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.

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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

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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.

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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

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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.

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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 25ºC 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.

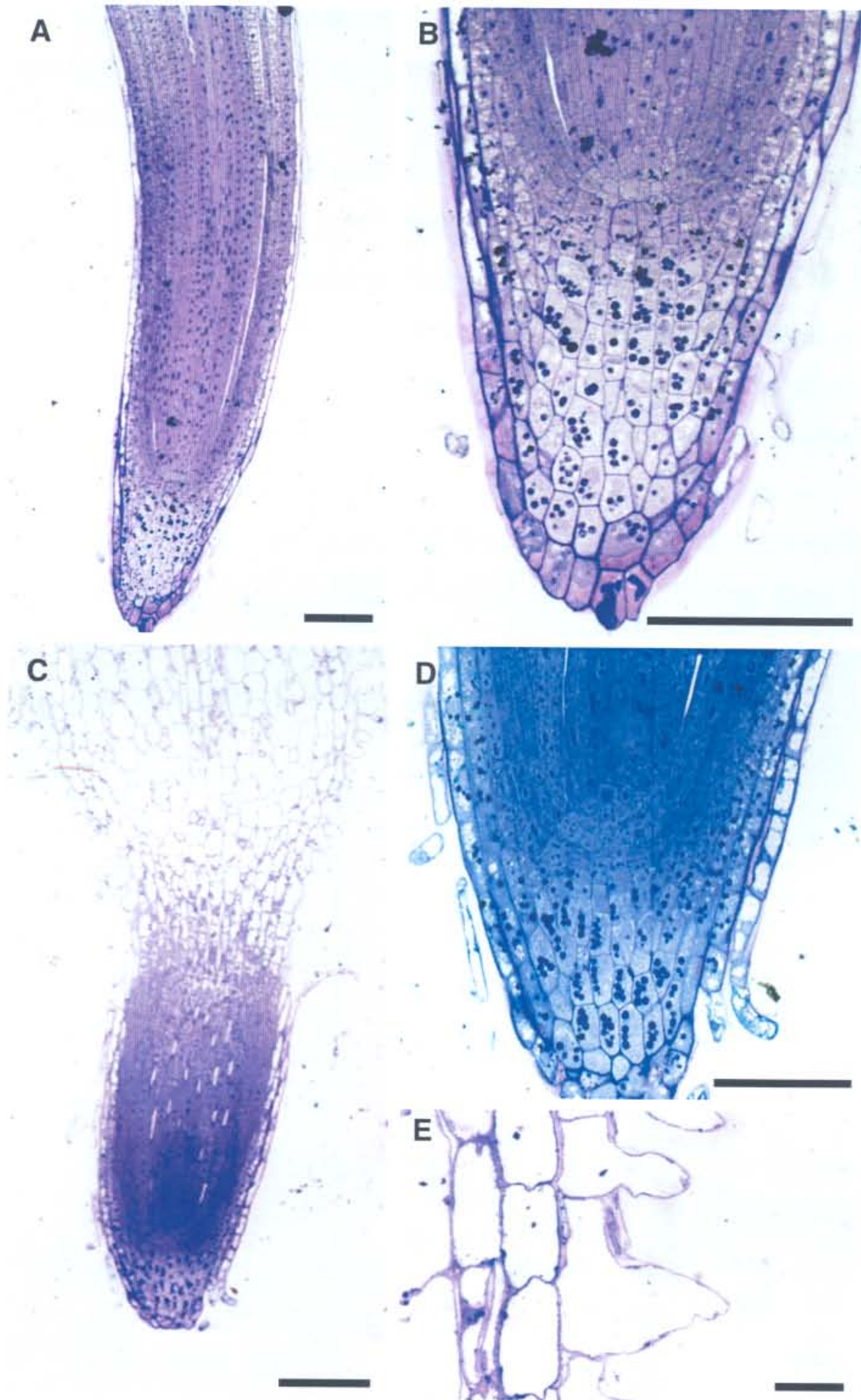

Figure 3.1 Longitudinal sections of the root tips of (A; B) a lettuce seedling not exposed to silverleaf nightshade foliar extract, and (C; D) a seedling exposed to the foliar extract – notice the abrupt transition from the meristematic zone to differentiated cells in the swollen area of the treated 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

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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.