Allelopathic potential of silverleaf nightshade

(*Solanum elaeagnifolium* Cav.)

by

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CONTENTS

Acknowledgements iii

List of abbreviations iv

List of tables v

List of figures viii

Abstract x

Uittreksel xii

Introduction 1

CHAPTER 1 Literature review

1.1 Introduction 3
1.2 Morphology and biology 4
1.3 Leaf anatomy 6
1.4 Interference of *S. elaeagnifolium* with crop species 7
1.5 Secondary metabolites of silverleaf nightshade 9
1.6 Biological activity of silverleaf nightshade 11
1.6.1 Antimicrobial activity 11
1.6.2 Phytotoxic activity 12
1.7 Overview of control strategies 13

CHAPTER 2 Evaluation of the bioassay technique

2.1 Introduction 15
2.2 Plant material 16
2.3 Extraction procedures 16
2.4 Choice of test species 17
2.5 Germination method 18

2.6 Sterilisation procedures 20
2.7 Osmotic interference 20
2.8 Concluding remarks 21

CHAPTER 3 Allelopathic potential of silverleaf nightshade foliar infusions and crude extracts

3.1 Introduction 22
3.2 Materials and methods 23
3.3 Results and discussion 26

CHAPTER 4 Chemical characteristics of phytotoxic compounds in silverleaf nightshade foliage

4.1 Introduction 31
4.2 Materials and methods 32
4.3 Results and discussion 36

CHAPTER 5 Potential link between leaf anatomy and allelopathic potential of silverleaf nightshade

5.1 Introduction 39
5.2 Materials and methods 40
5.3 Results and discussion 41

CHAPTER 6 General discussion and conclusion

6.1 Bioassay technique 49
6.2 Allelopathic potential of silverleaf nightshade foliage 50
6.3 Preliminary identification of active compounds 50
6.4 Leaf anatomy as linked to allelopathic potential 51
6.5 Conclusions and recommendations 51

Appendix A Abbreviated analysis of variance (ANOVA) tables 52

References 57

Summary 64

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LIST OF ABBREVIATIONS

m	:	meter
cm	:	centimeter
mm	:	millimeter
cm^3	:	cubic centimeter
µm	:	micrometer
µl	:	microlitre
PEG	:	polyethylene glycol
mOsm kg^{-1}	:	milliOsmol $kilogram^{-1}$
LSD	:	least significant difference
UV	:	Ultraviolet
TLC	:	Thin layer chromatography
^{1}H-NMR	:	^{1}H nuclear magnetic resonance

LIST OF TABLES

Table 3.1 The effect of increasing concentrations of a crude water extract of silverleaf nightshade foliage on germination, root and shoot lengths of cotton 26

Table 3.2 The effect of PEG-6000 solutions of increasing osmolality on germination, root and shoot lengths of cotton 27

Table 3.3 The effect of increasing concentrations of a water-soluble foliar extract of silverleaf nightshade on cumulative germination over three days, and mean root length of lettuce cv. Great Lakes 27

Table 3.4 The effect of PEG-6000 solutions of increasing osmolality on germination and mean root length of lettuce seedlings 28

Table 4.1 Effect of paper chromatography fractions on germination of lettuce over three days and mean root length of resulting seedlings 36

Table 4.2 Effect of Sephadex fractions on germination of lettuce over three days and mean root length of resulting seedlings 37

Table A1 Analysis of variance of percentage germination of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract, after angular transformation 52

Table A2 Analysis of variance of root length of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract 52

Table A3 Analysis of variance of shoot length of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract 52

Table A4 Analysis of variance of percentage germination of cotton germinated in increasing concentrations of PEG-6000 solution, after angular transformation 53

Table A5 Analysis of variance of root length of cotton germinated in increasing concentrations of PEG-6000 solution 53

Table A6 Analysis of variance of shoot length of cotton germinated in increasing concentrations of polyethylene glycol-6000 solution 53

Table A7 Analysis of variance of percentage germination (72 hours) of lettuce seedlings germinated in increasing concentrations of the water-soluble fraction of an organic silverleaf nightshade foliar extract, after angular transformation 54

Table A8 Analysis of variance of root length of lettuce germinated in increasing concentrations of the water-soluble fraction of an organic silverleaf nightshade foliar extract, after rank transformation 54

Table A9 Analysis of variance of percentage germination (72 hours) of lettuce germinated in increasing concentrations of PEG-6000 solution, after angular transformation 54

Table A10 Analysis of variance of root length of lettuce germinated in increasing concentrations of PEG-6000 solution 55

Table A11 Analysis of variance of percentage germination (72 hours) of lettuce germinated in paper chromatography fractions, after angular transformation 55

Table A12 Analysis of variance of root length of lettuce germinated on paper chromatography fractions 55

Table A13 Analysis of variance of percentage germination (72 hours) of lettuce germinated in Sephadex column fractions, after angular transformation 56

Table A14 Analysis of variance of root length of lettuce germinated in Sephadex column fractions 56

LIST OF FIGURES

Figure 3.1 Longitudinal sections of the root tips of (A; B) a seedling not exposed to a silverleaf nightshade foliar extract, and (C; D) a seedling exposed to the foliar extract; (E) differentiation of root hairs on the swollen area close to the root tip of an extract-treated seedling 29

Figure 4.1 TLC plates sprayed with Dragendorff reagents showing the presence of an alkaloid in fraction 4 from the silverleaf nightshade foliar extract Sephadex column 37

Figure 4.2 Preliminary ^{1}H-NMR spectral analysis of fraction 6 showing the presence of a relatively pure saponin 38

Figure 5.1 Transverse section of a young (A) and mature (B) silverleaf nightshade leaf, showing its isobilateral and amphistomatic nature and intrusive stellate trichomes 41

Figure 5.2 Transverse section of a silverleaf nightshade leaf showing crystals, most likely calcium oxalate, contained in an idioblast 42

Figure 5.3 Transverse sections through glandular trichomes on the silverleaf nightshade leaf surface showing the storied glandular trichome head and one stalk cell 43

Figure 5.4 Transverse section of a silverleaf nightshade leaf showing intrusive and non-intrusive variants of stellate trichomes and a stellate trichome in early stages of development 44

Figure 5.5 Scanning electron microscope images of the (A) adaxial surface of a 2 cm-leaf, (B) adaxial surface of a mature leaf and (C) abaxial surface of a mature leaf of silverleaf nightshade; (D) Removal of an intrusive trichome shows basal cells entering the leaf surface 45

ALLELOPATHIC POTENTIAL OF SILVERLEAF NIGHTSHADE (*SOLANUM ELAEAGNIFOLIUM* CAV.)

by

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ABSTRACT

Silverleaf nightshade is a serious problem weed in many semi-arid regions of the world. Although many research efforts have been devoted to this weed's interference with crop species, possible chemical interference (allelopathy) has not been thoroughly investigated yet. In the present study, allelopathic potential of silverleaf nightshade foliage was assessed by means of germination bioassays. Preliminary experimentation was necessary to evaluate procedures of the bioassay method to be used, as many different approaches are described in literature. Water infusions and crude water-soluble extracts of silverleaf nightshade foliage inhibited germination and root growth of cotton and lettuce respectively. Osmolalities of the infusions or extracts used were not inhibitory to germination or root growth of either cotton or lettuce. Preliminary exploration of the nature of the chemical substances implicated in this phytotoxic activity suggests that more than one compound is involved. This includes an alkaloid, a saponin and several flavonoidic constituents, implying the presence of a synergistic effect for crude extracts. An anatomical study was conducted in an attempt to link the allelopathic potential of silverleaf nightshade foliage to specific cells or structures in the leaves. It was considered that glandular trichomes, abundant on both leaf surfaces, might harbour phytotoxic secondary metabolites. It was furthermore speculated that stellate trichomes with intrusive basal cells observed to reach

the vascular bundles, might be involved in excreting alkaloids contained in the vascular bundle sheath onto the leaf surface. The results of this study represent the first step in showing the allelopathic potential of silverleaf nightshade and laid a foundation for continuing with field studies and more in-depth chemical analyses.

ALLELOPATIESE POTENSIAAL VAN SATANSBOS (*SOLANUM ELAEAGNIFOLIUM* CAV.)

deur

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UITTREKSEL

Satansbos is 'n probleem onkruid in baie semi-ariede dele wêreldwyd. Alhoewel heelwat navorsing oor hierdie onkruid se inmenging met gewasplante reeds uitgevoer is, konsentreer dit hoofsaaklik op kompetisie en is daar min bekend oor satansbos se potensiële chemiese inmenging (allelopatie) met gewasse. In hierdie studie is die allelopatiese potensiaal van satansbos loof geëvalueer deur middel van ontkiemingsbiotoetse. Voorlopige eksperimentering was nodig om die korrekte kombinasie van prosedures vir die ontkiemingsbiotoets te vind, aangesien daar uiteenlopende benaderings in die literatuur voorkom. Wateraftreksels en kru wateroplosbare ekstrakte van satansbos loof het ontkieming en vroeë wortelgroei van katoen en slaai, onderskeidelik, betekenisvol geïnhibeer. Die aftreksels en ekstrakte wat in die biotoetse gebruik is se osmolaliteite was nie inhiberend ten opsigte van ontkieming of wortelgroei van katoen of slaai nie. Voorlopige ondersoeke na die chemiese aard van die fitotoksiese verbindings, het aangedui dat meer as een chemiese verbinding betrokke is. Dit dui moontlik op die teenwoordigheid van 'n sinergistiese effek in die kru ekstrak. Die verbindings is voorlopig geïdentifiseer as 'n alkaloïed, 'n saponien en verskeie flavonoïed-bevattende fraksies. 'n Anatomiese ondersoek is onderneem om 'n moonlike verwantskap tussen spesifieke strukture of selle in satansbosblare, en die allelopatiese potensiaal van die loof te vind. Die kliertrigome, gevind op beide die adaksiale

en abaksiale blaaroppervlak, mag moontlik fitotoksiese sekondêre verbindings bevat. Verder is daar gespekuleer dat die stervormige trigome, met basale selle wat die blaarmesofiel binnedring en die vaatbondels bereik, moontlik betrokke mag wees by ekskresie van alkaloïede wat in die vaatbondelskede voorkom. Die resultate van hierdie studie is die eerste stap om die allelopatiese potensiaal van satansbos aan te toon, en lê sodoende 'n grondslag vir toekomstige veldstudies en meer in-diepte chemiese navorsing.

INTRODUCTION

Solanum elaeagnifolium, commonly known as satansbos or silverleaf nightshade, is currently a serious problem weed in many semi-arid regions of the world, including South Africa (Boyd & Murray, 1982; Boyd *et al.*, 1984; Wasserman *et al.*, 1988). It is regarded as one of the most troublesome perennial weeds in cotton (Green *et al.*, 1987; Cilliers, 1999). But how exactly does this weed interfere with crop species? Several studies have already been carried out on the competition aspect (Green *et al.*, 1988) and interference as a whole (Green *et al.*, 1987; Smith *et al.*, 1990), but little attention has been paid to the chemical aspect of interference, termed allelopathy.

Phytochemical studies on silverleaf nightshade foliage and fruit have revealed the presence of alkaloids (Guerreiro *et al.*, 1971), sapogenins and flavonoids (Chiale *et al.*, 1991), which are well known chemical groups implicated in allelopathic effects (Rice, 1984; Putnam, 1985). There have been reports of phytotoxic activity of unidentified saponins from silverleaf nightshade fruit (Curvetto *et al.*, 1976; Agüero & Boland, 1985), however no literature could be located on the phytotoxic or allelopathic potential of silverleaf nightshade foliage.

Silverleaf nightshade has already been described botanically by several authors (Wasserman *et al.*, 1988), and limited anatomical work on the leaves has been reported (Cosa *et al.*, 1998; Dottori *et al.*, 1998; Bruno *et al.*, 1999). An anatomical description of silverleaf nightshade leaves might assist in understanding the potential allelopathic interference originating from the foliage of this weed with crop species.

Wasserman *et al.* (1988) identified inadequate knowledge of the weed silverleaf nightshade itself as one of the reasons why eradication campaigns against this weed failed. Priorities in actions against the silverleaf nightshade problem, according to these authors, should therefore include in-depth ecological and physiological studies of the plant and its seed.

This study was therefore aimed at contributing to existing knowledge of silverleaf nightshade by determining the existence of any possible allelopathic influence that silverleaf nightshade foliage might exert on crop species. Specific objectives were: (a) to evaluate the phytotoxic activity of water infusions and water-soluble extracts of silverleaf nightshade foliage towards crop species, (b) to obtain some preliminary information on the chemical nature of the fraction(s) or compound(s) responsible for phytotoxic activity, and (c) to examine silverleaf nightshade foliage by light transmitting and scanning electron microscopy (LTM and SEM), in order to describe the leaf anatomically and identify possible cells or structures that might harbour allelopathic compounds.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Solanum elaeagnifolium Cav., commonly known as silverleaf nightshade, is native to the Americas (Boyd *et al.*, 1984), but has spread to many other semi-arid regions over the world. It has become a major problem weed in Australia, Argentina, Greece, India, Morocco, North America and South Africa (Heap & Carter, 1999). Silverleaf nightshade is known by many other vernacular names including meloncillo del campo, tomatillo, white horsenettle, bullnettle, silverleaf horsenettle, tomato weed, sand brier, trompillo, meloncillo, revienta caballo, silverleaf nettle, purple nightshade, whiteweed, western horsenettle, desert nightshade, morelle jaune and silverleaf bitter apple (Boyd *et al.*, 1984; Heap & Carter, 1999). It is also a declared weed in South Africa (Conservation of Agricultural Resources Act no. 43, 1983), where it is best known by its Afrikaans name, "satansbos" (Wasserman *et al.*, 1988).

Crops in which silverleaf nightshade have been reported a problem weed, were summarised by Boyd *et al.* (1984). These include alfalfa (*Medicago sativa* L.), cantaloupes (*Cucumis melo* L. var. *cantalupensis* Naud.), cotton (*Gossypium hirsutum* L.), grain sorghum (*Sorghum bicolor* (L.) Moench), peanuts (*Arachis hypogaea* L.), ragi (*Eleusine coracana* Gaertn.), rice (*Oryza sativa* L.), watermelons (*Citrullus lanatus* (Thunb.) Mansf.) and wheat (*Triticum aestivum* L.).

Research on silverleaf nightshade seems to have focused on chemical and biological control. Few studies have been dedicated to understanding ways in which this weed interferes with crop species. Interference comprises both competition and allelopathic interaction between plants (Putnam, 1985). Interference studies concerning

silverleaf nightshade, however, have primarily explored the competition aspect.

This literature survey will provide an overview of the research that has already been done on interference of silverleaf nightshade with crop species, in order to determine voids in current knowledge. The weed will also be described morphologically and anatomically as background for better insight into the mechanisms of interference. Finally, to establish how effective the battle against silverleaf nightshade is fought, a brief overview of control strategies employed against this weed species will be presented.

1.2 Morphology and biology

Following is a description of silverleaf nightshade as adapted from Cuthbertson *et al.* (1976), Symon (1981) and Boyd *et al.* (1984):
Silverleaf nightshade is an erect, clonal, herbaceous perennial up to 1 m, but more often 40 to 60 cm high with a silvery green appearance. The extensive underground root system is up to 3 m deep, producing usually annual vegetative growth in spring and summer. It has erect stems, branching towards the top. Acicular prickles or spines that are 2 to 5 mm long, straight, fine and often reddish, are usually present on stems, petioles and midribs of leaves. Older plants are sometimes nearly free of prickles. The 1 to 10 cm long leaves are oblong-lanceolate, distinctly sinuate-undulate and lower leaves are approximately 10 by 4 cm in size. The leaves and stems are covered with a close, dense, tomentum of sessile or shortly multiseriate-stalked, porrect-stellate trichomes with a medium or long central ray. The general aspect of these stellate trichomes is silvery-green, rarely rusty and slightly discolourous. According to Roe (1971) the hairs of *S. elaeagnifolium* are rather intermediate between porrect-stellate and peltate. These trichomes are responsible for the plant's dusky silvery appearance. Flowerbuds emerge singly or in small clusters at or near the tips of branches. The sepals may

also have prickles. The flowers are 2 to 3 cm in diameter and blue to violet of colour, rarely pale blue, white, deep purple, or pinkish colours are encountered. The fruit are smooth globular berries of 10 to 15 mm in diameter, each containing approximately 50 seeds. The average plant carries up to 60 berries, producing about 3 000 seeds per plant. The seeds are 3 by 2 mm in diameter, flat or biconvex, light to mid-brown in colour and smooth.

Silverleaf nightshade's high seed production and the reported 60% germination after 10 years of storage at room temperature, illustrate that seed of silverleaf nightshade are important propagules of this species (Boyd & Murray, 1982). However, despite high viability and longevity of seeds, high numbers of seedlings are observed only occasionally (Heap & Carter, 1999). Vegetative reproduction is extremely effective since all parts of the root system can regenerate if cut off or damaged by cultivation (Cuthbertson *et al.*, 1976). Regeneration from roots can occur from as deep as 50 cm in cultivated soils (Monaghan & Brownlee, 1979), and root fragments as short as 10 mm are able to regenerate (Richardson & McKenzie, 1981). Some root fragments can survive for 15 months with a capacity for regeneration (Fernandez & Brevedan, 1972).

Silverleaf nightshade is adapted to a wide range of habitats, a characteristic that contributes to its weediness in diverse regions around the world (Heap & Carter, 1999). The heaviest infestations occur on sandy soils with low organic matter content (Leys & Cuthbertson, 1977). Cool summers and high annual rainfall are important factors which may limit silverleaf nightshade distribution (Panetta & Mitchell, 1991). The plant appears to be susceptible to water-logging (Heap & Carter, 1999) and frost (aerial parts), and is highly resistant to drought (Wasserman *et al.*, 1988).

Detailed descriptions of phenology, seedling development, and seed morphology have been given by Economidou & Yannitsaros (1975).

1.3 Leaf anatomy

Literature discussing the leaf anatomy of silverleaf nightshade is extremely scarce and difficult to obtain.

The leaves of *S. elaeagnifolium* have been described as isobilateral, with an abundance of glandular and non-glandular trichomes on both sides of the leaf (Cosa *et al.*, 1998). Crystal-bearing idioblasts are encountered inside the mesophyll (Pilar, 1937; Bruno *et al.*, 1999).

According to Bruno *et al.* (1999) glandular trichomes consist of a bicellular stalk with a spherical "uni-pluricellular" head, and non-glandular trichomes can be simple or stellate. Stellate trichomes have two variants: one with a stalk emerging from the epidermis surface, and one with an intrusive base growing into the mesophyll. Epidermal stellate trichomes have been described for several *Solanum* species (Seithe, 1979), while the variant with intrusive base has only been described for *S. elaeagnifolium* (Pilar, 1937; Cosa *et al.*, 1998; Dottori *et al.*, 1998; Bruno *et al.*, 1999). Both variants have 8 to 16 horizontal unicellular radii and a prominent vertical apical cell. Apparently, early stages of development are similar in both variants (Bruno *et al.*, 1999). The intrusive variant's stalk cells start to grow, in general without dividing, causing the stalk to intrude aggressively between mesophyll cells, probably as a result of enzymatic disorganization of the middle lamella. Bruno *et al.* (1999) further states that the stalks cells of mature trichomes reach the vascular bundles, even surrounding them. At adult stage, the cell wall of both variants are uniformly thick, with the emerging part strongly lignified, whereas there is little or no lignification in the intrusive portion of the trichome.

Stellate hairs show the greatest range of topographical variants of the trichome types, especially between upper and lower leaf surfaces

(Seithe, 1979). Upper surface trichomes may have a longer central thorn, and sometimes fewer, shorter rays with shorter stalks.

Thus far, the function of these intrusive stellate trichomes has not been elucidated. Bruno *et al.* (1999) speculated that its morphology might be related to the water economy of the plant, as the cells of the stalk have very thick walls, which seem to penetrate to the point of making contact with the xylem. These authors also considered it likely that it has an important structural function, reducing the risk of collapse when the plant is stepped upon.

1.4 Interference of *S. elaeagnifolium* with crop species

Several studies have been conducted on the interference of silverleaf nightshade with crop species, mostly focusing on cotton (Green *et al.*, 1987; Green *et al.*, 1988; Smith *et al.*, 1990, Jacobsen *et al.*, 1994; Westerman & Murray, 1994). Silverleaf nightshade has also been considered one of the most troublesome perennial weeds in cotton (Green *et al.*, 1987; Cilliers, 1999), with reported lint yield reductions of up to 75% under semi-arid conditions with moderate silverleaf nightshade infestations (Abernathy & Keeling, 1979). Striking cereal yield reductions of 4 to 77% (mean 41%) have also been reported (Heap & Carter, 1999). Cuthbertson *et al.* (1976) reported a 12% wheat yield loss in southern New South Wales at silverleaf nightshade densities of 9 shoots m^{-2}, while greater reductions were recorded in Southern Australia where 3 to 5 shoots m^{-2} reduced wheat yield by up to 60% (Lemerle & Leys, 1991). Hackett & Murray (1982) reported a 47 to 65% yield reduction in peanuts. Interference and yield losses appear to be most severe on sandy soils and in seasons with low rainfall (Heap & Carter, 1999).

Green *et al.* (1987) determined the effect that silverleaf nightshade densities might exert on cotton height, lint yield, boll size and fiber

quality. Silverleaf nightshade plants used in the experiment were grown from seed, transplanted to the field at four- to six-true-leaf stage and left to grow for six weeks until a height of approximately 30 cm was reached. All silverleaf nightshade plants were then clipped near the soil surface immediately following cotton planting. Experiments were conducted under dryland and irrigation conditions. Cotton height was significantly reduced at silverleaf nightshade densities as low as four plants per 10 m of cotton row. At the highest weed density of 32 plants per 10 m of row, cotton height was reduced by 25% compared to plants without weed interference. Lint yield decreased as weed density increased, with dryland cotton showing significant decreases at lower weed densities than irrigated cotton. Therefore, it was concluded that irrigated cotton competed more effectively with the weed than dryland cotton, suggesting that soil water was a primary competition factor between silverleaf nightshade and cotton. Linear regression predicted a 1.54% lint yield reduction for each silverleaf nightshade plant per 10 m of cotton row. Increasing silverleaf nightshade density, starting at densities as low as two weeds per 10 m of row, progressively reduced boll size. Fiber properties were not affected.

Smith *et al.* (1990) continued research on the silverleaf nightshade plantings established by Green *et al.* (1987), to assess the influence of more mature silverleaf nightshade stands on cotton yield. The number of stems of one silverleaf nightshade plant per 10 m of cotton row, increased 10 fold after one year and up to 40 fold after two years of uncontrolled growth. As could be expected, yield reductions increased with increasing silverleaf nightshade stand maturity. Linear regression predicted a 9% yield loss for each additional 1 kg dry weight of silverleaf nightshade per 10 m of row for one-year-old stands. From two-year-old stands, a 21% yield loss was predicted for each additional 1 kg dry weight of silverleaf nightshade per 10 m of row. The competitive advantage of irrigated cotton over dryland cotton mentioned in initial experiments (Green *et al.*, 1987), was no longer evident.

Water is one of the primary growth factors that plants compete for. Competition between two plants usually begins when their root systems overlap in their exploration for water and nutrients, and is intensified under dryland and semi-arid conditions (Pavlychenko & Harrington, 1935). The competitive ability of weeds can be influenced by soil moisture conditions and, as mentioned above, young silverleaf nightshade plants apparently compete more effectively with dryland than with irrigated cotton (Green *et al.*, 1987). The ability of plants to extract soil moisture is partly dependent on their root distribution in the soil profile (Davis *et al.*, 1965). Silverleaf nightshade roots have been reported at depths of up to 3 m or more (Wasserman *et al.*, 1988), while the rooting depth of cotton seldom exceeds 1.5 m, with the upper 1 m of the soil profile being the principle soil moisture extraction region (Green *et al.*, 1988). Therefore, silverleaf nightshade has the potential to extract moisture from much greater depths in the soil profile than cotton.

Green *et al.* (1988) found that less water was available lower in the soil profile when silverleaf nightshade was grown with cotton. The largest differences in soil moisture depletion between cotton with and without silverleaf nightshade occurred during the flowering and early fruiting stages, a critical time for water demand and utilisation by cotton. Soil water was depleted more rapidly in plots with silverleaf nightshade during this period, except in irrigated, high rainfall conditions. Jacobson *et al.* (1994) confirmed that silverleaf nightshade began extracting soil water earlier in the growing season. In a wet year, soil water loss did not differ between cotton plots with and without silverleaf nightshade (Green *et al.*, 1988). However, even under these conditions cotton lint yield was reduced by 30% at the highest weed density of 32 plants per 10 m of cotton row. It was concluded that competition for available soil moisture is an important factor in silverleaf nightshade interference with cotton. However, there are other important factors that are not accounted for in current research.

1.5 Secondary metabolites of silverleaf nightshade

The Solanaceae contain many groups of secondary metabolites including flavonoids (Harborne & Swain, 1979), a number of triterpenes, tropane alkaloids, steroidal alkaloids, saponins and nicotine types (Seigler, 1981).

Phytochemical studies on silverleaf nightshade have revealed several interesting secondary metabolites produced by this species. The main publications have dealt with alkaloids and sapogenins (Maiti & Mathew, 1967; Guerreiro *et al.*, 1971; Kavka *et al.*, 1973; Hanna *et al.*, 1996b), while the presence of flavonoids (Chiale *et al.*, 1991) and several phytosterol components (Hanna *et al.*, 1996a) have also been reported.

The most publicised secondary metabolite of silverleaf nightshade is certainly the steroidal glycoalkaloid, solasodine, which is used to produce diosgenin, an important steroidal sapogenin in the manufacture of pharmaceutical corticosteroidal drugs (Heap & Carter, 1999). Maiti and Mathew (1967) reported yields of 3.2% of solasodine from berries (on a dry weight basis) in India, while lower amounts of 1.5% to 1.85% for green and ripe berries respectively, were reported in Egypt by Hanna *et al.* (1996b). Bradley *et al.* (1978) reported that green fruit of field-collected Australian silverleaf nightshade plants contained 1.6% and ripe fruit 1.7% solasodine, while no solasodine was present in leaves and stems. The saponin, diosgenin, and 3-deoxy-Δ^3-diosgenin were obtained as final products by processing *Solanum elaeagnifolium* foliage (leaves and twigs) collected during flowering, as well as from samples collected after the fruit had ripened (Guerreiro *et al.*, 1971). The percentage of both compounds in the first sample doubled the amount shown in the second sample. Unripe and seasoned fruit yielded both mentioned compounds as well as solasodine, and the alkaloid percentage in unripe fruit doubled the amount obtained from seasoned fruit.

Studies also revealed the presence of other steroidal glycoalkaloids, including solamargine, found in the unripe fruits of silverleaf nightshade (Guerreiro et al, 1973), solasurine, and solanelagnin, isolated from the stalks of this weed (Hanna *et al.*, 1996b). The presence of these compounds renders the plant even more valuable as a potential source of raw material for the steroid industry (Hanna *et al.*, 1996b).

There has been considerable research and interest in the domestication of silverleaf nightshade for commercial production of steroidal drugs in India and Argentina (Kanna & Singh, 1987; Heap & Carter, 1999). Maiti & Mathew (1967) considered silverleaf nightshade to be the most promising source of solasodine of 28 *Solanum* species studied, due to its high yield and few thorns. Research has even explored breeding qualities, as well as multiplication techniques and cultivation potential for this species (Khanna & Singh, 1987; Trione & Cony, 1988).

Chiale *et al.* (1991) first reported flavonoids from this species. These authors isolated the flavonoid kaempferol, and kaempferol 3-glucoside, as well as a new monoacylated flavonoid glucoside characterized as kaempferol 3β-D-(6″-*O*-*cis*-cinnamoylglucoside), all from the aerial parts of silverleaf nightshade.

Hanna *et al.* (1996a) found that the sterol content of the seeds and stems of *S. elaeagnifolium* composed mainly from squalene, campasterol, stigmasterol and β-sitosterol. A little amount of cholesterol (5.16%) was present only in the stems.

1.6 Biological activity of silverleaf nightshade

1.6.1 Antimicrobial activity

Hanna *et al.* (1996a) examined the antimicrobial activity of ethanolic extracts of *S. elaeagnifolium* seeds and stems. The test

organisms included gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, gram-negative bacteria, *Escherichia coli*, one species of pathogenic yeast, *Candida albicans*, and the pathogenic fungus *Aspergilus niger*. The seed extract exhibited very high activity against *E. coli*, while it inhibited *S. aeraus* moderately. The stem extract was highly active against both *E.coli* and *S. aureus*. Both seed and stem extracts showed moderate inhibition against *B. subtilis*, while no inhibition of *C. albicans* or *A. niger* was obtained by either extract.

1.6.2 Phytotoxic activity

The steroidal saponins, glycoside derivatives of steroids, of silverleaf nightshade have been reported to be involved in chemical interactions with other plant species.

The only report on allelopathic effects of silverleaf nightshade on other plant species was by Curvetto *et al.* (1976). They reported that an aqueous solution of the saponins extracted from silverleaf nightshade fruits, gradually reduced cucumber (*Cucumis sativa* L.) growth. They further discovered that fruit from which the pericarp had been removed, and were placed in petri dishes with soil, interfered with germination and seedling development of several crop and weed species. These saponins were not chemically identified.

Agüera & Boland (1985) obtained evidence suggesting that unidentified saponins from *S. elaeagnifolium* fruit extracts act directly on clover root membranes, altering their properties. After treatment with the saponin extract from silverleaf nightshade berries, Ca^{2+}-ATPase activity and ATP-dependent calcium uptake was inhibited *in vitro* in clover root fragments. During *in vivo* experiments pretreatment of clover seedlings with the steroidal saponin extracts, at concentrations equivalent to 15 µM diosgenin,

stimulated calcium uptake without significantly altering potassium and phosphate uptake. However, concentrations 10 times lower resulted in a selective inhibition of calcium transport.

1.7 Overview of control strategies

Over the decades a wide array of herbicides have been screened for efficacy in controlling silverleaf nightshade. The possibility of chemical control of silverleaf nightshade was investigated in the USA as early as the late thirties, while the first documented trials in South Africa started in 1952 (Wasserman *et al.*, 1988). Although instances of success have been recorded, there are few weeds which have withstood the onslaught of chemical research as effectively as silverleaf nightshade (Heap & Carter, 1999). Herbicides have been effective in killing shoots, however regeneration from roots occur during the following year (Lemerle & Leys, 1991). There is still no registered herbicide for control of silverleaf nightshade.

Because of the extensive root system, plants recover from conventional slashing and cultivation methods for weed control, and cultivation methods might even aid in spreading the weed (Cuthbertson *et al.*, 1976). The inability of cultural or chemical methods to control silverleaf nightshade has made it a major candidate for biological control in many countries including the USA, South Africa and Australia (Heap & Carter, 1999). Many research efforts have been dedicated to identifying and evaluating natural enemies of silverleaf nightshade as possible biocontrol agents.

Neither mechanical, chemical nor biological control methods have achieved wide-spread success in controlling silverleaf nightshade. Wasserman *et al.* (1988) identified the major reason for failed silverleaf nightshade eradication campaigns, to be inadequate knowledge of the

weed itself. Priorities in actions against the silverleaf nightshade problem according to these authors, include:

- Country-wide surveys on the present incidence of silverleaf nightshade and the views of farmers in infested areas.
- Registration of suitable herbicides.
- Efficient dissemination of existing knowledge on the control of silverleaf nightshade among producers.
- In-depth ecological and physiological studies of the plant and its seed.
- Intensification of efforts aimed at the biological control of this weed.

Currently integrated programmes of various control methods are recommended to limit the spread of this weed (Heap & Carter, 1999).

CHAPTER 2
EVALUATION OF THE BIOASSAY TECHNIQUE

2.1 Introduction

Preliminary bioassays to assess allelopathic potential are often performed by evaluating seed germination with leachates or extracts of allelopathic plants (Rice, 1984; Inderjit & Keating, 1999). Much criticism has been directed at this approach, as it does not simulate field conditions and the mere presence of allelopathic compounds in plant parts does not demonstrate allelopathy (Heisey, 1990). Inderjit & Keating (1999) also pointed out that there should not be sole reliance on seed germination as an indicator of allelopathic potential, as low inhibition during early seedling stages might have significant long-term impacts on the test species. Other arguments often brought against laboratory bioassays include the use of organic solvents and grinding of plant material for extraction, the use of artificially sensitive species (e.g. lettuce), exclusion of species naturally associated with the allelopathic plant, the use of only one test species, and lack of detail in control treatments (Inderjit & Keating, 1999).

Although laboratory bioassays have certain limitations, they are an integral part of allelopathic research (Leather & Einhellig, 1986). It is impossible to exactly simulate conditions in the field, but steps should be taken to avoid widening the gap between laboratory bioassays and field interactions (Inderjit & Keating, 1999). Properly executed bioassays for evaluating bioactivity of allelochemicals by its effect on germination and seedling growth are considered to be accepted parameters for indirect measurement of other physiological processes affected by chemical interaction (Macías *et al.*, 2000). In this way, a wide range of effects are covered, and such bioassays serve to select compounds that can be evaluated further in greenhouse and field studies.

As there are many different approaches in literature to execute germination bioassays in allelopathy studies, experimenting was necessary to decide which combination would prove to be adequately accurate and rapid for assessing allelopathic potential of silverleaf nightshade foliar extracts. This chapter will be presented in the form of discussions of relevant sections of the allelopathy bioassays employed in following chapters. Some shortcomings in experiments and obstacles encountered during the course of these trials will also be presented, as it might be of value for future students in the field of allelopathy. It was not deemed necessary to present statistical experimental results, as these are included in following chapters, but rather to discuss preliminary experiments that led to the selection of final bioassay procedures.

2.2 Plant material

Plant material used in all experiments included leaves and twigs from young plants collected at the onset of flowering. The highest content of inhibitors is usually present in the leaves of a plant (Roshchina & Roshchina, 1993). It has also been observed that certain phytotoxic constituents of silverleaf nightshade foliage are present at much higher concentrations in young leaves collected during flowering, than in older leaves picked when the fruit has already ripened (Guerreiro *et al.*, 1971). Therefore, the plant material used in these studies was probably very rich in potential inhibitors. However, older foliar material collected after fruit has ripened, still contained enough inhibitors to inhibit cotton growth at 1% dry material per kilogram of soil (Mkula, unpublished).

2.3 Extraction procedures

In allelopathy studies the use of organic solvents and grinding of plant material for extraction, have received much criticism. Therefore, silverleaf nightshade extracts for initial experiments were prepared by soaking frozen, intact, juvenile foliage in distilled water for 24 hours. However, the fact that plant material was frozen prior to soaking, most

likely caused disruption of cell membrane integrity leading to larger quantities of chemical constituents leaching out, than would have been the case with fresh material. Dilutions of the dark brown infusion were used in experiments and distilled water was included as a control treatment.

In later trials, organic solvents were used for extracting, dissolving and fractionating chemical constituents of silverleaf nightshade. In control treatments the specific solvent was always incorporated in equal amounts and left to evaporate as in the other treatments, to assess whether it might be residues from the solvent itself, rather than the extract, causing germination or growth inhibition. After evaporation, the organic solvents ethyl acetate, methanol and acetic acid did not show any negative effects towards germination or growth of the test species. Residues of butanol used during preparative paper chromatography, however, inhibited seedling growth to such an extent that experiments needed to be repeated with other solvents.

2.4 Choice of test species

Concerns regarding the test species in bioassays include the use of sensitive species (e.g. lettuce) and exclusion of species naturally associated with the allelopathic plant (Inderjit & Keating, 1999). Despite all the criticism against the use of lettuce as a test species, it was chosen by Macías *et al.* (2000) as the most desirable target species for allelopathic bioassays from several dicots and monocots evaluated. This was as a result of its fast germination, and the fact that lettuce showed the highest level of homogeneous growth, improving reproducibility of the bioassay and increases the statistical level of acceptability. Blum (1999) also regarded the use of sensitive species (e.g. lettuce) appropriate, provided the actual field species are also included in trials for comparison.

For initial bioassays in this study cotton (*Gossypium hirsutum* L.), cv. Delta Opal, was chosen as the test species, as silverleaf nightshade is a serious problem weed in this crop (Abernathy & Keeling, 1979; Green *et al.*, 1987; Cilliers, 1999). Therefore, the choice of cotton as test species renders bioassay results more applicable to the field situation. However, as cotton germination and growth proved exceptionally variable causing statistical difficulties, it was decided to continue bioassays with lettuce (*Lactuca sativa* L.) cv. Great Lakes. In addition to the advantages mentioned by Macías *et al.* (2000), the small lettuce seeds also permitted a larger number of seeds to be included in each treatment, further increasing the statistical level of acceptability. Furthermore, less test solution was needed in each petri dish, which proved especially valuable when only small volumes of test solutions were available as in the case of fractions obtained from column chromatography.

2.5 Germination method

In assessing germination potential of crop cultivars, paper germination rolls, also known as "rag dolls", are mostly used. It was decided to compare this germination method with the use of petri dishes lined with filter paper, as a technique for assessing allelopathic potential of plant extracts.

The standard technique was applied by wetting four layers of germination paper with 100 ml of test solution. The top layer was removed, 50 seeds of the test species (cotton cv. Delta Opal) were arranged in the same direction, after which the fourth paper layer was added on top. The paper was rolled up carefully and lightly tied with an elastic band, after which it was placed in a plastic bag that was lightly tied at the end so as to still allow gas exchange. The paper rolls were placed upright (with the radicle end of the seed facing downwards) in a growth chamber at 25°C for four days. Each treatment was replicated four times, totaling 200 seeds that had to be measured for each treatment.

In the case of petri dish bioassays, 9 cm petri dishes were lined with one layer of 9 cm Whatman no. 1 filter paper. Five cotton seeds and 5 ml of test solution were added to each petri dish. Petri dishes were placed in a growth chamber at 25°C for four days in the dark. Each treatment was replicated five times, totaling 25 seeds that had to be measured for every treatment. Because of the variable germination and growth of cotton, the seeds in each replication were increased to 10 (two petri dishes each containing five seeds), therefore totaling 50 seeds per treatment.

Bioassays in petri dishes resulted in significant and much more pronounced differences between treatments, whereas only trends and no significant differences were observed in the paper rolls for the cotton cultivar Delta Opal. Apparently other cotton cultivars do show significant differences when using paper rolls, however, still not as distinct as in petri dishes (Mkula, unpublished). This inconsistency of results between the two methods could probably, at least partly be explained by moisture differences. In the paper rolls all the moisture was absorbed by the four layers of paper, while there was a considerable amount of free solution or water in petri dishes with only one layer of filter paper. To test this theory, petri dishes were lined with two layers of filter paper and wetted with the same amount of aqueous test solution, resulting in much less free solution. As expected, the differences between treatments were no longer as distinct as in petri dishes with one layer of filter paper. Therefore, more pronounced inhibition occurred in a wet environment where free test solution was available, as opposed to a moist environment with the same amount of solution added but absorbed by the paper.

It was decided to employ the petri dish germination method in all following experiments, as it showed differences more distinctly, required less test solution, were easier to evaluate (less seedlings to measure per treatment) and allowed successful germination of seeds requiring light for germination, e.g. lettuce.

2.6 Sterilisation procedures

When using cotton as a test species, fungal contamination did not seem to be a significant problem, as commercial seed with a fungicidic seed dressing was used. However, when using lettuce seeds in the bioassays, fungal contamination occurred to such an extent that it was nearly impossible to measure germination and growth. Hence it was decided to introduce measures to exclude microbe contamination that might cloud results.

Only pre-sterilised petri dishes were used in experiments. Filter paper, sealed and wrapped in aluminium foil, and distilled water was autoclaved at 121°C for 30 minutes for sterilisation. All experiments were conducted in a laminar flow cabinet, where surfaces and instruments were sterilised with 70% ethanol and flaming. Lettuce seeds were surface-sterilised with a 10% solution of commercial bleach (0.35% of available sodium hypochloride) for five minutes, then rinsed three times with sterile distilled water and air-dried inside the cabinet. Lettuce seeds were added to each petri dish lined with sterilised filter paper. All test solutions were passed through Whatman Puradisc polyethersulfone membrane filters with 0.2 μm pore size. This method is ideal to remove fungal spores and even vegetative bacterial cells from solutions that could be heat sensitive and therefore cannot be autoclaved (Cloete, 1994). After four days in a growth chamber at 22ºC, petri dishes were still free from visible contamination, therefore these sterilisation procedures proved adequate for eliminating pathogens in bioassays.

2.7 Osmotic interference

Few researchers take the osmotic potential of test solutions into account when reporting on allelopathic potential of plant species. Extreme osmotic potentials of test solutions in bioassays inhibit germination and growth of many plant species (Haugland & Brandsaeter, 1996). Therefore it is important to determined osmotic potentials of extracts to

be tested for allelopathic effect and to ensure that it is indeed chemical compounds in the test solution, rather than a high osmotic potential, causing germination inhibition, or decreased growth of the test species.

An osmotic range was prepared by dissolving polyethylene glycol (PEG-6000) in distilled water. In preliminary experiments the concentrations of PEG that would give a proper osmotic range for these studies were determined. Subsequently it was decided to use 12.5, 25, 50 and 75 g of PEG-6000 per liter of water. Osmolality of the PEG solutions were measured using a Roebling digital micro-osmometer measuring freezing point depression. The readout is displayed in mOsm kg^{-1}, which can be diverted to pressure units using the Van't Hoff equation. This instrument was chosen as it uses small sample volumes (100 µl) and produces accurate and extremely reproducible readings (±0.5% with 100 µl samples). It was decided not to test osmolalities higher than the 75 g l^{-1} PEG solution (54 mOsm kg^{-1}), as no osmolalities of extracts used in the allelopathy experiments exceeded this. These osmotic solutions were evaluated on both cotton and lettuce to determine whether results obtained from allelopathy bioassays could be considered reliable (Experiment presented in Chapter 3).

2.8 Concluding remarks

Laboratory germination bioassays can be valuable for close examination of carefully isolated components of the complex natural system. Hence it is considered a useful tool in assessing allelopathic potential of plant species, provided that bioassays are planned and conducted properly. In the current study it was attempted to execute all allelopathy bioassays in such a way as to ensure reliable results. Although there might still exist some deficiencies in the technique, there is confidence that bioassays were sufficiently accurate to make dependable conclusions on the allelopathic potential of silverleaf nightshade.

CHAPTER 3
ALLELOPATHIC POTENTIAL OF SILVERLEAF NIGHTSHADE FOLIAR INFUSIONS AND CRUDE EXTRACTS

3.1 Introduction

Interference studies on silverleaf nightshade have thus far focused on the competition aspect of interference (Green *et al.*, 1987; Green *et al.*, 1988; Smith *et al.*, 1990), while research exploring potential allelopathic interference of this weed with crop species, is extremely limited.

Phytochemical studies of silverleaf nightshade have revealed the presence of alkaloids (Guerreiro *et al.*, 1971), sapogenins and flavonoids (Chiale *et al.*, 1991) in foliage and fruit of this species. These types of secondary metabolites have all been implicated in allelopathic interactions (Rice, 1984; Putnam, 1985). The steroidal saponins of silverleaf nightshade have reportedly been involved in chemical interactions with other plant species. Cucumber (*Cucumis sativa* L.) growth was gradually reduced by the saponins extracted from silverleaf nightshade fruits, while the fruit pericarp incorporated into soil, interfered with germination and seedling development of several crop and weed species (Curvetto *et al.*, 1976). Agüera & Boland (1985) obtained evidence suggesting that saponins from *S. elaeagnifolium* fruit extracts act directly on clover root membranes, altering their properties and interfering with calcium uptake. However, no published research exploring allelopathic potential of the foliage of silverleaf nightshade could be located.

The objective of this study was to investigate the possibility that crude foliar extracts of silverleaf nightshade interferes in an allelopathic manner with seed germination and early seedling growth of crop species. This was evaluated by means of germination bioassays, aiming at minimising interfering factors, e.g. osmotic concentration of extracts and pathogenic

organisms. The effect of these foliar extracts on the roots of the test species was also examined microscopically.

3.2 Materials and methods

Plant material:

For all experiments, foliage (leaves and twigs) of flowering silverleaf nightshade plants was collected on arable or disturbed land on the Hatfield experimental farm of the University of Pretoria as well as near Settlers in the Northern Province.

Crude extract experiments:

After collection, foliage was frozen as soon as possible. Infusions for bioassays were prepared by soaking the frozen plant material in distilled water for 24 hours (100 g of fresh weight per 1000 cm^3 of water). The infusion was drained through a sieve to remove plant material and then through Whatman no. 1 filter paper to remove the abundance of stellate trichomes washed from the foliage. The full strength infusion was then diluted with distilled water to give 25, 50, 75 and 100% strength infusions. Osmolalities of the solutions were measured with a Roebling digital micro-osmometer measuring freezing point depression.

Cotton was chosen as the test species for initial experiments. Five fungicide-coated seeds of the cultivar Delta Opal were placed per 90 mm petri dish, lined with one layer of 90 mm Whatman no. 1 filter paper. To this, 5 ml of test solution was added, and distilled water was used as a control. Treatments were replicated five times and each replication consisted of two petri dishes (10 seeds). Petri dishes were placed in growing chambers and left to germinate at 25°C in the dark. After four days the number of germinated seeds for each treatment was counted and the root and shoot lengths of seedlings were measured to the nearest millimeter. Seeds with a radicle length of 2 mm or greater were considered successfully germinated, and only the root and shoot lengths of germinated seeds were taken into account in statistical analyses.

Chemical extraction:

Foliar material of silverleaf nightshade was left to dry in the dark at room temperature for three weeks. The dried plant material was homogenized in ethyl acetate using an anti-explosion electrical blender. The water-soluble fraction was removed from the ethyl acetate extract by partitioning in a separating funnel. The original water-soluble fraction was diluted with distilled water to give a concentration range with osmolalities of 5, 10, 20, 30, 35, 44 mOsm kg^{-1}, measured with a Roebling digital micro-osmometer.

In initial experiments it proved necessary to work under sterile conditions. Lettuce cv. Great Lakes seeds were surface-sterilised in a 10% dilution of commercial bleach (0.35% available sodium hypochlorite) for five minutes, followed by three rinses with sterilised distilled water. Sterile 90 mm petri dishes were lined with a single layer of 90 mm Whatman no. 1 filter paper, previously sterilised by autoclaving at 121°C for 30 minutes. To each petri dish 20 lettuce seeds and 2.5 ml of extract were added. The extract was sterilised by passing it through a millipore filter (Whatman Puradisc 25AS 0.2 μm pore size), after stellate trichomes were removed by filtering the extract through Whatman no. 1 filter paper. Sterilised distilled water was used as a control. Each treatment was replicated five times. Petri dishes were sealed with Parafilm® to prevent unnecessary loss of moisture and put in growth chambers to germinate at 22°C in light. The number of germinated seeds was counted at 24-hour intervals, and after three days root lengths of germinated seedlings were measured and the experiment terminated. Seeds with a radicle length of 2 mm or greater were considered successfully germinated, and only root lengths of germinated seeds were taken into account for statistical analyses. All bioassay experiments were performed inside a laminar flow cabinet.

Osmotic inhibition:

In order to exclude the possibility of osmotic inhibition in both experiments, solutions of polyethylene glycol (PEG-6000) were prepared with distilled water to give concentrations of 1.25, 2.5 and 7.5% PEG (on a weight basis). Osmolalities of these solutions were measured with a Roebling digital micro-osmometer and the PEG solutions were applied to cotton and lettuce seeds and evaluated in the same manner as the plant infusions and extracts.

Microscopic evaluation of roots exposed to extracts:

The root tips of three-day-old lettuce seedlings from the control and 20 mOsm kg^{-1} foliar extract treatments were excised and fixed in 2.5% glutaraldehyde in a 0.1M $NaPO_4$ buffer (pH 7.4) for two hours. Glutaraldehyde fixation was followed by three rinses (10 minutes each) in the same buffer. Dehydration was done in a series of ethanol:water dilutions comprising of 30, 50, 70, 90 and 100% ethanol for 10 minutes each. After dehydration the material was infiltrated and embedded in LR White medium grade resin. Longitudinal sections of 0.5 to 1.0 µm were made with glass knives using a Reichert Ultracut E ultramicrotome and stained for 20 seconds at 60°C in 0.5% Toluidine blue O dissolved in 0.5% Na_2CO_3 in distilled water (Van der Merwe, 2000). The sections were mounted in immersion oil and viewed with a Nikon Optiphot light microscope using transmitted light. Images were recorded digitally using a Nikon DXM 1200 digital camera.

Statistical analysis:

Analysis of variance (ANOVA) was done using the statistical program GenStat (2000). A completely randomised design was used in all experiments. Analysis of variance was used to test for differences between treatments. Root length data for lettuce exposed to the extract was subjected to rank transformation, otherwise the shoot and root data was acceptably normal with homogeneous treatment variances. In the case of germination percentages, angular transformation was used to stabilise variances. Treatment means were separated using Tukey's

studentised range test least significant difference (LSD) at the 5 % level of significance.

3.3 Results and discussion

Phytotoxicity of crude extracts on cotton:

Cotton germination, root length and shoot length were inhibited significantly, compared to the control treatment, with increasing extract concentration (Table 3.1). Germination and shoot length were significantly inhibited from an infusion concentration of 23 mOsm kg^{-1}, while significant reduction in root length occurred from 35 mOsm kg^{-1}.

Table 3.1 The effect of increasing concentrations of a crude water extract of silverleaf nightshade foliage on germination, root and shoot lengths of cotton (ANOVA in Tables A1, A2 and A3, Appendix A)

Extract concentration	Osmolality (mOsm kg^{-1})	Germination percentage	Root length (mm)	Shoot length (mm)
0%	0	78 a	50.57 a	39.72 a
25%	12	52 ab	47.17 a	34.71 ab
50%	23	36 b	41.15 a	25.00 bc
75%	35	34 b	25.88 b	23.63 bc
100%	48	40 b	12.18 b	12.56 c

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)

When considering the influence of osmotic concentration of extracts on cotton germination and early seedling growth, PEG experiments showed that no significant inhibition of germination or root growth occurred up to 53 mOsm kg^{-1}, the highest osmolality tested (Table 3.2). However, shoot length seems to have been more sensitive, as significant inhibition already occurred at the lowest osmolality tested (3 mOsm kg^{-1}). As osmolalities of extracts ranged from 12 to 48 mOsm kg^{-1}, only germination and root length results were considered reliable parameters, as osmotic inhibition of shoot growth may have occurred to some extent.

Table 3.2 The effect of PEG-6000 solutions of increasing osmolality on germination, root and shoot lengths of cotton (ANOVA in Tables A4, A5 and A6, Appendix A)

PEG-6000 conc. ($g\ l^{-1}$)	Osmolality ($mOsm\ kg^{-1}$)	Percentage germination	Root length (mm)	Shoot length (mm)
0	0	76 a	30.13 a	15.03 a
12.5	3	54 a	24.77 a	11.72 b
25	8	52 a	26.75 a	11.21 b
50	25	64 a	31.51 a	7.57 c
75	53	54 a	21.43 a	5.23 c

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)

Phytotoxicity of extracts on lettuce:

Increasing concentrations of the water-soluble fraction of the organic foliar extract progressively inhibited lettuce germination as well as seedling root growth (Table 3.3). Root length was significantly inhibited even at the lowest extract concentration (5 $mOsm\ kg^{-1}$) when compared to the untreated control. After three days the percentage germination was significantly lower from the 10 $mOsm\ kg^{-1}$ treatment until virtually no germination occured at 30 $mOsm\ kg^{-1}$.

Table 3.3 The effect of increasing concentrations of a water-soluble foliar extract of silverleaf nightshade on cumulative germination over three days, and mean root length of lettuce cv. Great Lakes (ANOVA in Table A7 and A8, Appendix A)

Concentration ($mOsm\ kg^{-1}$)	Percentage germination			Mean root length (mm)
	24 h	48 h	72 h	
0	97 a	99 a	99 a	13.59 a
5	80 b	91 b	95 ab	8.92 b
10	39 c	76 b	87 b	5.79 c
20	1 d	7 c	20 c	2.98 d
30	0 d	0 d	2 d	N/A

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)

Osmolalities of the extracts tested ranged from 5 to 44 mOsm kg^{-1}. Experiments with PEG solutions of increasing osmolality indicated that no osmotic interference with germination or root growth occurred for the 0 to 53 mOsm kg^{-1} osmotic range tested (Table 3.4). Therefore, results for all extract concentrations used, were not distorted by osmotic effects.

Table 3.4 The effect of PEG-6000 solutions of increasing osmolality on germination and mean root length of lettuce seedlings (ANOVA in Tables A9 and A10, Appendix A)

PEG-6000 conc. ($g\ l^{-1}$)	Osmolality ($mOsm\ kg^{-1}$)	Percentage germination	Root length (mm)
0	0	98 a	15.40 b
12.5	3	97 a	17.40 ab
25	8	100 a	17.20 ab
50	24	98 a	17.74 a
75	53	96 a	18.69 a

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)

Effect of extracts on root histology:

Lettuce seedlings exposed to the foliar extract of silverleaf nightshade exhibited severe swelling of the roots just behind the root tip. In longitudinal sections of these roots the cell division and elongation zones appeared noticeably shorter than in seedlings not exposed to the extract, suggesting inhibition of cell division and elongation (Fig. 3.1). The non-treated roots had a gradual transition from the meristematic zone to differentiated cells. In contrast, the swollen area of treated roots, marked an abrupt transition from meristematic to differentiated cells with root hairs starting to differentiate very close to the root tip. Aliotta *et al.* (1993) also observed this apical shift of root hair differentiation and inhibited cell elongation of the differentiating zone, when investigating radish germination and subsequent root growth in response to treatment with several phenylpropanoids and coumarins.

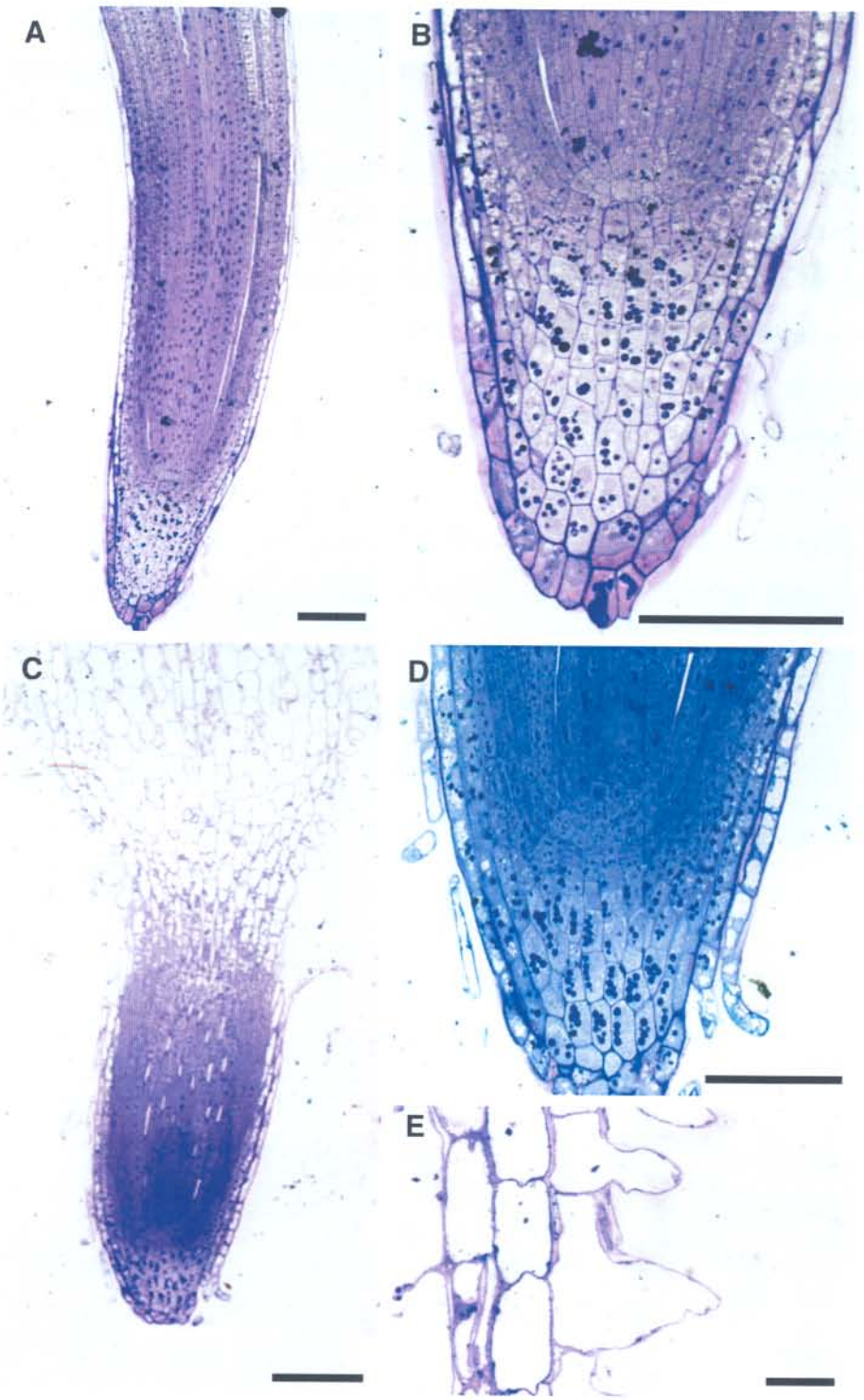

Figure 3.1 Longitudinal sections of the root tips of (A; B) a lettuce seedling not exposed to silverleaf nightshade foliar extract, and (C; D) a seedling exposed to the foliar extract – notice the abrupt transition from the meristematic zone to differentiated cells in the swollen area of the treated root as compared to the gradual transition in the non-treated root; (E) differentiation of root hairs on the swollen area close to the root tip of an extract-treated seedling; Scale bars: A – D = 100μm, E = 10μm

The two germination bioassays illustrated that silverleaf nightshade foliage contains compound(s) that are phytotoxic to cotton and lettuce germination and early seedling growth. Since possible pathogen interference was ruled out and PEG experiments confirmed the absence of osmotic inhibition, the presence of probably water-soluble allelochemicals in the foliage of silverleaf nightshade is indicated. Although the results obtained in this study do not confirm allelopathy, it represents the first step in showing the allelopathic potential of silverleaf nightshade. Pot experiments conducted at the University of Pretoria have already confirmed the phytotoxic activity of silverleaf nightshade foliage on cotton in the soil environment (Mkula, unpublished). Possibilities for future research include evaluation of the sensitivity of other crop species associated with silverleaf nightshade towards these phytotoxins, and validation of bioassay results in field experiments.

CHAPTER 4
CHEMICAL CHARACTERISTICS OF PHYTOTOXIC COMPOUNDS IN SILVERLEAF NIGHTSHADE FOLIAGE

4.1 Introduction

Phytochemical studies on *Solanum elaeagnifolium* have revealed several interesting secondary metabolites produced by this species. The main publications have dealt with alkaloids and sapogenins (Maiti & Mathew, 1967; Guerreiro *et al.*, 1971; Kavka *et al.*, 1973; Hanna *et al.*, 1996b), while the presence of flavonoids (Chiale *et al.*, 1991) and several phytosterol components (Hanna *et al.*, 1996a) have also been reported.

The glycoalkaloid, solasodine, found in the fruit of silverleaf nightshade, is an important steroidal alkaloid in the manufacture of pharmaceutical corticosteroidal drugs (Heap & Carter, 1999). No solasodine is present in leaves and stems of this plant (Bradley *et al.*, 1978). Other glycoalkaloids reported for silverleaf nightshade, include solamargine, found in the unripe fruits (Guerreiro et al, 1973), solasurine and solanelagnin, isolated from the stalks of this weed (Hanna *et al.*, 1996b). The saponin diosgenin, and 3-deoxy-Δ^3-diosgenin were obtained from silverleaf nightshade foliage (leaves and twigs) collected during flowering, as well as from samples collected after the fruit had ripened (Guerreiro *et al.*, 1971). These compounds were also encountered in unripe and ripe fruit of this species. The percentage of both compounds in the younger leaves and fruit doubled the amount revealed in leaves and fruit after fruit ripening (Guerreiro *et al.*, 1971).

Chiale *et al.* (1991) isolated the flavonoid kaempferol and kaempferol 3-glucoside, as well as a new monoacylated flavonoid glucoside characterised as kaempferol 3β-D-(6″-*O*-*cis*-cinnamoylglucoside), all from the aerial parts of silverleaf nightshade.

The types of secondary metabolites reported for silverleaf nightshade have all been implicated in allelopathic interactions (Rice, 1984). The steroidal saponins (glycoside derivatives of steroids) from silverleaf nightshade fruit have reportedly been involved in chemical interactions with other plant species (Curvetto *et al.*, 1976; Agüera & Boland, 1985). No publications could be located on research investigating the possible allelopathic or phytotoxic properties of the silverleaf nightshade foliage.

In Chapter 3 it was illustrated that foliar infusions and crude water-soluble foliar extracts of silverleaf nightshade inhibit germination and seedling root growth of cotton and lettuce. The objective of this investigation was to obtain preliminary information about the chemical nature of the compound(s) responsible for the phytotoxic activity exhibited by foliar extracts of silverleaf nightshade. It has to be emphasised that complete purification and identification of chemical constituents was not intended, as this falls outside the scope of this dissertation.

4.2 Materials and methods

Plant material:

The foliar material of *S. elaeagnifolium* was collected near Settlers in the Northern Province at anthesis, and left to dry in the dark at room temperature for three weeks.

Extraction of active compounds:

Dried plant material was homogenised in ethanol using an anti-explosion electrical blender and left one week for extraction on a stirrer. This process was repeated twice. The total ethanolic extract was combined after filtration and the solvent was evaporated under reduced pressure at 40°C. The total extract obtained was dissolved in 80% ethanol and partitioned with ethyl acetate to yield two fractions, an ethyl acetate fraction (A), and aqueous ethanolic fraction (B).

Isolation and preliminary detection of the nature of active compound(s):
It was decided to continue further fractionation only with the water-soluble fraction (B), as this probably contained the chemical constituents more likely to be available in a natural agricultural situation.

For preparative paper chromatography fraction B, dissolved in methanol, was applied to Whatman 3MMChr chromatography paper and developed in 15% acetic acid. After drying, the chromatogram was inspected under short wave and long wave ultraviolet (UV) light and bands were marked with pencil, cut out and bioassayed.

The most phytotoxic fraction was extracted from the paper and subjected to Sephadex LH-20 column chromatography using methanol as eluent. Phytotoxicity of the obtained fractions was evaluated with a lettuce germination bioassay. The active fractions were collected and subjected to preliminary chemical identification by preparing thin layer chromatography (TLC) plates of the active fractions in various solvents and spraying these with different chemical reagents specific for detecting different types of natural products.

For flavonoids, the following reagents were used: (a) Ammonia solution and (b) 5% $AlCl_3$ solution in methanol. In each case colours were recorded in day light and under UV (short and long wavelength), before and after spraying.

For alkaloids, the following reagents were used: (a) Ninhydrin solution (0.3% solution in BuOH), followed by heating the chromatogram to 100°C, and (b) Dragendorff reagents (Stahl, 1965). With the latter reagents alkaloids appear as stained orange spots on a yellow background. The sensitivity of the detection can be increased appreciably by subsequent spraying with 10% HCl.

For terpenoids vanillin in sulfuric acid was used as reagent.

Preliminary spectral analysis (^{1}H-NMR) of nearly pure fractions was done by the Chemistry Department at the University of Pretoria.

Lettuce germination bioassay:

Paper chromatography : Bands of different components on the dried chromatogram were cut into sections of approximately 32 cm². These sections were placed in sterile petri dishes, 20 lettuce seeds cv. Great Lakes and 2 ml of sterilised distilled water were added to each. A clean piece of identical paper, subjected to the same eluent, was used as a control. The chromatogram could not be sterilised in an autoclave due to the possibility of breakdown of the chemical constituents by exposure to high temperatures. Each treatment was replicated five times. Petri dishes were sealed with parafilm, whereafter they were placed in a growth chamber at 22°C in light for three days. The number of germinated seeds for each treatment was counted daily, and on the third day (72 hours) radicle lengths were measured. A radicle length of 2 mm or greater was considered as successful germination, and only root lengths of successfully germinated seeds were taken into account in statistical analysis.

Sephadex fractions : The solvent of fractions from the Sephadex column was evaporated under reduced pressure at 40°C. Fractions were weighed and dissolved in methanol and diluted in such a way that 1 ml contained 10% of the amount eluted from the column. Of these dilutions 0.8 ml was applied to one disc of 90 mm Whatman no. 1 filter paper, pre-sterilised by autoclaving for 30 minutes at 121°C. Therefore, the fractions were applied in the ratio in which it occurred in the plant. For the control treatment the same volume of pure solvent was applied to the filter paper. The solvent was allowed to evaporate from the treated filter paper. After solvent evaporation, the filter papers were placed in sterile 90 mm petri dishes, 10 lettuce seeds cv. Great Lakes and 2 ml of sterilised distilled water were added to each. Treatments were replicated

three times. Petri dishes were sealed with parafilm®, placed in a growth chamber and evaluated in a similar way as paper chromatography bioassays.

All bioassay experiments were performed inside a laminar flow cabinet.

Statistical analysis:

Analysis of variance (ANOVA) was performed using the statistical program GenStat (2000). A completely randomised design was used in all experiments. Analysis of variance (ANOVA) was used to test for differences between treatments. The root data was acceptably normal with homogeneous treatment variances. In the case of germination percentages, angular transformation was used to stabilise variances. Treatment means were separated using Tukey's studentised range test least significant difference (LSD) at the 5 % level of significance.

4.3 Results and discussion

Bioassays on the paper chromatogram of the water-soluble fraction indicated that the most polar fraction (5) was the most active, with pronounced inhibition of germination and root length of seedlings when compared to the control treatment (Table 4.1). Reddish colours obtained by the Dragendorff spraying reagents suggested that this fraction contained an alkaloid.

Table 4.1 Effect of paper chromatography fractions on germination of lettuce over three days and mean root length of resulting seedlings (ANOVA in Tables A11 and A12, Appendix A)

Fraction	Percentage germination			Root length
	24 h	48 h	72 h	(mm)
Control	69 a	85 a	92 a	11.87 ab
1	66 a	91 a	96 a	12.27 a
2	72 a	92 a	99 a	8.79 c
3	82 a	96 a	98 a	9.20 c
4	86 a	94 a	98 a	10.06 bc
5	0 b	3 b	8 b	1.33 d

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)
Fractions are arranged in order of increasing polarity

Fractions 2, 3 and 4 also inhibited root growth of lettuce seedlings significantly, although germination was not affected. These compounds were probably flavonoidic constituents judging by the colours that were observed under UV light.

Of the fractions obtained from the Sephadex column, fraction 4 significantly inhibited germination of lettuce seeds (Table 4.2). However, no inhibition of root growth occurred. This fraction gave all the characteristics of an alkaloidal compound in both ninhydrin and Dragendorff reagents (Fig. 4.1). The concentration at which this fraction

was applied, however, is approximately five times the recommended concentration as suggested by Rimando *et al.* (2001). Fraction 6 significantly inhibited root growth of lettuce seedlings, although germination was not affected (Table 4.2). Preliminary ^{1}H-NMR analysis confirms the presence of a saponin in this fraction (Fig. 4.2). The concentration of this fraction applied, falls into an accepted range (Rimando *et al.*, 2001).

Table 4.2 Effect of Sephadex fractions on germination of lettuce over three days and mean root length of resulting seedlings (ANOVA in Tables A13 and A14, Appendix A)

Treatment	Concentration (mg/ml)	Percentage germination						Root length (mm)	
		24 h		48 h		72 h			
Control	-	100	a	100	a	100	a	11.03	a
1	2.00	96.7	a	96.7	a	96.7	a	9.19	ab
2	1.40	100	a	100	a	100	a	8.10	ab
4	5.60	10	c	16.7	c	26.7	b	8.07	ab
5	1.00	53.3	b	76.7	b	90	a	8.65	ab
6	0.72	100	a	100	a	100	a	6.43	b

Means in each column followed by different letters are significantly different according to Tukey's studentised range test LSD ($P < 0.05$)

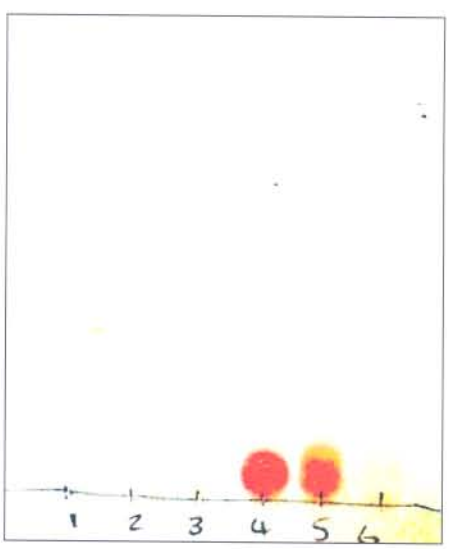

Fig. 4.1 TLC plates sprayed with Dragendorff reagents showing the presence of an alkaloid in fraction 4 from the silverleaf nightshade foliar extract Sephadex column

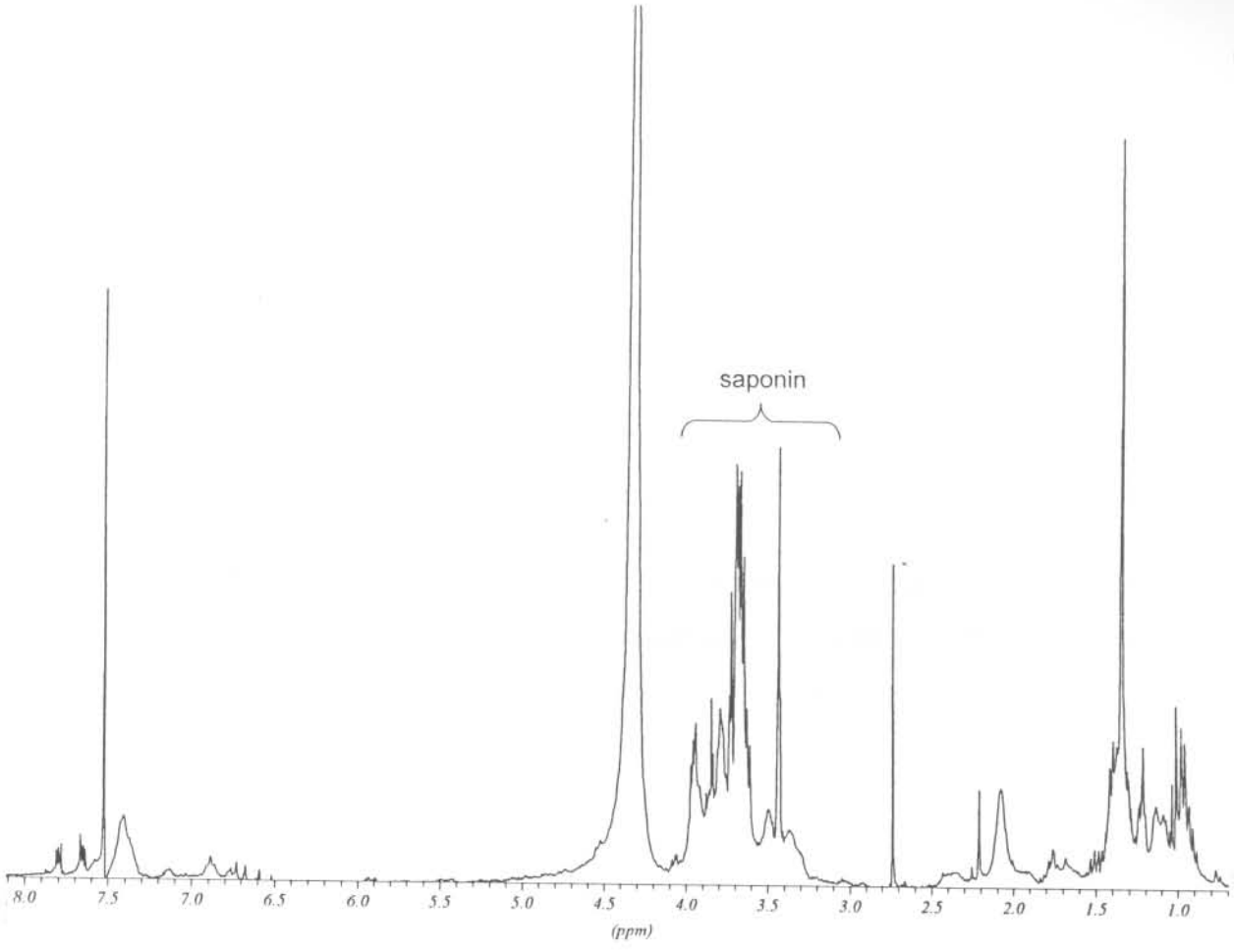

Figure 4.2 Preliminary ^{1}H-NMR spectral analysis of fraction 6 indicating the presence of a relatively pure saponin

The phytotoxic activity observed with the fractions from the Sephadex column, was much less pronounced than activity obtained with the crude water-soluble extract at very low concentrations. Significant phytotoxic activity was observed with at least two of the fractions from the Sephadex column, as well as with possible flavonoidic compounds from the paper chromatogram. This suggests a synergistic effect, where not one specific compound is responsible for inhibition of lettuce germination and growth, but a combination of these various constituents in the crude extract. However, further investigation is necessary to confirm this hypothesis. Furthermore, purification and complete spectral analysis for these compounds are still required for their identification.

CHAPTER 5
POTENTIAL LINK BETWEEN LEAF ANATOMY AND ALLELOPAHTIC POTENTIAL OF SILVERLEAF NIGHTSHADE

5.1 Introduction

Anatomical studies on silverleaf nightshade were first performed by Pilar (1937) and only in the late 1990's the leaf anatomy of this plant was further investigated by Cosa *et al.* (1998), Dottori *et al.* (1998) and Bruno *et al.* (1999). Interesting anatomical features described for the leaves, include two variants of stellate non-glandular trichomes. One variant has a stalk emerging from the epidermis surface, and the other has an intrusive base growing into the mesophyll. Glandular trichomes are plentiful on both leaf surfaces, and there is an abundance of crystals in the leaves.

It was established in Chapters 3 and 4 that silverleaf nightshade foliage may be phytotoxic to crop species and that the allelopathic activity shown by foliar extracts are caused by a synergistic effect of several compounds. The active compounds in the most phytotoxic fraction were characterised as an unidentified alkaloid and a saponin, while less pronounced growth inhibition of the test species also occurred as a result of three flavonoidic fractions. In plants the highest content of phytotoxic compounds is found in leaves and these plant inhibiting substances may be contained in specialised structures (Roshchina & Roshchina, 1993). A number of plants have specific idioblasts that contain tannins, alkaloids or glucosinolates (Wink, 1999). More often, secondary metabolites are concentrated in trichomes or glandular hairs, stinging hairs or the epidermis itself (Wink, 1999). Kanchan & Jayachandra (1980) found that growth inhibitors contained in soft, fine trichomes of *Parthenium hysterophorus* L. resulted in allelopathic growth inhibition of 10-day-old wheat (*Triticum aestivum* L.) seedlings. Also, several sesquiterpene lactones from the glandular trichomes on sunflower leaves (*Helianthus*

annuus L.) cv. VYP® influenced root and shoot growth of lettuce (*Lactuca sativa* L. var. *nigra*), tomato (*Lycopersicon esculentum* Mill.), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) seedlings (Macías *et al.*, 1996). However, it has also been shown that significant relationships between the number of glandular trichomes on leaves and either the release or phytotoxic activity of allelochemicals do not necessarily exist (Nilsson *et al.*, 1998).

In this study the leaf anatomy of silverleaf nightshade was examined in an attempt to relate specific cells or structures to the allelopathic potential exhibited by foliar infusions and extracts. The study was also conducted to confirm and contribute to previous anatomical studies on the leaves of this plant. The focus was on the various trichome types, but other anatomical features of the leaves will also be discussed briefly.

5.2 Materials and methods

Plant material:

Leaves of *Solanum elaeagnifolium* were collected from plants at anthesis on the Hatfield experimental farm of the University of Pretoria. Leaves were selected from four different size classes – 1 cm, 2 cm and 5 cm in length, as well as mature leaves (approximately 7 to 8 cm in length). Plant material was collected early in the morning and kept in water until preservation.

Scanning electron microscopy (SEM):

Sections of 3mm by 3mm, excised from the middle of the lamina between the mid rib and leaf margin, were fixed in 2.5% glutaraldehyde in a 0.1M $NaPO_4$ buffer (pH 7.4) for two hours. Glutaraldehyde fixation was followed by three rinses (10 minutes each) in the same buffer. Post-fixation was done with 1% aqueous OsO_4 for two hours. OsO_4 was removed with three rinses (10 minutes each) of distilled water. Dehydration was done as described for root tips in Chapter 3. This was followed by critical point drying in a Polaron critical point drier. The dried

samples were mounted on aluminium stubs and made conductive by exposing them to vapour from a 0.5% RuO_4 solution (Van der Merwe & Peacock, 1999). Specimens were viewed with a JOEL 840 scanning electron microscope operated at 5 kV and images were recorded digitally.

Light transmitting microscopy (LTM):

Sections of 1mm by 1mm, excised from the middle of the lamina between the mid rib and leaf margin, were fixed in glutaraldehyde, dehydrated and embedded in LR White medium grade resin as for LTM in Chapter 3. Transverse sections were made, stained and images recorded as described for root tips in Chapter 3.

5.3 Results and discussion

General:

The leaves of silverleaf nightshade are isobilateral and amphistomatic, with a uniseriate adaxial and abaxial epidermis covered by a variaty of numerous trichomes (Fig. 5.1). This corroborates findings of Cosa *et al.* (1998).

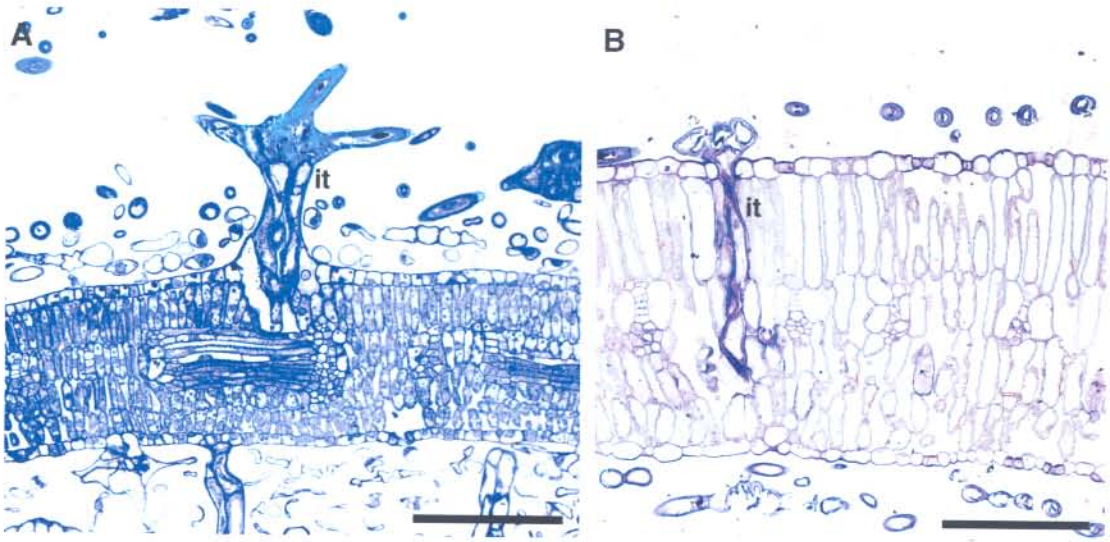

Figure 5.1 Transverse section of a young (A) and mature (B) silverleaf nightshade leaf, showing its isobilateral and amphistomatic nature and intrusive stellate trichomes (it); Scale bars = 100 μm

Crystals:

There is an abundance of crystals contained in idioblasts in the leaves, especially close to the vascular bundles (Fig. 5.2). Bruno *et al.* (1999) also noted the presence of these crystals in the leaves of silverleaf nightshade. The crystalline inclusions observed in silverleaf nightshade leaves are most probably small pyramidal crystals of calcium oxalate monohydrate known as "crystalline sand" (Cody & Horner, 1985). The most frequent crystal mineral salt deposition in idioblasts is calcium oxalate, often present as crystalline sand (Roshchina & Roshchina, 1993). According to Cody & Horner (1985) crystal sand is found in 36 angiosperm families and is most prevalent in the Amaranthaceae, Rubiaceae, and Solanaceae.

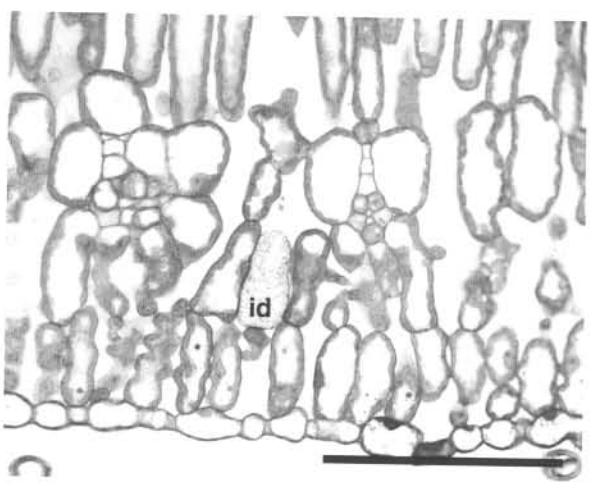

Figure 5.2 Transverse section of a silverleaf nightshade leaf showing crystals, most likely calcium oxalate, contained in an idioblast (id); Scale bar = 100 μm

Trichomes:

Seithe (1979) listed eight recognisable trichome types that occur on mature *Solanum* plants. Of these, it appears that silverleaf nightshade leaves have three types, namely stellate hairs, multicellular glands and multicellular prickles.

- Glandular trichomes:

  Multicellular glands are present on the adaxial and abaxial leaf surfaces, and seem to be more prevalent on younger leaves. Bruno

et al. (1999) described the glandular trichomes as consisting of a bicellular stalk with a spherical "uni-pluricellular" head. In this study, however, only a single stalk cell could be observed for glandular trichomes (Fig. 5.3), which is in agreement with Seithe (1979) who stated that the stalk of glandular trichomes in the genus *Solanum* always remains unicellular. According to the categorisation of Seithe (1979), the glandular trichomes on silverleaf nightshade leaves can be classified as storied glands, with two or three storeys of cells, each with two or more glandular cells.

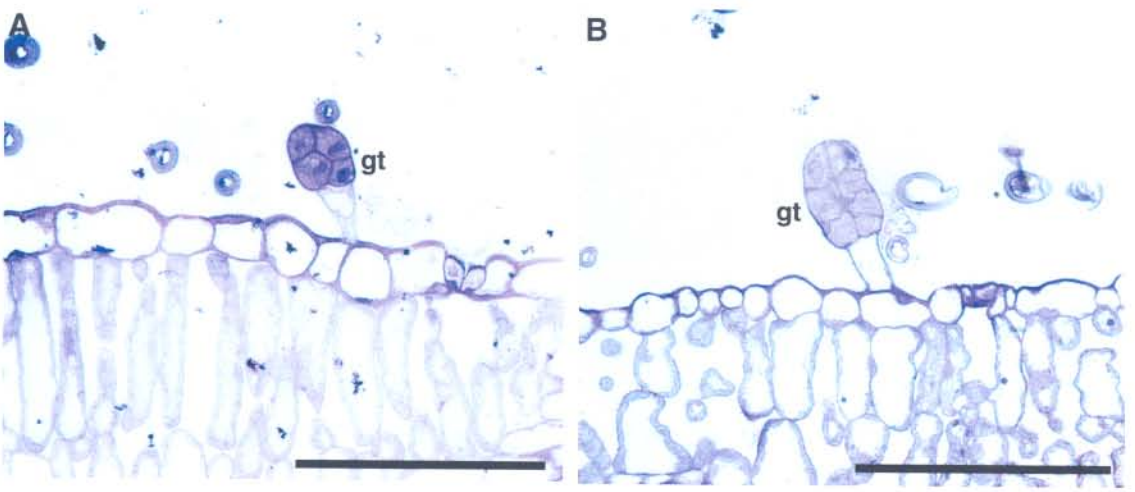

Figure 5.3 Transverse sections through glandular trichomes (gt) on the silverleaf nightshade leaf surface showing the storied glandular trichome head and one stalk cell; Scale bars = 100 μm

- Non-glandular trichomes:

  As also found by Bruno *et al.* (1999), two variants of non-glandular porrect-stellate trichomes could be distinguished on both sides of the silverleaf nightshade leaf. One has a multicellular stalk emerging from the epidermis surface, while the other variant has fewer but much larger intrusive stalk cells growing into the mesophyll (Fig. 5.4). Epidermal stellate trichomes have been described for several *Solanum* species (Seithe, 1979), while the variant with the intrusive base has only been described for *S. elaeagnifolium* (Pilar, 1937; Cosa *et al.*, 1998; Dottori *et al.*, 1998; Bruno *et al.*, 1999). In both variants the stellate head consists of 8 to 16 horizontal unicellular

radii and one prominent vertical apical spine cell (Fig. 5.5) with a large basal lumen (Fig. 5.4B), borne on a biseriate stalk. These silverleaf nightshade trichomes were described by Roe (1971) as being rather intermediate between porrect-stellate and peltate. From a surface view it is not possible to distinguish between intrusive and non-intrusive variants (Fig. 5.5)

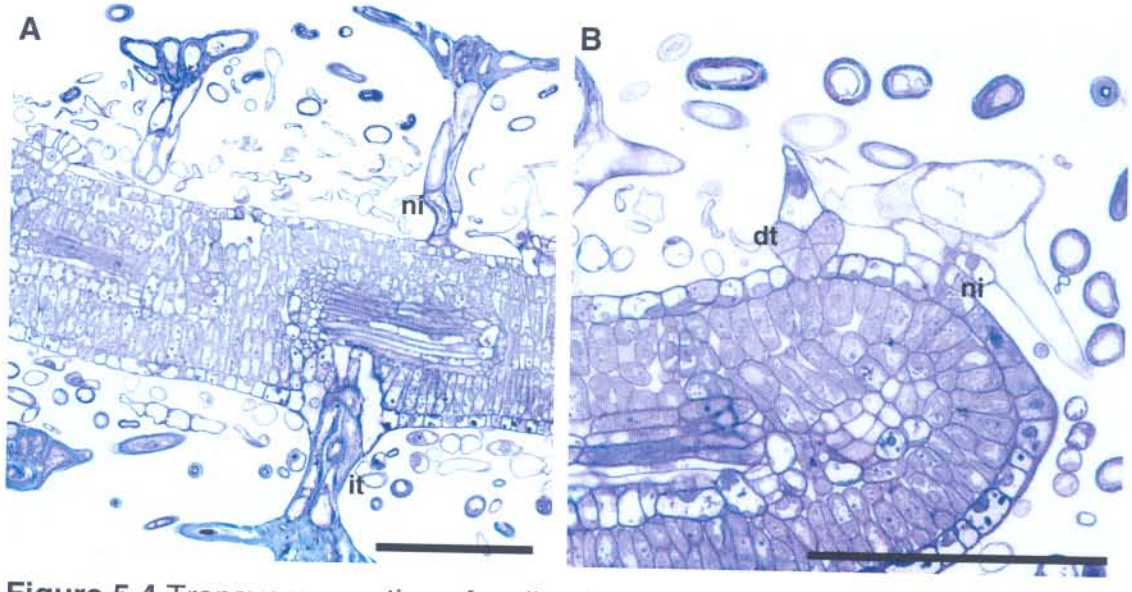

Figure 5.4 Transverse section of a silverleaf nightshade leaf showing intrusive (it) and non-intrusive (ni) variants of stellate trichomes and a stellate trichome in early stages of development (dt); Scale bars = 100 μm

Stellate trichomes are extremely closely spaced on young leaves, however, density of trichomes decreases dramatically as leaves mature. On fully mature leaves the stellate trichomes are markedly denser on the abaxial side of the leaf than on the adaxial side (Fig. 5.5).

Bruno *et al.* (1999) illustrated that the early stages of development are similar in both stellate trichome variants. Basal cells start to grow, in general without dividing, and cause the stalk to intrude aggressively between mesophyll cells, probably as a result of enzymatic disorganization of the middle lamella. When mature, the stalks seem to reach the vascular bundles. At adult stage, the cell

walls of both variants are uniformly thick; the emerging part is strongly lignified whereas there is little or no lignification in the intrusive portion of the trichome. By differential staining with Toluidine Blue O (0.5% in a sodium carbonate buffer) the same could be observed in this study.

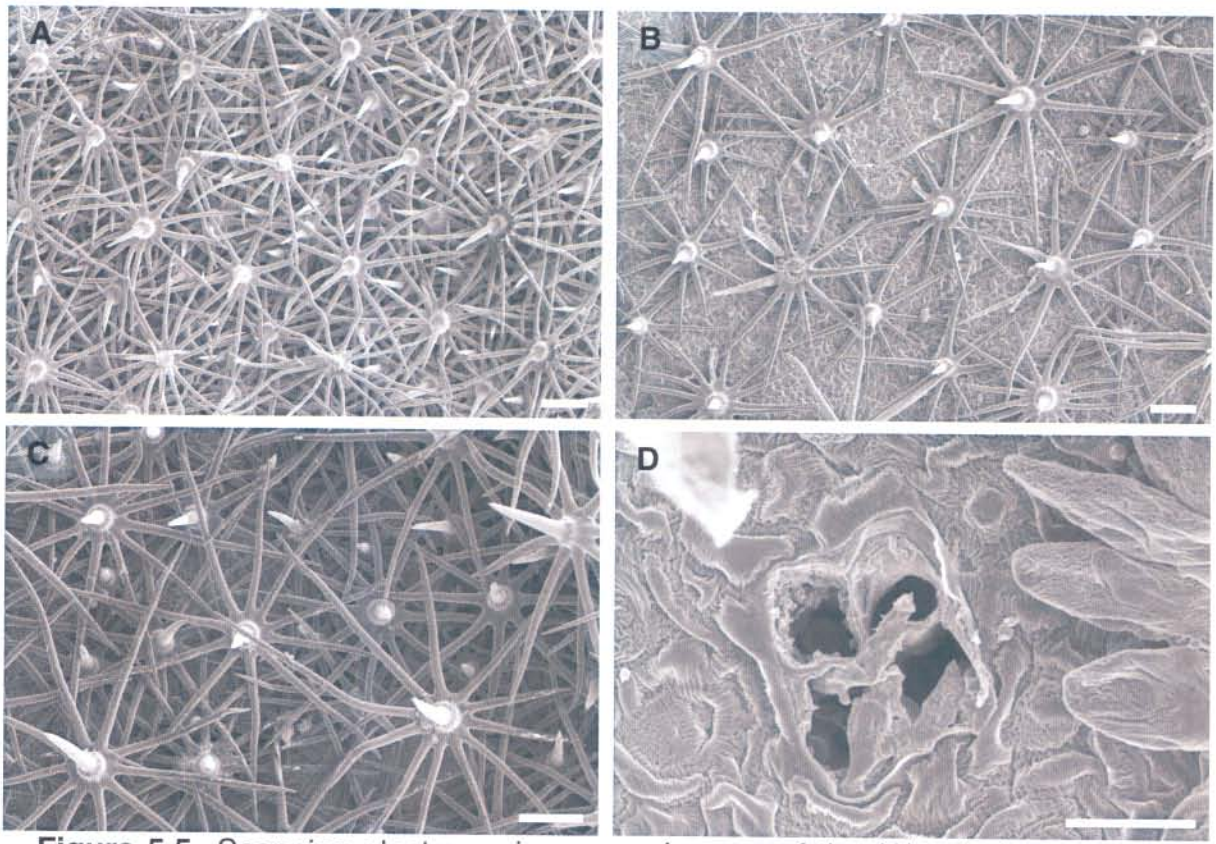

Figure 5.5 Scanning electron microscope images of the (A) adaxial surface of a 2 cm-leaf, (B) adaxial surface of a mature leaf and (C) abaxial surface of a mature leaf of silverleaf nightshade; (D) Removal of an intrusive trichome shows basal cells entering the leaf surface; Scale bars: A – C = 100 μm, D = 10 μm

According to evolutionary tendencies proposed by Seithe (1979) for stellate trichomes of *Solanum*, those of silverleaf nightshade would belong to the more evolved group, since they cumulate the characteristics of a short non-glandular apical cell, many horizontal non-glandular radii, and the presence of a biseriate stalk.

The tendency to develop either a stellate hair or a prickle seems to be determined according to the "pattern scheme" described by Bünning (1948), known previously for stomata (Seithe, 1979). In certain instances the stimulus for extending cells to form stellate hairs is replaced by another stimulus which promotes cell divisions and turns hairs into prickles. The same mechanism might possibly determine development of either epidermal or intrusive stellate hair types.

Currently, the function of these stellate trichomes with its intrusive bases is unclear. Bruno *et al.* (1999) speculated that its morphology might possibly be related to the water economy of the plant, since the stalk cells, with its very thick walls, seem to penetrate to the point of making contact with the xylem. However, before any conclusions of involvement in water transport can be made, the presence of pits and the nature thereof has to be established. Furthermore, Bruno *et al.* (1999) raised the possibility that, as observed with sclereids in the epidermis of other xerophytic species (Martinez, 1995), the thick-walled intrusive cells of these trichomes might have an important sustaining function, reducing the risk of collapse when the plant is stepped on.

The only other species in which intrusive non-glandular trichomes have been reported, is the rootless epiphytic terrestrial species of *Tillandsia* in the Bromeliaceae (Gibson, 1996). They have broad peltate trichomes, which are flexed upward under dry conditions, absorb water into the dead cells when wetted, and then flatten on the water film drawing water into live stalk cells intruding into the mesophyll. However, it is not likely that a relationship exists between these trichomes and those of silverleaf nightshade, as all cells of the intrusive stellate trichomes in the latter seem to be dead.

Bruno *et al.* (1999) also mentioned the presence of simple and non-glandular trichomes. In this study, however, no simple hairs were

detected by SEM or light microscopy on any of the samples examined.

Xeromorphic adaptations:

Except for its extensive underground root system, silverleaf nightshade has highly specialised leaves adapted to the xerophytic environment in which it normally grows. The amphistomatic leaves with isolateral palisade mesophyll are typical of non-succulent desert shrubs (Gibson 1996). The abundance of crystals in the leaves can also be considered of adaptative value to xerophytic conditions (Fahn & Cutler, 1992). Furthermore, the wealth of silvery stellate trichomes on the leaf surface contributes to silverleaf nightshade's drought resistance (Bruno *et al.*, 1999), as dead non-glandular trichomes may significantly reduce absorbed infrared radiation and thereby leaf temperature (Gibson, 1996). The presence of dense trichomes has also been explained as a way to reduce leaf transpiration (Gibson, 1996).

Structures possibly linked to allelopathic potential:

Although higher plants are capable of producing literally thousands of secondary metabolites, it is interesting to note that trichomes generally contain the most active constituents, even though it contains less amounts and kinds of secondary metabolites as compared to the whole leaf or other plant tissues (Wink, 1999). Therefore, when considering cells or structures that might play a role in the allelopathic potential of silverleaf nightshade leaves, there are two obvious features to consider. Firstly the glandular trichomes and secondly the non-glandular stellate trichomes with intrusive basal cells.

The secretion of alkaloids by glandular hairs of two Solanaceous species, potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.) have been described (Roshchina & Roshchina, 1993). It is likely that glandular trichomes on *S. elaeagnifolium* leaves may harbour the alkaloidal compound responsible for the allelopathic effects of silverleaf nightshade foliar extracts, and even the phytotoxic saponin or flavonoids. The basal

cells of the intrusive stellate trichome variant seem to reach the vascular bundles, and one of the main concentration sites of alkaloids in plants is the vascular bundle sheath (Roshchina & Roshchina, 1993). It can therefore be considered a possibility that the intrusive stellate trichomes might be involved in excreting alkaloidal compounds situated in the vascular bundle sheath of silverleaf nightshade leaves. These theories, however, still need to be confirmed by further chemical investigation of the exact contents of these trichomes.

Own experiments to evaluate allelopathic potential of silverleaf nightshade made use of foliar extracts where either frozen material was soaked or dried foliage was homogenised in organic solvents for extraction. Concurrent experiments at the University of Pretoria aimed at evaluating phytotoxicity of silverleaf nightshade foliage in soil, made use of ground, dry foliar material incorporated into potted soil in which the test species was sown (Mkula, unpublished). All these methods resulted in extraction or availability of most of the compounds in the entire leaf. Further studies should explore the phytotoxic activity of a leaf wash of fresh intact foliage with water or organic solvents (Duke *et al.*, 1994). Such a leaf wash would contain most, if not all, substances present on the leaf surface, enabling the assessment of whether allelochemicals in silverleaf nightshade are indeed located in structures on the leaf surface or are excreted onto the leaf surface.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Research investigating interference of silverleaf nightshade with crop species has mainly explored the competition aspect, while literature concerning its possible allelopathic interference with crop species is extremely limited. Published research concentrate solely on phytotoxic activity of saponins in silverleaf nightshade fruit (Curvetto *et al.*, 1976; Agüero *et al.*, 1985). Due to its importance as a weed, it would be of great value to know and understand all mechanisms by which silverleaf nightshade interfere with crops, however significant voids still exist in knowledge concerning the allelopathic potential of this weed. The experiments conducted in this study were aimed at contributing to knowledge of the allelopathic potential of the foliage of silverleaf nightshade.

6.1 Bioassay technique

Numerous different approaches for the execution of germination bioassays in allelopathy, are encountered in literature. Some important conclusions were reached while experimenting to find an appropriate bioassay for evaluating the allelopathic potential of silverleaf nightshade foliage (Chapter 2):

- The use of organic solvents in extraction or fractionation of an extract necessitates evaluation of phytotoxicity of these solvents' residues after evaporation.
- The importance of choosing relevant test species in a bioassay was highlighted.
- It was established that the use of germination paper rolls, known as rag dolls, is not an effective method for evaluating allelopathic potential of extracts on seed germination and seedling growth.
- Maintaining aseptic working conditions are essential in germination bioassays with extracts in Petri dishes, as it prevents fungal growth which may distort results.

- Osmolalities of extracts used in bioassays need to be monitored to exclude the possibility of osmotic inhibition.

6.2 Allelopathic potential of silverleaf nightshade foliage

Previous studies showed the phytotoxicity of compounds in the fruit of silverleaf nightshade towards cucumber and clover (Curvetto et al., 1976; Agüera & Boland, 1985). This study revealed the presence of phytotoxic substances in the foliage of silverleaf nightshade as well. Germination and early root growth of cotton and lettuce were inhibited by crude foliar infusions and water-soluble extracts at low concentrations where osmotic inhibition was not present (Chapter 3). While this does not prove allelopathy, it indicates that foliage of silverleaf nightshade does have the potential to exert allelopathic inhibition on crop species. Roots of lettuce seedlings exposed to the phytotoxic extracts exhibited severe swelling, marking a rapid transition from the meristematic zone to differentiated cells, close to the root tip. Cell division and elongation were inhibited in the root tip and an apical shift of root hair differentiation occurred.

6.3 Preliminary identification of active compounds

In Chapter 4 it was established that more than one chemical fraction of the foliar silverleaf nightshade extract inhibited germination and/or early root growth of the test species. Three fractions, probably flavonoids, on the preparative paper chromatogram significantly inhibited root growth. More pronounced inhibition of root growth as well as germination were caused by the most polar fraction containing alkaloidal compound(s). This fraction proved to contain two phytotoxic compounds, an unidentified alkaloid and a saponin. All these phytotoxic compounds probably act together in the crude extract resulting in a synergistic effect where more pronounced inhibition of germination and growth occurs than is observed with more pure fractions. This theory, however, needs to be substantiated with further chemical investigation.

6.4 Leaf anatomy as linked to allelopathic potential

There are many reports on the occurrence of phytotoxic substances in glandular trichomes of plants (Roshchina & Roshchina, 1993; Duke *et al.*, 1994). It was considered likely that the glandular trichomes observed on the isobilateral, amphistomatic leaves of silverleaf nightshade might contain some of the phytotoxic compounds found in the foliar extract. It was furthermore speculated that the intrusive stellate trichomes, described in Chapter 5, might be involved in excreting alkaloids from the vascular bundle sheath, one of the main alkaloid concentration sites in plants, onto the leaf surface. These speculations, however, still needs to be confirmed by means of chemical analysis of the exact contents of the various trichomes.

6.5 Conclusions and recommendations

Although the results of this study are an indication of the allelopathic potential of silverleaf nightshade, it does not prove allelopathy. Analysis of world literature on allelopathy (Rice, 1984) indicates that there are few examples of verifiable allelopathic influence of intact plants on the growth of other plant species. Therefore, many questions have yet to be addressed in further investigations before more definite conclusions may be drawn. Follow up studies at the University of Pretoria have already established that phytotoxicity of foliage remains intact in the soil environment, resulting in growth inhibition of cotton seedlings (Mkula, unpublished). The next step would be to verify whether the same reaction occurs under field conditions. Proper purification and identification of compounds responsible need to be performed and it should be ascertained whether these compounds are present in soil where silverleaf nightshade interferes with crops.

APPENDIX A

Abbreviated analysis of variance (ANOVA) tables

Table A1 Analysis of variance of percentage germination of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract, after angular transformation (Table 3.1)

Source	DF	MS	F-value	Pr > F
Concentration	4	618.7	6.18	0.002
Error	20	100.0		
Corrected total	24			
CV (%)	22.9			
R^2	0.55			

Table A2 Analysis of variance of root length of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract (Table 3.1)

Source	DF	MS	F-value	Pr > F
Concentration	4	1289.52	23.14	0.0001
Error	20	55.73		
Corrected total	24			
CV (%)	21.10			
R^2	0.82			

Table A3 Analysis of variance of shoot length of cotton germinated in increasing concentrations of silverleaf nightshade foliar extract (Table 3.1)

Source	DF	MS	F-value	Pr > F
Concentration	4	556.16	12.60	0.0001
Error	20	44.14		
Corrected total	24			
CV (%)	24.50			
R^2	0.72			

Table A4 Analysis of variance of percentage germination of cotton germinated in increasing concentrations of PEG-6000 solution, after angular transformation (Table 3.2)

Source	DF	MS	F-value	Pr > F
Concentration	5	837.09	12.68	0.0001
Error	24	66.00		
Corrected total	29			
CV (%)	17.6			
R^2	0.73			

Table A5 Analysis of variance of root length of cotton germinated in increasing concentrations of PEG-6000 solution (Table 3.2)

Source	DF	MS	F-value	Pr > F
Concentration	5	89.97	2.97	0.0327
Error	23	30.28		
Corrected total	28			
CV (%)	21.08			
R^2	0.39			

Table A6 Analysis of variance of shoot length of cotton germinated in increasing concentrations of PEG-6000 solution (Table 3.2)

Source	DF	MS	F-value	Pr > F
Concentration	5	82.30	31.63	0.0001
Error	23	2.601		
Corrected total	28			
CV (%)	17.27			
R^2	0.87			

Table A7 Analysis of variance of percentage germination (72 hours) of lettuce germinated in increasing concentrations of the water-soluble fraction of an organic silverleaf nightshade foliar extract, after angular transformation (Table 3.3, 24h and 48h not shown)

Source	DF	MS	F-value	Pr > F
Concentration	4	6622.16	111.07	0.0001
Error	20	59.62		
Corrected total	24			
CV (%)	14.5			
R^2	0.96			

Table A8 Analysis of variance of root length of lettuce germinated in increasing concentrations of the water-soluble fraction of an organic silverleaf nightshade foliar extract, after rank transformation (Table 3.3)

Source	DF	MS	F-value	Pr > F
Concentration	3	208.33	83.33	0.0001
Error	16	2.50		
Corrected total	19			
CV (%)	15.06			
R^2	0.94			

Table A9 Analysis of variance of percentage germination (72 hours) of lettuce germinated in increasing concentrations of PEG-6000 solution, after angular transformation (Table 3.4, 24h and 48h not shown)

Source	DF	MS	F-value	Pr > F
Concentration	4	58.43	1.32	0.295
Error	20	44.13		
Corrected total	24			
CV (%)	7.9			
R^2	0.21			

Table A10 Analysis of variance of root length of lettuce germinated in increasing concentrations of PEG-6000 solution (Table 3.4)

Source	DF	MS	F-value	Pr > F
Concentration	4	7.20	5.94	0.0026
Error	20	1.21		
Corrected total	24			
CV (%)	6.37			
R^2	0.54			

Table A11 Analysis of variance of percentage germination (72 hours) of lettuce germinated on paper chromatography fractions, after angular transformation (Table 4.1, 24h and 48h not shown)

Source	DF	MS	F-value	Pr > F
Concentration	5	4514.86	50.95	0.0001
Error	24	88.62		
Corrected total	29			
CV (%)	13.3			
R^2	0.91			

Table A12 Analysis of variance of root length of lettuce germinated on paper chromatography fractions (Table 4.1)

Source	DF	MS	F-value	Pr > F
Concentration	5	78.93	63.78	0.0001
Error	24	1.24		
Corrected total	29			
CV (%)	12.47			
R^2	0.93			

Table A13 Analysis of variance of percentage germination (72 hours) of lettuce germinated in Sephadex column fractions, after angular transformation (Table 4.2, 24h and 48h not shown)

Source	DF	MS	F-value	Pr > F
Concentration	6	1461.06	21.14	0.0001
Error	14	69.12		
Corrected total	20			
CV (%)	10.6			
R^2	0.90			

Table A14 Analysis of variance of root length of lettuce germinated in Sephadex column fractions (Table 4.2)

Source	DF	MS	F-value	Pr > F
Concentration	6	7.74	4.30	0.0115
Error	14	1.80		
Corrected total	20			
CV (%)	15.09			
R^2	0.65			

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SUMMARY

ALLELOPATHIC POTENTIAL OF SILVERLEAF NIGHTSHADE (*SOLANUM ELAEAGNIFOLIUM* CAV.)

1. The possibility that silverleaf nightshade foliage might possess allelopathic properties was researched in this study. This was done by means of germination bioassays conducted under laboratory conditions. The bioassay technique used in allelopathy experiments first needed to be refined to ensure reliable results. An anatomical study on the leaves of silverleaf nightshade was also conducted to confirm current knowledge and to relate specific leaf structures or cells to the allelopathic potential of the foliage.

2. Important factors highlighted during experiments to refine the bioassay technique include the importance of eliminating clouding factors such as osmotic inhibition, pathogenic microorganisms and phytotoxic residues of organic solvents used for extraction or fractionation.

3. Crude water infusions and water-soluble extracts of silverleaf nightshade foliage inhibited germination and early root growth of the two test species, cotton and lettuce. The osmolalities of the extract concentrations used were not inhibitory to either germination or root growth of the test species. Roots of lettuce seedlings exposed to the foliar extract were severely swollen close to the root tip. Cell division and elongation were inhibited in the meristematic and elongation zones, and an apical shift of the root hair differentiation zone occurred.

4. It was established that more than one chemical compound is responsible for the phytotoxic activity of silverleaf nightshade foliar extracts. Indications are that the phytotoxic substances are three flavonoidic compounds, an alkaloid and a saponin.

5. Interesting features observed in the anatomical study of silverleaf nightshade leaves include crystal harbouring idioblasts, various trichomes covering both leaf surfaces including glandular trichomes and two variants of porrect stellate trichomes. One variant emerges from the epidermis surface, while the other variant has intrusive basal cells entering the mesophyll seeming to connect with vascular tissue. It was speculated that glandular trichomes might harbour the phytotoxic substances present in the foliar extracts, or that intrusive stellate trichomes might excrete alkaloids contained in the vascular bundle sheath onto the leaf surface.

6. Further experiments at the University of Pretoria confirmed the phytotoxicity of silverleaf nightshade foliage in a soil environment. Studies confirming silverleaf nightshade allelopathy under field conditions and proper isolation and identification of allelochemicals are still required.