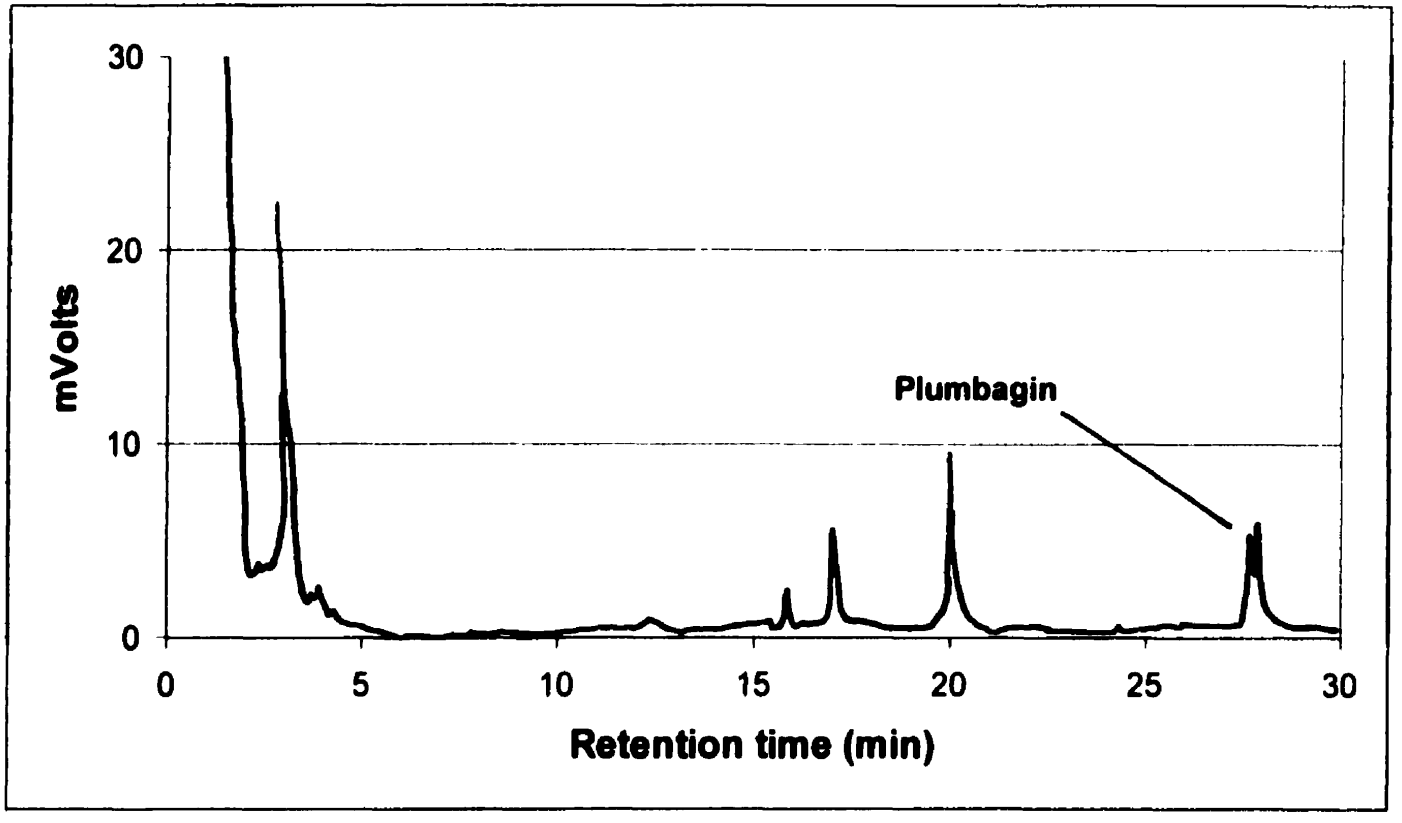


Figure 4: Gas phase chromatogram of *D. adelae* extract purified using the Chem-Elute™ method.

reconstituted standard of 10 ηg/μl plumbagin) to ensure that the method would allow quantification of plumbagin at the low concentrations found in sundew.

4.4.3.3. Interference of the Solvents with Quantification of Plumbagin

The Chem-Elute™ procedure was carried out substituting H_2O (distilled and filtered with a Barnstead Nanopure II PCS system) in place of extract in order to see if any of the solvents that make up the cartridge caused interference with the plumbagin peak. GC analysis of the water purified by Chem-Elute™ cartridge revealed interference from one or more solvents causing the plumbagin peak to be split and wide, a characteristic that makes quantification less accurate. For these reasons, a second GPC column of intermediate polarity (DB1701, J. & W. Scientific) was tested under the under the same temperature conditions. The retention time of plumbagin in the DB1701 column was 24.49 minutes and the linearity ranged from 0 to 25 ηg/μl. The interference was less using the DB1701 column than when using the SPB1 GPC column (used for the non-purified extracts). In the case of the SPB1 column, the interference caused by Chem-Elute™ solvents coinciding with the plumbagin peak was greater than when using the DB1701 column. For this reason, purified samples were quantified using the same GPC settings as the non-purified samples, but the DB1701 column was used instead of the SPB1 column. The interference of the Chem-Elute™ cartridge was measured as 0.22 μg (peak equivalent to 0.22 μg of plumbagin at the retention time of plumbagin even when water was used instead of extract) on the DB1701 column, but since the samples were compared only to others analyzed using the same column, this interference was disregarded.

4.4.4. Statistical Analysis

Gas chromatography data for purified samples were analyzed separately from those that had not been purified. Within each of these categories, the number of samples varied among blocks, and among treatments within blocks, so SAS PROC GLM was used. In an initial analysis, block*treatment interactions were tested. These were not significant ($P>0.05$), so blocks were ignored. The analyses were conducted considering each set of data (purified and non-purified) as a completely randomized design using

PROC GLM, including the number of new side-shoots as a covariate to compensate for any effect of young tissue. Since the data set contained some missing values due to unbalanced design, least square means were computed for each treatment. Differences between the treatments were tested using a t-test at the 0.05 level of significance, interpreting only the linearly independent comparisons between levels of each factor.

4.5. Identification of Medicinal Compounds by Mass Spectrometry

A non-purified extract (method of obtaining this extract described in section 3.4.3.1) of *D. adelae*, and a plumbagin standard of 10 ηg/μl were each subjected to mass spectrometry. This analysis was conducted to determine whether *D. adelae* contains naphthoquinones reported in other *Drosera* spp., by comparison with the spectra described by Bonnet et al. (1984) and the spectrum of the plumbagin standard. A Varian gas chromatograph, model No. 3400, fitted with a septum programmable injector adjusted to 40°C was used. The injector temperature was increased at a rate of 180°C per minute until it reached a final temperature of 230°C. The column was a DB1701, of intermediate polarity, 30 m in length, with an internal diameter of 0.25 mm and a stationary phase thickness of 0.25 μm. The oven had an initial temperature of 55°C, which was increased by 1°C per minute until it reached 240°C. The final temperature was maintained for 5 minutes. The carrier gas was helium at a pressure of 15 p.s.i. (103.45 kPa). The line of transfer was fixed at 270°C. The ion trap mass spectrum detector (Varian model Saturn II) was adjusted to 220°C and the masses acquired were of 40 to 450 m/z (mass to charge ratio) in electron ionization mode. The chromatogram of *D. adelae* extract is illustrated in Figure 5. Scans of the spectra of peak 4825 (suspected to be plumbagin) and of peak 5074 (the second compound) from this chromatogram were compared with the National Institute of Technology's Spectra Library 92 (NIST 92). This software tool contains an extensive database of spectra. Using the software, a search component calls up the chemical information from the database corresponding to the compounds with spectra that most closely match the sample.

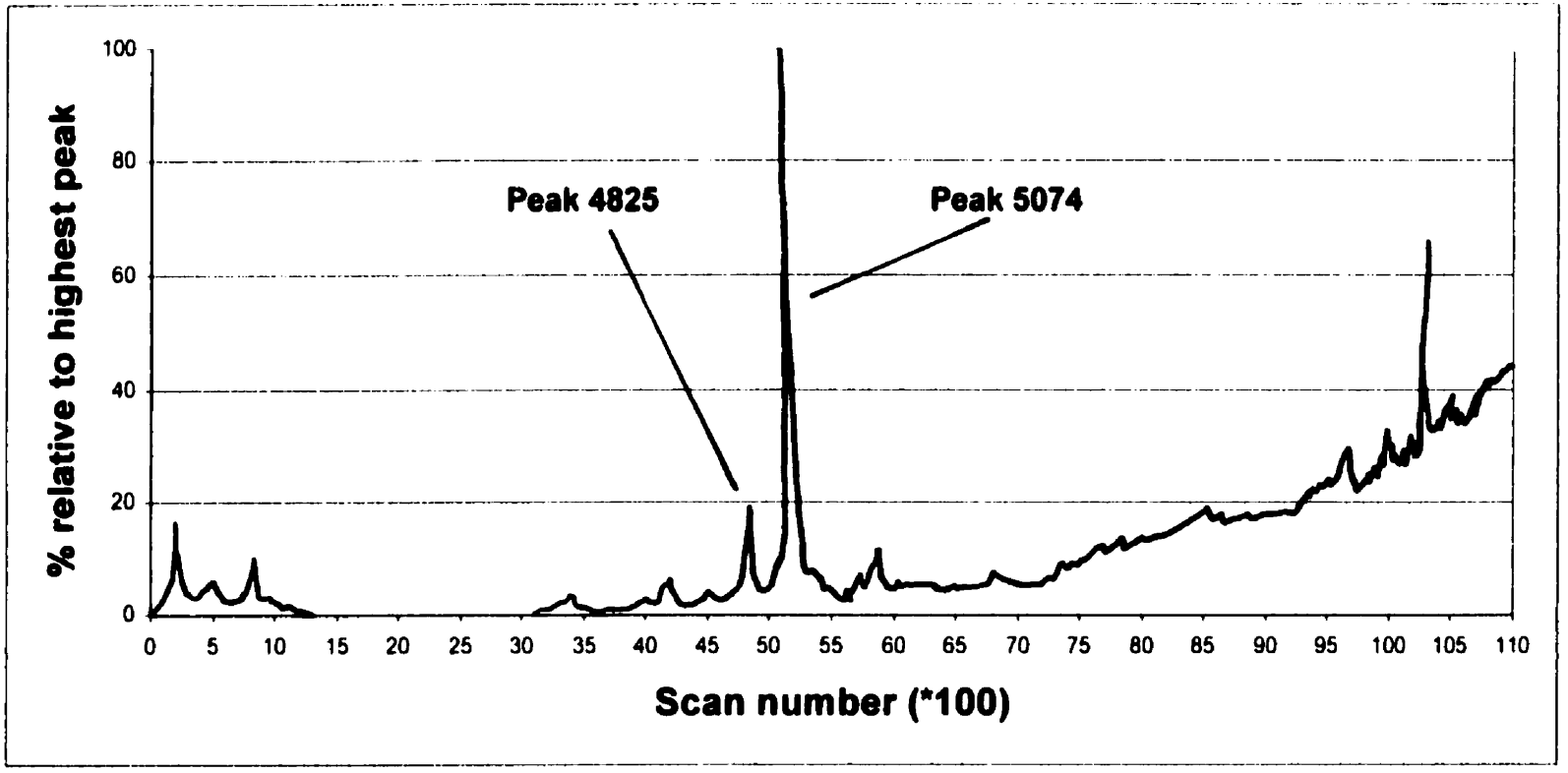


Figure 5: Complete chromatogram of non-purified *D. adelae* extract obtained using gas phase chromatography with ion trap mass spectrometry.

5. Results

5.1. Experiment 1

5.1.1. Effects of Nitrogen

Fresh weights of shoots and plants of *D. adelae* were higher for the treatment with no added nitrogen than for the treatments with 5.1, 25.5 or 51.0 mg/L added nitrogen (Table 4). For root fresh weight, root dry weight and shoot dry weight there was no significant differences among the nitrogen levels. Nevertheless, the total plant dry weight was slightly higher for the treatment without added nitrogen than for the treatments with 5.1 or 51.0 mg/L nitrogen levels. Growth rate of plants grown with no added nitrogen was greater than plants grown with added nitrogen (Figure 6).

5.1.2. Effects of Harvesting Method

All plant variables for *D.adelae* demonstrated the lowest weights in the sequential harvest treatment, although shoot dry weight and total plant dry weights were also similarly low in the treatment harvested after two months of growth (Table 5). Root, shoot and total plant fresh weight were the greatest in the two-month harvest treatment, as was growth rate (Figure 7). Growth rate was lowest in the sequentially harvested treatments. Harvest method did not affect root dry weight.

5.1.3. Flowering

Four *D. adelae* plants flowered in Experiment 1. Two of these were grown using no added nitrogen, one using 5.1 mg/L added nitrogen, and one using 25.5 mg/L added nitrogen. All flowering occurred before the first harvesting date.

5.2. Experiment 2

5.2.1. Effects of Nitrogen and Substrate

In Experiment 2, main effects of nitrogen and substrate were significant for root fresh weight (Tables 6 and 7). Root fresh weight was lower when plants unfertilized or were grown using the highest level of nitrogen fertilizer (51.1 mg/L) than when grown using 5.1 or 25.5 mg/L nitrogen (Table 6). Plants grown in rockwool had higher root

Table 4: Least square means of root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, total plant fresh weight and total plant dry weight for *D. adelae* plants grown at four levels of added nitrogen (0.0, 5.1, 25.5, 51.1 mg/L) in Experiment 1. Within columns, values with the same letter are not significantly different from each other at P≤0.05.

Added nitrogen (mg/L)	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)	Total plant fresh weight (g)	Total plant dry weight (g)
0.0	0.68a	0.10a	1.13a	0.16a	1.82a	0.26a
5.1	0.64a	0.15a	0.88b	0.13a	1.53b	0.22b
25.5	0.63a	0.17a	0.97b	0.14a	1.61b	0.23ab
51.0	0.61a	0.14a	0.88b	0.12a	1.52b	0.21b

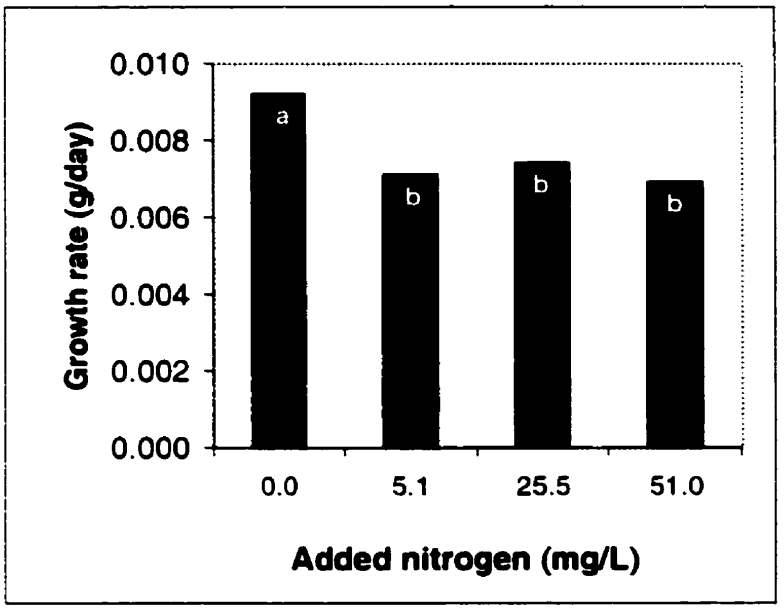


Figure 6: Least square means of growth rate of *D. adelae* plants grown at four levels of added nitrogen (0.0, 5.1, 25.5 or 51.1 mg/L) in Experiment 1. Means with the same letter are not significantly different from each other (P>0.05).

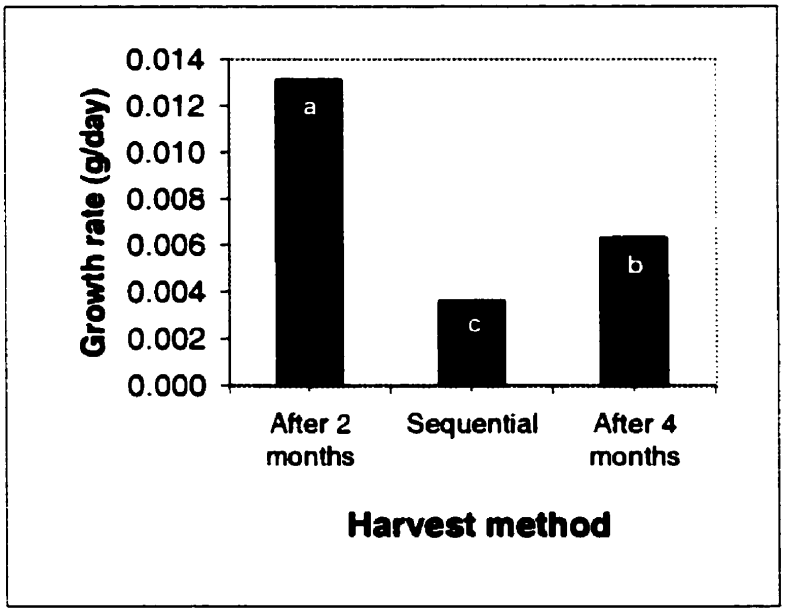


Figure 7: Least square means of growth rate of *D. adelae* plants harvested using three methods (after two months, after four months or sequential harvest at two, and four months) in Experiment 1. Means with the same letter are not significantly different from each other (P>0.05).

Table 5: Least square means of root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, total plant fresh weight and total plant dry weight for *D. adelae* plants harvested using three methods (after two months, after four months or sequential harvest at two and four months) in Experiment 1. Within columns, values with the same letter are not significantly different from each other (P>0.05).

Harvest method	**Root fresh weight (g)**	**Root dry weight (g)**	**Shoot fresh weight (g)**	**Shoot dry weight (g)**	**Total plant fresh weight (g)**	**Total plant dry weight (g)**
after 2 months	0.81a	0.12a	1.08a	0.13b	1.90a	0.21b
sequentially, after 2 and 4 months	0.51c	0.11a	0.75b	0.12b	1.27c	0.20b
after 4 months	0.60b	0.19a	1.08a	0.17a	1.70b	0.29a

Table 6: Least square means of root fresh weight for *D. adelae* plants grown at four levels of added nitrogen (0.0, 5.1, 25.5 or 51.1 mg/L) in Experiment 2. Means with the same letter are not significantly different from each other (P>0.05).

Added nitrogen (mg/L)	Root fresh weight (g)
0.0	0.22ab
5.1	0.30a
25.5	0.28a
51.0	0.20b

Table 7: Least square means of root fresh weight for *D. adelae* plants grown in three different substrates (long fibre sphagnum, peat moss or rockwool) in Experiment 2. Means with the same letter are not significantly different from each other (P>0.05).

Substrate	Root fresh weight (g)
Long fibre sphagnum	0.21b
Peat moss	0.26ab
Rockwool	0.29a

fresh weight than those grown in long fibre sphagnum (Table 7). All of the other variables exhibited significant interactions between the two factors nitrogen level and substrate. When analysed for simple effects, differences in root dry weight were not significant. Rockwool, although yielding similar shoot fresh weight to that of peat when no nitrogen was added, resulted in fresh shoot weights comparable to those of long fibre sphagnum when both received higher levels of nitrogen fertilization (Figure 8). However, dry shoot weight was unaffected by substrate at all levels of added nitrogen, only demonstrating that rockwool gave the highest dry weight and long fibre sphagnum the lowest when no nitrogen was added. Total plant fresh weight showed differences at two levels of nitrogen fertilization. At 5.1 mg/L nitrogen, long fibre sphagnum produced higher total plant fresh weights than rockwool did, whereas at 51.0 mg/L, peat gave higher yields than either long fibre sphagnum or rockwool. Total plant dry weight was lowest when no nitrogen was added to plants grown in long fibre sphagnum. When 25.5 mg/L nitrogen was added, peat gave higher yields than rockwool. A general trend pointed to fresh weight decreasing with increasing additions of nitrogen, while dry weights did not exhibit any trend.

5.3. Gas Phase Chromatography

5.3.1. Purified Samples

Plumbagin concentrations in the purified samples from *D. adelae* ranged from 46 to 833 mg/kg dry matter. The covariate (number of new side shoots) had no significant effect. A second compound, closely related to plumbagin was detected but could not be quantified in the purified samples because this compound was largely lost during the purification process. No treatment differences in the concentration of plumbagin were detected ($P>0.05$) (Table 8).

5.3.2. Non-Purified Samples

Plumbagin concentrations were higher in samples from *D.adelae* from the treatment without added nitrogen and with the lowest level of added nitrogen (5.1 mg/L) than in samples from the treatments with higher levels of added nitrogen (Table 8).

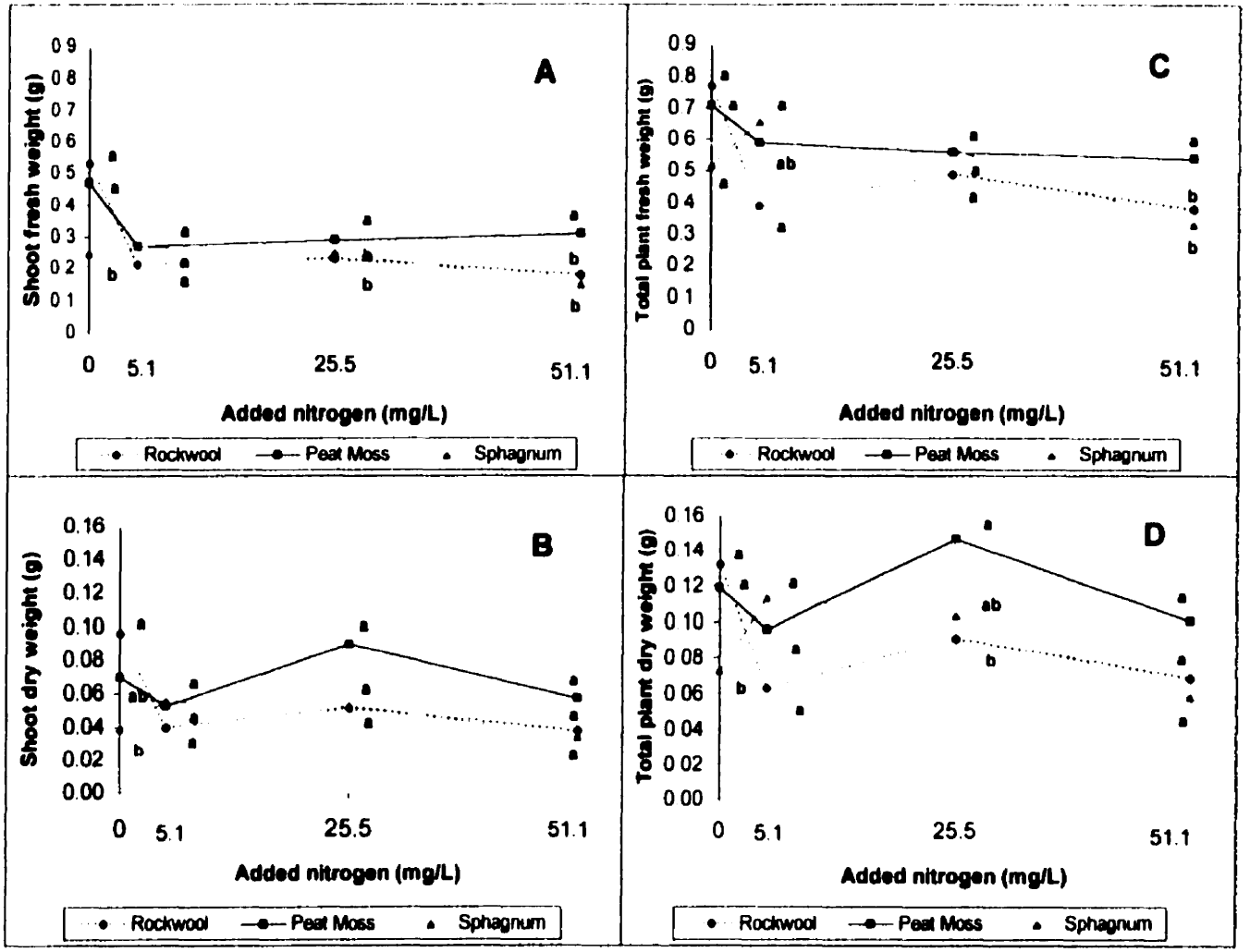


Figure 8: Least square means of shoot fresh weight (A) shoot dry weight (B), total plant fresh weight (C) and total plant dry weight (D), for *D. adelae* plants grown in three substrates (rockwool, peat moss or long fiber sphagnum) at four levels of added nitrogen (0.0, 5.1, 25.5 or 51.1 mg/L) in Experiment 2. Within nitrogen levels, values with the same letter are not significantly different from each other. ($P>0.05$).

Table 8: Least square means of the concentrations of plumbagin and the second, unidentified compound in purified and non-purified extracts from *D. adelae* fertilized at four rates of added nitrogen (0.0, 5.1, 25.5 or 51.0 mg/L). Within columns, values with the same letter are not different significantly different from each other (P>0.05).

Added	Plumbagin				Second compound	
nitrogen	Non-purified		Purified		Direct	
(mg/L)	Ls mean	Std err	Ls mean	Std err	Ls mean	Std err
0.0	288.49a	30.80	257.83a	103.95	54.05a	46.56
5.1	276.76a	25.20	457.24a	151.47	117.21a	35.39
25.5	94.02b	35.41	327.53a	130.22	90.49a	53.39
51.0	152.91b	21.70	151.19a	210.69	55.36a	32.80

Concentrations of the second (unidentified) closely related compound ranged from 0 to 347 mg/kg, with no significant differences among treatments. The covariate (number of side shoots) had no significant effect.

5.4. Mass Spectrometry

Figures 9 and 10 show the spectra obtained from scans 4825 and 5074, respectively, from the chromatogram of non-purified *D. adelae* extract (Figure 5, section 3.5). The spectrum of scan 4825 differs from the spectrum of scan 5074 in several aspects. Notably, the spectrum of scan 5074 does not contain the fragments 120-121 m/z that are present in the spectrum of scan 4825. It does contain 106 m/z and 134 m/z which are both missing from the spectrum of scan 4825. A comparison of the spectrum of 4825 with the spectrum of the 10 ηg/μl plumbagin standard (Figure 11) shows common fragments of 173 m/z, 160 m/z, 145 m/z, 131 m/z, 120 m/z, 103m/z, 92 m/z, 77 m/z, 63 m/z and 61 m/z. The heights of fragment peaks are characterized as percentages relative to the highest peak. A visual inspection of the spectrum of the plumbagin standard and the spectrum of scan 4825 shows that the heights of the peaks are similar in both cases, relative to the height of fragment 131 m/z, which was the highest in both spectra.

The close match of the spectrum of the plumbagin standard and the spectrum of scan 4825 indicated that scan 4825 represented plumbagin in the extract of *D. adelae*. This was also confirmed by allowing the NIST 92 library software to search for a match to the spectrum of scan 4825 in its database of spectra of chemical compounds. The spectrum of 4825 matches the library's spectrum of plumbagin, and the software returned plumbagin as the most likely match to scan 4825, as reported in Table 9. From these matches, it was concluded with certainty that scan 4825 represents plumbagin, and that *D. adelae* contains plumbagin..

The product represented by scan 5074 could not be identified with certainty. While this product was suspected to be ramentaceone, no ramentaceone standard could be obtained. The spectrum of scan 5074 was introduced into the NIST 92 software'

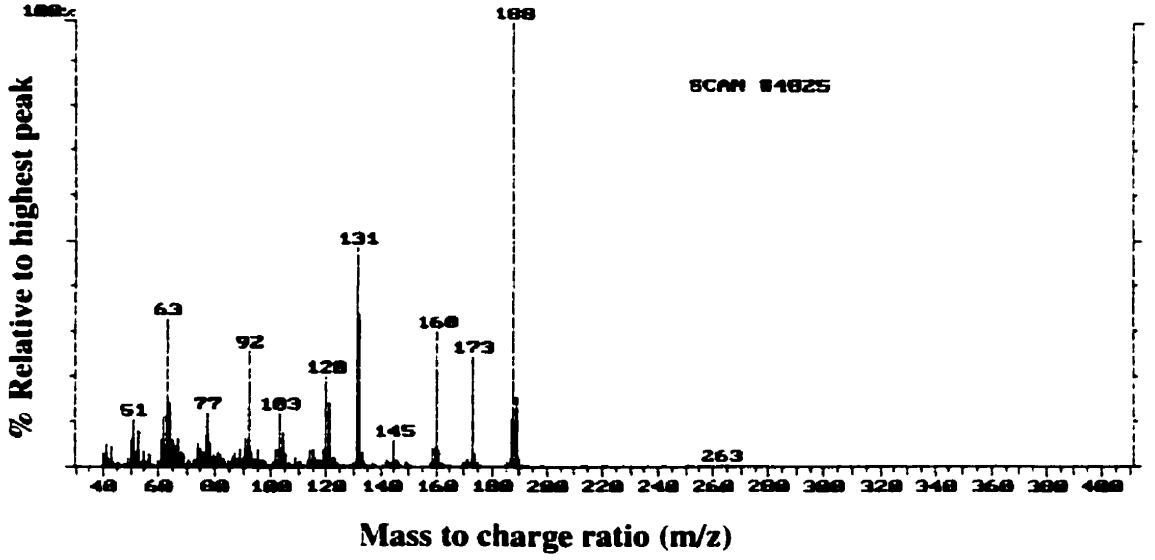


Figure 9: Spectrum of peak 4825 from the complete chromatogram of non-purified *D. adelae* extract.

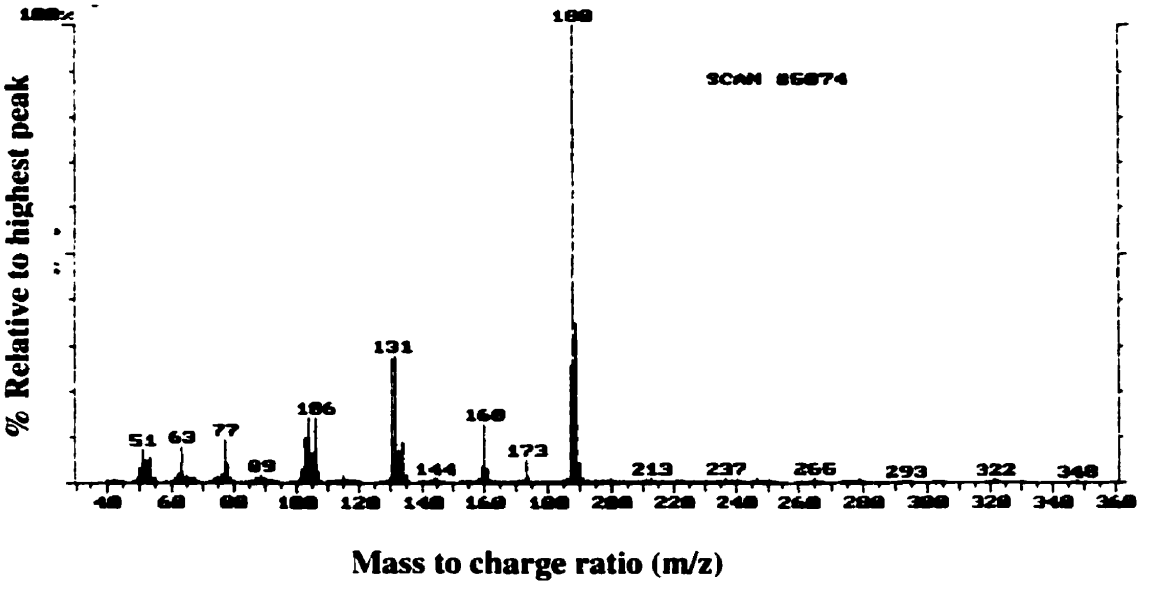


Figure 10: Spectrum of peak 5074 from the complete chromatogram of non-purified *D. adelae* extract.

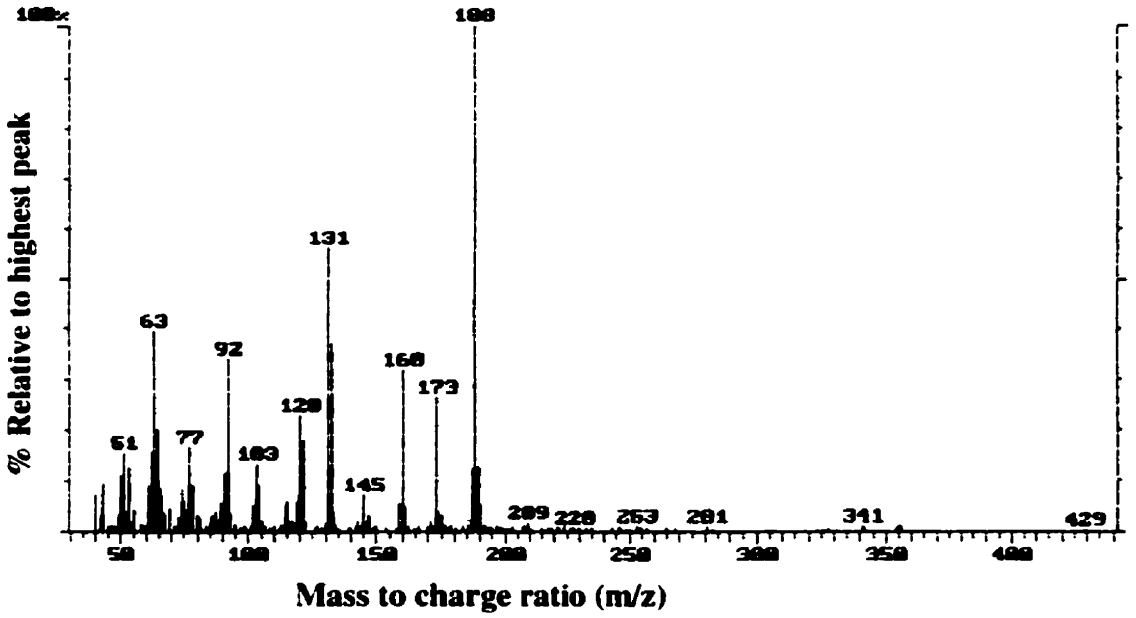


Figure 11: Spectrum of plumbagin standard (10 ηg/μl).

Table 9: Closest matches obtained from a database search for spectra matching scan 4825 and scan 5074 in the NIST 92 Library, the fit, purity and likelihood that the product is contained in the library (Rfit).

NIST match parameter	Scan 4825	Scan 5074
Most likely match	1,4-Naphthalenedione, 5-hydroxy-2-methyl- (Plumbagin)	1,4-Naphthalenedione, 2 - hydroxy-3-methyl-
Fit (on 1000)	978	980
Purity (on 1000)	949	696
Rfit (on 1000)	957	696

search function, and the most likely match was found to be 2-hydroxy-3-methyl-1,4-naphthelenedione. This product is a naphthoquinone, closely related to plumbagin, however it is not ramentaceone. The library reported this as a close match, as reported in Table 9, but ranked the product as relatively impure (696 on 1000). It also reported a low Rfit of 696 on 1000, which is a measure of the likelihood that the product is contained within the library. In other words, it appears that the spectrum of scan 5074 had many similarities with 1,4-naphthelenedione, 2-hydroxy-3-methyl-, yet it may have contained additional peaks. The library interpreted this as either the presence of impurities, or another product, the spectrum of which is not contained in the library.

6. Discussion

6.1. Experiment 1

6.1.1. Nitrogen

6.1.1.1. Biomass

Nitrogen is often the most limiting nutrient in plant production. Most plants contain between 1 and 5% nitrogen by weight (dry matter basis), which they absorb through their roots as nitrate or ammonium (Tisdale et al., 1993). Incorporated into amino acids, and subsequently into proteins and nucleic acids, nitrogen also forms the centre of each of four porphyrin rings in the chlorophyll molecule, the primary absorber of light energy in photosynthesis. All plants require nitrogen for growth.

Because nitrogen is such an important macro-nutrient and is generally required in large amounts by crop plants, a common approach to developing a cropping system is to begin by investigating the response of a crop to nitrogen and from there developing a workable fertilization plan. This is frequently accomplished by fertilizing the crop with different rates of nitrogen and examining the yield response. In general, yield of crop plants increases with increasing nitrogen fertilization. The rate of yield increase with respect to added nitrogen eventually decreases, yields level off, and then at even higher levels of nitrogen the yield begins to decline. This pattern is widely recognized as a typical yield response to increasing nitrogen.

In contrast to this typical positive response to nitrogen, sundew plants appear to be negatively affected by added nitrogen. Like most carnivorous plants, sundews are adapted to habitats with nutrient-poor soil (Givinish et al., 1984). Sundew plant populations are also known to decline when given even modest applications of fertilizer (Redbo-Tortensson, 1994). Hobbyists indicate that sundew plants should never be fertilized because this may cause them to die (Kondo et al., 1976). While sundews are known to supplement nitrogen fertilization from insect prey, it is also known that leaves that trap too many prey, or make too frequent a catch, die.

In the case of sundew plants in the wild, studies show that added nitrogen may in fact decrease sundew growth, not by harming the plants themselves, but by favouring the growth of more competitive grass species (Wilson, 1985). This does not explain, however, the effects found by hobbyists who often grow sundews in the absence of competitors. Brewer (1998, 1999) comments that competitors may reduce the germination of seeds and inhibit seedling establishment, but do not affect the growth of established plants. No explanation can be found in the literature for the reason why sundews may be negatively affected by even modest nitrogen fertilization. No investigation has sought to resolve whether negative effects could be due to toxicity to the plant parts or to antagonism among different nutrients.

Experiment 1 dealt with *D. adelae* growing in rockwool, undergoing four levels of nitrogen fertilization, ranging from 0 mg/L added nitrogen, to 51.1 mg/L nitrogen. Rockwool was expected to be an excellent choice of substrate for investigating the effects of nitrogen fertilization because the medium takes on the characteristics of the hydroponic solution. Few significant differences ($P \leq 0.05$) were found among the treatments. Roots showed no effects and neither did the shoot dry weights. Shoot fresh weights, and total plant dry and fresh weights showed that no added nitrogen resulted in the best yield, followed by the 3rd nitrogen treatment with 25.5 mg/L. While these results were statistically significant ($P \leq 0.05$), it does not necessarily follow that the absence of nitrogen fertilizer is the cause of greater yield. It should be noted here that plants in the treatment without added nitrogen were watered with tap water only (adjusted to pH 5.8), while plants in the other treatments received nutrient solutions with different levels of nitrogen. Therefore, it is not possible to conclude that the nitrogen itself depressed plant growth: the effect could have been due to other components of the nutrient solution. In retrospect, it would have been preferable to include a control treatment of the "complete-minus-nitrogen" nutrient solution.

However, three of the four nitrogen treatments did contain the "complete-minus-nitrogen" solution. Among the three treatments involving nutrient solution no significant

differences occurred for any variable. The range of nitrogen concentrations used was wide, but may have been outside (above or below) the range in which *D. adelae* would respond to added nitrogen. Perhaps at levels below 5.1 mg/L or above 51.0 mg/L, a yield response might have been observed, either positive or negative. Given the greater growth in the no added nitrogen treatment, it may be that the three other levels represented the concentrations typical of the nitrogen response curve where, having levelled-off, it begins to decline.

According to Resh (1987), dry peat contains about 1% nitrogen by weight. Dykyjova and Ulehlova (1998) report that less than 2% of dry organic mass from raised peat bogs was nitrogen. Nitrogen in the form of organic matter would only become available to the plant after microbial mineralization. This process occurs slowly in peat because the acidic environment is not optimum for the mineralizing microbes. In a study conducted on a peat bog, Regina et al. (1998) described NO_3^- in the range of 1.7 mg/kg to 67 mg/kg, and NH_4^+ in the range of 4.5 to 220 mg/kg in dry peat depending on the time of year and the depth of the sample. The mineralization rate of nitrogen in the peat bog environment was found to be 87 kg nitrogen per hectare annually. The range of nitrogen fertilizer selected for this experiment appears to be appropriate. Clearly, the no added nitrogen treatment presents a suitably low value, while the 51.0 mg/L upper range is within the range encountered in natural settings for sundews. The range selected was lower than those found in standard hydroponic solutions for growing vegetables. Cucumbers are often grown using 230 mg/L nitrogen, herbs using 210 mg/L nitrogen, lettuce using 200 mg/L nitrogen, peppers using 175 mg/L nitrogen and tomatoes using 200 mg/L nitrogen (Benton-Jones, 1997). Common nutrient solutions for hydroponic production include Hoagland and Arnon with 210 mg/L nitrogen and Cooper with 200 mg/L nitrogen.

The "complete-minus-nitrogen" fertilizer solution was developed by following the ratios encountered in the literature in fertilizer regimes of sundews and other carnivorous plants (Murashige and Skoog, 1962; Pringsheim and Pringsheim, 1962; Anthony, 1992;

Bobak et al., 1995). Designed to be injected at a rate adequate for use with the highest level of nitrogen fertilization, the concentration of "complete-minus-nitrogen" nutrient solution remained constant among treatments using the three levels of added nitrogen.

In Experiment 1, significant differences between nitrogen treatments occurred for only shoot fresh weight, total plant fresh weight and total plant dry weight. Shoot dry weight did not exhibit any significant response to nitrogen fertilisation. Therefore, the significant differences in shoot fresh weights were due to moisture levels. Plants that had higher fresh weights dried to weights similar to those of plants with lower fresh weights. Plants undergoing the treatment with no added nitrogen had a higher moisture concentration than those undergoing the other two treatments.

One explanation for this effect may be that the plants were osmotically adjusting to a salt stress introduced by the higher fertilizer levels. A common environment for plants is one in which solutions surrounding the roots have lower concentrations of salts than do the cells of the plant tissues. Water enters the root by mass flow from the higher exterior concentration to the lower interior concentration. In situations of higher salt concentration outside than inside cells, the cells may accumulate salts to maintain the gradient that will allow water to flow in. Accumulation of organic metabolites or inorganic ions inside the cells thus protects the plant from low water potential in the medium by decreasing the water potential of the plant (Greenway and Munns, 1980). While the accumulation of these compounds within the plant might lead to higher dry weights than in plants not accumulating these compounds (not under salt stress), Sagi et al. (1997) pointed out that the transport and accumulation process is very energy-demanding and, therefore, often leads to a decrease in biomass. Graifenberg et al. (1996) noted that the ability of plants to survive under salt stress is dependent on the ability to develop an equilibrated osmotic gradient between the soil, root and shoot, and to exclude salt ions from actively growing tissues where deleterious effects might arise. In a study of fennel (*Foeniculum vulgare*), increasing salt stress caused fresh weights to decrease because roots could not retain adequate salt ions, which were instead transported to

growing shoots, causing damage (Graifenberg et al., 1996). If osmotic adjustment is to be used to explain the results of Experiment 1, it follows that while the plants in the treatments with added nitrogen accumulated osmotically-mediating compounds contributing to dry weight, the plants in the no added nitrogen treatment readily absorbed more water from the medium and consequently lost more weight when they dried. The growth rate of plants in Experiment 1 was greatest when no added nitrogen was used. This supports the notion that plants were osmotically adjusting and that the energetically expensive task of accumulating organic compounds or ions, caused the decrease in growth.

A second explanation to support the results may be that plants in the no added nitrogen treatment had larger or more numerous drops of mucilage than plants fertilized with added nitrogen. The mucilage is largely made up of water, so plants with more mucilage would have smaller dry weights than plants with similar fresh weights, but less mucilage. Why would plants with no nitrogen fertilization produce larger mucilage drops than plants fertilised with nitrogen? It would be a logical survival mechanism for these plants to produce more mucilage in the case of low nitrogen availability in order to better trap insect prey for nitrogen supplementation. In the course of carrying out these experiments, it became evident that some sundew plants not used in the experiments, without cup covers, trapped insects. However, it was also noted that some insects were able to escape the mucilage traps of the sundews. Thum (1989) also noticed that some insects were able to walk away from the sundew plant leaves, and found that ants often carried away the sundew's prey for their own uses. Perhaps, larger mucilage drops contribute to more effective prey capture.

The speculation that unfertilized plants may have produced more mucilage is supported by the insignificant differences in the variables root fresh weight and root dry weight. The roots of *D. adelae* are very tough and fibrous, and completely lack the glandular hairs that hold drops of mucilage near the leaf surfaces. Roots do not have

mucilage drops and, therefore, the fresh weight of roots would be much less variable than the fresh weight of shoots.

Plants in this study grown without added nitrogen were more moist, or more "succulent" than those grown using higher levels of nitrogen. However, the accepted phenomenon of succulence is one in which low nitrogen levels lead to carbohydrate deposition on cell walls, whereas high nitrogen levels leads to an increase in highly hydrated protoplasm production (Tisdale, 1993). The situation in this study appears to be the opposite and, therefore, this definition of succulence cannot be offered in explanation.

6.1.1.2. Flowering

High rates of nitrogen fertilization often lead to greater vegetative growth, and a delay or absence of flowering in many crop species. This is often explained as a survival mechanism. When nutrients are abundant, the plant need not invest in reproductive structures. In times of nutrient stress, this investment may be necessary since the parent plant may die, leaving only seeds as propagules. Redbo-Tortensson (1994) concluded in a study of nitrogen fertilization of sundews, "There is a narrow range of nutrient availability required for both floral initiation (low N) and maximum seed production (higher N)". It was further noted that plants receiving too little nitrogen produce fewer flowers and seeds compared with those receiving higher levels of nitrogen, yet at even higher levels of nitrogen, flower initiation was prevented.

In this study, flowering was an exceedingly rare occurrence, possibly because plants were young. Redbo-Tortensson (1994) found that of 57% of sundews that flowered within four years, only 10.4 % flowered within the first year and were not necessarily one-year-old plants. Rather, these plants constituted a wild population of varying ages.

How important is it to investigate flowering? While Denoël (1949) noted that the flowers of both *D. rotundifolia* and *D. intermedia* contained high levels of naphthoquinones relative to other plant parts, the biomass of the flowers alone relative to the leaves of the plant were very small. Why would the naphthoquinone concentration of

flowers be greater than the concentration in shoots? Durand and Zenk (1974b) suggested that the napthoquinones evolved as anti-microbial agents in a protective role. Salisbury and Ross (1992) mentioned that developing flowers and young fruits have a great ability to draw mineral salts, sugars and amino acids, presumably from leaves, since the concentrations of these constituents decrease in the other plant parts (Salisbury and Ross, 1992). Plumbagin and ramentaceone are formed from the breakdown of an amino acid (tyrosine) to acetate for use in the biosynthesis of naphthoquinones (Durand and Zenk, 1974b). Future work could be done on the role flowering plays in the concentration of naphthoquinones in other plant parts. If the concentration in fact decreases due to flowering, more research is required to determine whether there is a net gain or loss of naphthoquinones, taking into account not only concentration but also biomass production. As Salisbury and Ross (1992) noted, removing flowers is a technique gardeners have employed for centuries, to stimulate vegetative growth. Perhaps in sundew plants , such a practice might serve a second purpose by preventing a decrease in shoot naphthoquinone levels.

Another reason for pursuing studies on flowering in sundew plants is that many sundew species are threatened or endangered, and more species may become so in the future. As Redbo-Tortensson (1994) reported, deposition of atmospheric nitrogen pollutants is a major threat to the flora of many European countries, particularly the flora (including sundew) of ombrotrophic bogs. Clearly, seed production of sundew should be studied and optimized in order to develop a genetic pool for use in the event that some of these species disappear from their natural habitats.

6.1.2. Harvest Method

A hydroponic system for growing sundew, although seemingly appropriate for this wetland denizen has not been reported in literature. In addition, research on any sort of agronomic production system is very sparse, and tests of different harvest methods are equally lacking. Experiment 1 of this study investigated the effects of three harvest methods on the growth of D. adelae. Two of the methods were chosen to determine

whether plants were best harvested after two months or after four months. A sequential harvest method was also investigated. Various sundews form adventitious buds from roots and leaves that touch wet substrates (Kondo et al., 1976), so it was possible that these plants could recover from an aggressive partial harvest, and that buds released from the apical dominance of the main shoot might grow with vigour.

While analysis of these methods focussed on the biomass accumulation, further evaluation is required in a cost-benefit analysis to determine which harvest method is economically most advantageous. That discussion is beyond the scope of this study.

Harvest method had a significant influence on many variables. Table 5 reveals that while root fresh weight differed significantly ($P \leq 0.05$) at the different harvest dates , root dry weight did not significantly differ ($P > 0.05$). Shoot fresh weights harvested after four or after two months were identical, but the four-month method resulted in a significantly higher dry weight ($P \leq 0.05$). It appears from both roots and shoots that the younger plants have a higher water concentration than older plants. Succulence may be the reason for this difference. Young plants are actively growing and have a large proportion of new, expanding cells. Older plants may have cells that are becoming thickened with additional deposits of structural carbohydrates. Sequential harvesting resulted in the lowest dry and fresh yields, as well as the lowest growth rate, suggesting that sequential harvest is of no advantage. The new shoots that had formed on some plants in the sequential harvest treatment grew well after the first cut, whereas other plants became stressed and fared poorly, or even died. Still others had not formed new shoots at the time of first harvest and did not form any before the second. On average the plants in this treatment had the lowest shoot biomass, and the effects were evident in the roots as well. Some of the roots of sequentially harvested plants died back and were decaying at the four-month harvest date. It is possible that others used root reserves to put forth the new shoots once the main shoot had been cut away, thus reducing the weight of healthy roots.

Harvest method greatly affected growth rate (Figure 4). The highest growth rate occurred for the two-month harvest date. In fact, all of the plants would have experienced this growth rate for the first two months of treatment since all were subjected to the same conditions. On comparison of the growth rate of plants undergoing the four-month harvest method, it appears that sundews under the same conditions grow more slowly after the first two months. Plants undergoing the sequential harvest method had the lowest growth rate, as well as the lowest shoot fresh weights. At first, it seems peculiar that plants in the sequentially harvested treatment would not have fresh shoot weights at least as large as those in the two-month harvest treatment since the first sampling occurred at the same date for both treatments. However, it should be noted that in the case of the sequentially harvested plants, only the main shoot was removed at the two-month harvest. Some of the new shoots that were left growing may have subsequently died.

6.2. Experiment 2

6.2.1. Nitrogen and Substrate

In Experiment 2 the same nitrogen levels were tested as in Experiment 1, but this time plants were grown on rockwool, peat or long fibre sphagnum. This resulted in potentially greater nutrient availability to plants growing on either peat or sphagnum, although Regina et al. (1998) showed an overall decrease in nitrogen mineralization in peat fertilized with nitrogen. Kondo et al. (1976) advised sundew hobbyists not to worry about nutrient deficiency when growing sundew in long fibre sphagnum moss, warning that plants should not be fertilized at all. Long fibre sphagnum does contain nitrogen, but much of this nitrogen is bound in organic matter, and similar to peat moss, the acidity of the material prevents rapid mineralization of organic nitrogen for plant use.

Rockwool was selected in Experiment 1 as the substrate because it provided the possibility of examining the effects of nitrogen fertilization treatments without any confounding effects of the substrate. In Experiment 2, rockwool was tested against peat and long fibre sphagnum, to assess its potential as a growing medium for sundew in

hydroponic culture. Rockwool held transplant roots in place better than either peat or sphagnum substrates, resulting in better medium-root contact.

Peat and rockwool share some characteristics that make them good substrates for the growth of sundew. Both retain water well, and can be arranged to result in good contact with roots. The long fibre sphagnum has good water-holding ability, but it does not hold the transplants in place well. A drawback of peat and long fibre sphagnum is that they may contain spores of fungi, bacteria, or even insect eggs. While it is rare that commercial grade peat and long fibre sphagnum contain pathogenic organisms, it is a possibility. While irrigation water can introduce a host of organisms such as algae, fungal spores and bacteria to rockwool, the medium starts off without any of these contaminants. While no disease or insect pest problems arose in this study, it should be noted that the warm, humid environment, combined with the succulence of the plants and readily available fertilizer, make ideal conditions for these types of problems.

Nitrogen and substrate interacted in Experiment 2, meaning that the effects of nitrogen on the plant growth varied, depending on the substrate used. This was expected, since the characteristics of the substrates influence the concentration of various nutrients available for plant growth. The rockwool is inert and, therefore, the fertilizer treatments remain as applied, and the rockwool takes on the pH of the applied solution. Peat and long fibre sphagnum, however, contain nitrogen as well as other plant nutrients, and have their own pH that may or may not be influenced by applied fertilizer. Organic matter, a large constituent of peat and long fibre sphagnum, binds some compounds. In a peat bog, organic matter acts as an acid cation exchanger, binding divalent ions more strongly than univalent ions (Procter, 1995). The carbon to nitrogen ratio of peat is high, leading to nitrogen immobilization during microbial decomposition. Procter (1995) commented that in peat bogs, the activity of the microorganisms is often limited by phosphorus availability. In the case of both peat and long fibre sphagnum, it is clear that the fertilizer solutions will not remain unchanged as in the case of rockwool. In fact, nitrogen may or may not become available from the organic matter, ions may or may not be held to the

organic matter, and the pH may or may not change with added fertilizer. In Experiment 2, the exact nature of the changes in fertilizer solution in peat and long fibre sphagnum were not investigated, and the nitrogen treatments were treated categorically rather than quantitatively.

Shoot fresh weight, but not shoot dry weight, increased when added nitrogen levels were 5.1 mg/L and 25.5 mg/L. At these higher nitrogen levels, plants had higher fresh weights in peat moss. The dry weight of plants in peat moss was also higher at these levels, but not significantly so. When no nitrogen was added, plants performed best in rockwool and peat moss, and poorly in sphagnum.

Sphagnum gave significantly higher yields of total plant fresh weight at the 5.1 mg/L nitrogen level than rockwool, however at the highest nitrogen level (51.1 mg/L), long fibre sphagnum and rockwool resulted in lower yields than peat did. Total plant dry weight showed rockwool and peat to be the substrates of choice when no nitrogen was added, whereas peat was clearly better than rockwool when 25.5 mg/L was added. The response of plants to nitrogen when grown in long fibre sphagnum indicates increasing growth with nitrogen until 5.1 mg/L, followed by a decrease in growth. This pattern follows the course of a typical response curve to nitrogen. In contrast, the yield of plants in rockwool was negatively affected by increasing nitrogen. Peat-grown plants had high yields when 25.5 mg/L nitrogen was added. This suggests that nitrogen is lacking in long fibre sphagnum, but that fertilization with 5.1 mg/L nitrogen is in the range of the top values of tolerable nitrogen for the plant in this environment. Peat, apparently, does not contain adequate amounts of nitrogen for peak yield unless fertilized with nitrogen in the range of 25.5 mg/L. Finally, rockwool seems to already contain adequate nitrogen for peak growth. This final statement runs contrary to the expectation that nitrogen is required for growth since rockwool itself contains no available nitrogen, and irrigation water levels of NO_3^- were at just 0.02 mg/L. No plug was carried over in the transplantation process. Plant roots were washed in warm water until no visible traces of peat were left. Although plants were acclimated for one month with the highest level of

nitrogen fertilization, this solution should have been completely flushed from the rockwool cubes during the first application of treatment solutions. Rockwool is inert, so nitrogen should not bind to it, and the quantity of treatment solutions applied was adequate to completely replace all solution in each block at every watering. The only explanation that can be offered is that the range of fertilizer may not have been wide enough. Perhaps a positive yield response to nitrogen would have been observed at higher levels of nitrogen.

The relatively small range of growth exhibited by plants in these experiments points to one of two possibilities. Either the nutrient solutions or some other environmental condition was insufficient for greater growth, or the plants are insensitive to environmental conditions. Proctor (1995) states that bog plants often show little response in nutrient-addition experiments on peat and that this reflects the "...inherently slow growth rate and efficient nutrient retention characteristics of wild species adapted to nutrient–poor habitats."

6.3. Gas Chromatography

The method used for GPC of the extracts of D. adelae was largely novel work, based loosely on the results obtained by Bonnet et al. (1984), who used a direct GPC method without purification of the extracts. This method served quite well for many of the samples, but for some, often those of larger weight, purification of the sample was required. Without purification these samples blocked the GPC column. Two peaks, identified as plumbagin and a closely related product, were the focus of the analyses. Several methods of purification were attempted before settling on the Chem-Elute™ method. When the various tests were done to identify the interference of solvents, and the recovery rates achieved in purified samples, it became apparent that the process removed many of the components of the non-purified extract and notably diminished the peak corresponding to the second compound. The exact reason for the loss of the second components is unknown, but it is likely that the internal environment of the cartridge or column was involved. While this situation was far from ideal, time constraints were such

that the decision was made to continue with the Chem-Elute™ method, since it resulted in a recovery rate of plumbagin similar to that of the non-purified samples. Nonetheless, the resulting data were difficult to analyze since results obtained using one method could not justifiably be grouped with results obtained using the other method. The use of a different column for the non-purified samples (SPB1) and the purified samples (DB1710) was another factor that precluded combining the data for the analysis.

Due to limited resources, only 40 samples were analysed using GPC. This meant that results of concentrations of naphthoquinones in plants from different substrates and from different harvesting methods went unexamined. However, the results of the samples that were analysed were very encouraging.

Purified samples yielded a wide range of plumbagin concentrations, but the nitrogen treatments did not affect the plumbagin concentration. These results of this statistical analysis are, however, very unreliable. The data were unbalanced in design. With only 17 purified samples and four nitrogen treatments, some treatments were represented by only two samples. The standard error was very large for the various means. In the case of the no added nitrogen treatment, the standard error was almost as large as the mean (Table 8)! Therefore, although no significant treatment differences were found, this is not conclusive evidence that nitrogen does not affect plumbagin content.

Nitrogen had an effect on the plumbagin concentration of D. adelae in the non-purified samples, but the standard error of the means was very large. Although the results indicate that the lowest level of nitrogen fertilization (5.1 mg/L) and no nitrogen fertilization produced plants with higher concentrations of plumbagin than the higher nitrogen fertilzation levels did, further studies should be conducted, with larger sample sizes to verify this result.

The concentration of the second compound was exceedingly variable. Because of the small sample size, the standard error was larger than the mean in some treatments. Again, although no differences were detected, this does not preclude a relationship between nitrogen level and the concentration of this second naphthoquinone compound.

With such variability and such a small sample size, it may be more useful to consider the range of concentrations produced, disregarding the nitrogen treatments. A wealth of literature exists on the levels of naphthoquinones found in certain *Drosera* species. However, of over 90 species, these reports detail the contents of fewer than 20. Caniato et al. (1989) made recommendations that more species of sundew be investigated with respect to production possibilities and naphthoquinone content. In addition, they claimed that the European species frequently studied contained low levels of naphthoquinones compared with South African and Australian species, which are larger in size. This study examined one of the largest of the Australian sundews. While the mean concentrations obtained by GPC in this study ranged from 94 to 457 mg/kg for plumbagin and 55 to 117 mg/kg for the second compound, these results represent only 76 % of the actual concentration (the recovery rate, Table 3). If these values are converted to account for this low recovery rate, concentrations vary from 124 to 602 mg/kg for plumbagin 73 to 154 mg/kg for the second compound. This highest level of plumbagin is approximately 0.06% of the dry weight. Given a roughly tenfold difference between fresh weights and dry weights, this value would correspond to 0.005% of fresh weight. These values are similar to those of Finnie and van Staden's (1993); they found that plumbagin constituted 0.0045% of the fresh weight of D. capensis grown in vivo. D. capensis is a South African sundew known as the cape sundew, and is much smaller in stature than D. adelae. Finnie and van Staden (1993) also report plumbagin concentrations of 0.0005% of fresh weight in D. capensis grown in vitro, 0.002 % of fresh weight in D. natalensis grown in vivo, and 0.001% of fresh weight in D. natalensis grown in vitro. Crouch et al. (1990) reported plumbagin concentrations of 0.01% of fresh weight in Plumbago auriculata, roughly double the highest concentration of plumbagin found in this study. Denoël (1949) reported that the concentration of total naphthoquinones in D. rotundifolia

ranged from 0.224% of dry weight in the glandular leaf hairs to 1.34% of dry weight in the flowers. As Bonnet et al. (1984) pointed out, Denoël's colorimeteric approach was not able to distinguish between plumbagin and other naphthoquinones. Bonnet et al. (1984) found that D. intermedia contained naphthoquinones in the concentration 1.99 % of dry weight when collected from the wild, and of 9.86% of dry weight in plants grown in vitro. The latter figure is by far the highest concentration reported. It is unclear whether the source of this value is a single plant or a mean value from numerous extracts. Blehova et al. (1995) investigated ramentaceone levels in D. spathulata and found that the concentration ranged from 0.057% of dry weight to 1.655% of dry weight.

While the findings of this research do not support the statement by Caniato et al. (1989) that Australian sundews have higher levels of naphthoquinones than European species, it appears that D. adelae has plumbagin levels similar to those found in other sundews. This, combined with the large size of D. adelae, makes it an interesting possibility as a species for cultivation.

6.4. Mass Spectrometry

Zenk et al. (1969), in a survey of the naphthoquinone contents of 17 species of sundew, found that most species contained either plumbagin or ramentaceone, but very few contained both. Finnie and van Staden (1993) explained that these are not the only naphthoquinones in sundew. In fact, various sundews contain a complex mixture of naphthoquinones, including rossoliside (1,4,5-trihydroxy-7-methylnaphthalene glycoside), rametone (2-methyl naphtharazin), droserone (3-hydroxy plumbagin) and others. While herbals have listed the sundew plant with its cocktail of compounds, as useful medicine, modern science and medicine have concentrated on the importance of only plumbagin and ramentaceone. It seems likely, that many of these closely related compounds may be responsible, either in themselves or in combination, for some of the therapeutic effects of sundew plants.

No reports on the naphthoquinone contents of D. adelae could be found in the literature, so this may well be the first to confirm the presence of plumbagin in D. adelae. Comparison of the spectrum of the peak 4825 (Figure 8) of a scan of D. adelae extract with the spectrum of plumbagin standard (Figure 9) revealed a close match. In addition, a high degree of fit was obtained when the mass spectrum of the peak 4825 was compared with plumbagin in the NIST 92 library. Further, the spectrum obtained matches the plumbagin spectrum obtained by Bonnet et al. (1984), as well as that of Zenk et al. (1969) and Caniato et al. (1989). The presence of plumbagin in D. adelae is expected, since the Australian sundews investigated to date, have all contained plumbagin (Caniato et al. , 1989).

The second compound presented a challenge to identify. The NIST 92 Library identified the compound as 2-hydroxy-3-methyl 1,4-naphthalenedione, which is a naphthoquinone, although not ramentaceone. However, this identification is not certain. The Library suggested that this identification fits well with the spectrum of the second compound, but it suggested that the peak may not represent a pure substance (purity 696 on 1000). Furthermore, the RFit, a measure of the likelihood that the true identity is contained within the library, is quite low. The NSIT Library is searchable, and in fact, a search revealed that ramentaceone is one compound that is not included in the library.

The spectrum of the second compound has many of the same fragments as ramentaceone as reported by Bonnet et al. (1984). In common are the fragments 188 m/z, 187 m/z, 160 m/z, 134 m/z, 132 m/z, 131 m/z, 106 m/z, 77 m/z, and 51 m/z. With the exception of 39m/z, the spectrum of this second compound contains all of the peaks that Bonnet et al. (1984) reported in the spectrum of ramentaceone. However, additional peaks appear in the spectrum of this second compound as well. It is possible that the second compound is ramentaceone mixed with co-eluted material.

While the report by Bonnet et al. (1984) indicated the peaks in the ramentaceone spectrum, it provided no details of the magnitude of these peaks. Both intensity and m/z

fragment are required to identify a compound with certainty. One way to compare the magnitude of the peaks would be to compare the spectrum of the second compound with the spectrum of a ramentaceone standard. This was not done because no standard was available. A standard would have to be synthesized, at a cost outside the range of funding for this work.

The second compound is a naphthoquinone, and it may well have medicinal qualities, regardless of whether it is ramentaceone. Therefore, further research should be carried out to identify and characterize this compound with certainty.

6.5. Production System

Much work has been done on the possibilities of producing sundew plants in vitro. These studies have focussed on optimizing in vitro conditions, organogenesis and callus production, methods of surface sterilization of explant material, and the effects of growth regulators on the growth and medicinal compound yield of sundew plants (Crouch and van Staden, 1988; Kukulczanka and Czastka, 1988; Anthony, 1992; Blehova et al., 1995; Bobak et al. 1995). While Kukulczanka and Czastka (1988) feel that great amounts of sundew plant material can be produced for the hobby market using in vitro methods, should in vitro propagation be the sole method of production of sundew to fill the medicinal market? Finnie and van Staden (1993) point to sundew's low plumbagin levels as evidence that all hopes for commercial propagation of sundew in any manner should be discarded because Plumbago spp. present a much richer source of plumbagin.

This approach neglects the other compounds in sundew, not only the ramentaceone, but numerous other potentially beneficial compounds. Synergisms or additive effects that are unknown may be responsible for some of sundew's therapeutic effects. Finnie and van Staden's (1993) analysis is appropriate only in the context of sundew as a source plant for the extraction and purification of plumbagin. These same researchers indicated that in vivo-grown plants have higher concentrations of plumbagin than do in vitro-grown plants. Blehova et al. (1995) reported that the amount of

ramentaceone in tissues increases with differentiation. It is logical that tissue culture techniques can provide an excellent source of transplant material for production in vivo, which is the approach this study took.

Numerous methods of cultivation in the greenhouse might allow sundew plants to grow well. This study has suggested two substrates that support sundew growth well, rockwool and peat. The third substrate, long fibre sphagnum, was a less attractive alternative. Fertilizer use is low in these plants, and many species grow well in low light conditions. The plants were well suited to the hydroponic system. The only demanding characteristics of these plants are warm temperature and high humidity.

6.5.1. Best Management Practices for Hydroponic Sundew Production

The following best management practices for the hydroponic production of *D. adelae* are based on this research alone, and are open to refinement as further study is carried out. Micropropagated plants should be in good health, and free from pests, particularly aphids which can avoid the mucilage drops by living at the leaf surface. The plants benefit from high humidity through all stages of development, but are negatively affected by free water on leaf surfaces. Coverings that allow light penetration and limited air circulation should be used to maintain high relative humidity around the plants. Plant transplants into rockwool or peat that has been thoroughly soaked with water. The transplants should have two or more well-developed leaves. Care must be taken to avoid damaging the roots. Water the plants frequently; the substrate should be constantly wet to the touch but not saturated. Do not fertilize. The plants are not demanding of light, and do not require supplemental lighting. They survive well in shaded conditions.

7. Conclusion

This study demonstrated that micropropagated *D. adelae* can be transferred successfully *ex vitro* and can grow in a hydroponic system. Nitrogen fertilization (0 to 51.1 mg/L) had little effect on the dry weight of plants, but affected fresh weights more. Plants fertilized without or with low levels of nitrogen had higher water contents than other plants, and the effect was more pronounced for shoot weight than for total plant weight. This could have been due to osmotic regulation in response to salt stress; plants in the higher N treatments may have accumulated salts and therefore had relatively higher dry weights. Another possibility is that plants in increased mucilage production in response to low levels of nitrogen, with the benefit of increased nitrogen nutrition through better prey capture. Mucilage is largely water, and could well be responsible for the variation in water concentrations. Nitrogen fertilization interacted with substrate, which was expected since two of the substrates (long fibre sphagnum and peat) have characteristics that may influence fertigation solutions. Rockwool and peat resulted in better growth than long fibre sphagnum did when no nitrogen fertilizer was used; they out-yielded sphagnum by more than one and a half times in the case of shoot dry and fresh weight, and total plant fresh and dry weight. However, the total plant dry and fresh weights were highest at the 5.1 mg/L nitrogen fertilizer level when sphagnum was the substrate. A study of harvest methods showed that the growth of the plants slowed dramatically after two months, resulting in a much lower growth rate of plants harvested after four months versus after two months of growth. The plants did not respond well to sequential harvest at two and four months and sustained significant damage leading to the lowest growth rate among the treatments. Sundew plants grow slowly. In this study, plants grew at a rate of only 0.008 g/day under the best nitrogen treatment (0.0 mg/L added N) and only 0.013 g/day early in their youngest, most productive phase (harvested at two months). Assuming the best growth rate achieved in this study, and that 100 plants could be grown in 1 m^2, over a 60 day period, the gain in fresh weight would be 78g/m^2 or 780 kg/ha, corresponding to approximately 78 kg/ha gain in dry weight.

Plumbagin was positively identified in *D. adelae* through gas chromatography-mass spectrometry. The presence of plumbagin was expected since it has been found in other Australian sundews. A second closely related compound was also investigated. The characteristics bore considerable similarity to reports of ramentaceone found in some other sundew species, but the identity could not be confirmed. The effect of nitrogen on the medicinal quality of *D. adelae* remains inconclusive. The range of concentrations of plumbagin found in *D. adelae* was similar to those reported in the literature for other sundew species. While the concentrations of plumbagin in *D. adelae* (124 to 602 mg/kg dry weight) are not among the highest reported, *D. adelae* is among the largest sundews. Its quality combined with its large size makes it a good candidate for future studies on large-scale production of sundew.

8. References

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