EXPERIMENT 1

Effect of Seed Meal Derived Glucosinolates on Germination and Early Growth of Wheat.

INTRODUCTION

Since seed of rapeseed has known levels of glucosinolates, seeds can be used as a source of glucosinolates and breakdown products. Seeds contain a number of glucosinolates as well as myrosinase enzyme, responsible for the production of breakdown products. Endogenous breakdown of seed glucosinolates generally leads to the production of isothiocyanates, thiocyanates or nitriles as the major primary products (Ettlinger and Kjaer 1968, Van Etten Daxenbichler and Wolff 1969, Cole 1976, Fenwick et al. 1982, Hanley et al. 1983, Slominski and Campbell 1989b).

Boiling of seed prior to processing (grinding to produce meal) can be employed to denature the endogenous myrosinase enzyme thus leaving intact glucosinolates present in the meal. Kjaer (1960) has determined that endogenous myrosinase has a temperature optimum of below 40°C. This technique may be used to produce meal containing either intact glucosinolates or breakdown products. In this way it is possible to investigate whether glucosinolates or their breakdown products are inhibitory to germination.

In this experiment a single rapeseed variety was used with boiling of seed as a main treatment. Wheat seed were germinated in the presence of differing amounts of rapeseed seed meal.

METHODS

These experiments were carried out in mid 1988 at Wagga Wagga in NSW.

Rapeseed (cv. "Wesbrook") seeds were used as a source of glucosinolates. Half the seed was boiled prior to being ground in a coffee grinder to produce meal. Boiling of seed for 5 minutes was used to denature endogenous enzymes. Seed not boiled was ground at room temperature.

Wheat (cv. "Owlet") seeds (surface sterilized to remove micro organisms which may have affected germination) were germinated in petri dishes in the presence of rapeseed meal of varying quantities
per dish. 20 wheat seeds were placed on two Whatman No 1 filter papers and 5 ml per dish of de-ionised water added to each dish. 4 replicates were used. Dishes were germinated in the dark at 15°C with the longest seminal root (root length) measured on day 6.

Wheat was germinated in the presence of quantities of rapeseed meal of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0g/dish.

Data were analysed using Analysis of Variance techniques.

RESULTS

Figure E1.1 The effect of rapeseed meal on early growth of wheat

Heat treatment
LSD (P<0.05) = 1.85 mm, (1%) = 2.5 mm

Amount of material per dish
LSD (P<0.05) = 6.28mm (1%) = 8.55mm

Two heat treatments at same amount of meal. LSD (P<0.05) = 5.78mm.

Root length of wheat seedlings was significantly reduced (P<0.01) as higher amounts of seed meal were added to the dishes.
DISCUSSION

Boiling of the seed prior to grinding to produce meal was seen to produce significantly less inhibition of root growth than using seed meal ground at room temperature (P<0.01). A degree (5%) of interaction was present where the difference between the heat treatments became larger at higher levels of meal.

The reduction in seminal root length in the presence of rapeseed meal may have been due to substances in the seed meal leading to reduced radicle growth. It would be expected that rapeseed meal would contain a variety of compounds, some of which may be inhibitory to root extension. These compounds may act alone or in combination.

While meal from both boiled and unboiled seed was inhibitory, meal from seed which had been boiled was seen to be less inhibitory than seed ground without this treatment. Prior boiling would be expected to denature and endogenous enzymes present in seed, so leaving intact compounds which may otherwise be broken down to other compounds with the mixing of seed contents in the grinding operation. Specifically, boiling may denature endogenous myrosinases, thus, leaving endogenous glucosinolates intact. Where meal was produced without boiling mixing of enzyme and glucosinolates would produce breakdown products.

Similarly, boiling of seed may have led to many enzymes and other compounds being altered so as to produce a mix of compounds in meal different to that produced when contents are mixed during grinding at room temperature.

Whatever the effect of boiling on the eventual components of the meal produced, the boiling treatment produced meal containing less inhibitory agents than meal produced at room temperature.

Enzymic breakdown of glucosinolates may produce products more toxic to (wheat) seed germination and early growth than intact glucosinolates, this being apart from effects from other compounds present in meal. It is possible that glucosinolates are inhibitory, but that breakdown products are moreso. There may also be some other compounds in rapeseed seed which is responsible for the inhibition seen apart from glucosinolates or breakdown products and that this is reduced by boiling of seed prior to meal production.
EXPERIMENT 2

Effects on Germination of Indicator Species of Glucosinolate containing Rapeseed Meal from two Varieties.

In this experiment similar treatments as in experiment 1 were employed with the added factor of using two varieties of rapeseed seed as the source of meal. Varieties containing low and high seed glucosinolate levels were used.

METHODS

This experiment was carried out in mid 1988 in Wagga Wagga, NSW.

Similar boiling and meal production procedures were followed as described in Experiment 1. Varieties of rapeseed seed were cv. "Maluka" and cv. "Jumbuck".

Wheat (cv. "Owlet") seeds were germinated in petri dishes as per experiment 1, in the presence of rapeseed meal with the same quantities of seed meal used per dish. Other experimental details were also as per experiment 1.

RESULTS AND DISCUSSION.

1) Effect of seed meal addition to wheat root growth.

The general response of wheat root growth to addition of rapeseed meal is shown in Figure 2-1 below. The addition of meal was seen to dramatically decrease root length. Data shown plots root length (mm) against log of the amount of meal per dish.

All results were analysed using Analysis of Variance techniques. Means sharing the same letter are not significantly different.
2) Effect of cultivar of rapeseed as the source of meal.

Figure E2-2 (below) shows the effect of seed (meal) variety on germination of wheat. While both cvs. Jumbuck and Maluka were significantly inhibitory to wheat germination as measured by root length, Jumbuck was seen to be more inhibitory than Maluka (P<0.05). This may be due to the higher glucosinolate content in Jumbuck giving rise to more inhibitory compounds with breakdown. Both seed varieties may be also considered to be inhibitory regardless of glucosinolate content, again suggesting that a number of seed contained compounds may be active.

It may be that glucosinolates (and breakdown products) are among of a number of compounds contained in rapeseed seed which may be inhibitory to wheat seed germination in these experiments.
Figure E2.2: The effect of variety of rapeseed meal on wheat root length

Means sharing the same letter are not significantly different (P<0.05).

3) Boiling treatment of seed meal.

There was no significant effect of prior boiling of the rapeseed meal on root length of germinating wheat when averaged over both varieties. Both treatments produced meal which was seen as inhibitory when compared to the water control. See Figure E2-3 below.
4) Interaction between boiling treatment of seed and variety.

Boiling of Jumbuck seed prior to grinding produced less inhibition than seed having not been boiled. Maluka seed meal, either boiled or unboiled gave similar degrees of inhibition. See figure E2.4 below.

Means sharing the same letter are not statistically different (P<0.01).
Where seed meal was boiled prior to meal production both varieties showed similar effects on radicle length. Where meal was produced without boiling Jumbuck was more inhibitory (P<0.01) than Maluka.

Meal produced with no prior seed boiling would be expected to contain glucosinolate breakdown products, with these possibly contributing to the greater inhibition seen. Seed from Jumbuck is known to be higher in glucosinolates than Maluka (Wratten, pers comm). If breakdown products are more inhibitory to seed germination than intact glucosinolates, and Jumbuck being higher in glucosinolate content, this may explain any greater inhibition with seed meal from Jumbuck. Maluka seed being of lower glucosinolate content would produce less (toxic) breakdown products.

The smaller difference between varieties where boiling occurred may indicate glucosinolates as being of weak inhibitory ability, thus there being little difference between high and low levels.

The fact that meal produced from both boiled and unboiled seed was more inhibitory than control treatments again suggests that other components of rapeseed seed (meal) may be inhibitory to germination. It is possible that glucosinolate breakdown products, where present, are an additional inhibitory factor.

It should be noted that the wheat used in this experiment was used solely as an indicator species, and where inhibition of germination or early growth occurred with rapeseed meal it is not an indication that wheat should not be grown after canola, but rather that wheat provided a ready source of seed of known germination quality for use as a test species.
EXPERIMENT 3

THE EFFECT OF FRESH RAPESEED MATERIAL ON GERMINATION AND EARLY GROWTH OF THREE INDICATOR SPECIES.

1. INTRODUCTION

Green rapeseed plant material may provide a source of active compounds, notably compounds derived from the indole glucosinolates, which predominate in stem and leaf material (Sang et al. 1984). The quantities and availability (for example, soluble cell contents) present in large green rapeseed plants of these compounds make the use of green material a possible source of these compounds. In this experiment green rapeseed material was used as a source of potentially active compounds.

Green rapeseed plants (large plants at flowering) applied as residues to germinating indicator species may show growth effects on these species. Any effects seen could be due to leachates from the rapeseed residue applied, derived from compounds in the plant or breakdown products from these.

It is also likely that applying green plant material to germinating seed in pots would produce some stimulation from addition of nutrients (C, N, P, etc.) although it is also possible that other compounds may be produced as the plant material breaks down and that some of these may be inhibitory. Fresh material would be likely to contain high levels of compounds (including glucosinolates) which may be important as precursors for breakdown products produced at senescence.

This experiment examined whether green rapeseed material contained compounds which may be inhibitory to germinating plants, or compounds which form inhibitory compounds with breakdown and/or senescence. Green material may be in itself stimulatory, and any inhibition seen could indicate a possible greater activity of the responsible compounds if applied alone.

The experiment also served to identify possible sensitive indicator species to any inhibitory agents present.

It was also thought that finely ground plant material may have yielded a greater availability of active substances as cell contents would be well mixed, and available for reaction and/or breakdown.
Material left substantially intact may have yielded less of any active compounds as cells would be less disrupted.

**MATERIALS AND METHODS**

This experiment was conducted during late 1988 at Wagga Wagga in NSW.

Rapeseed plants (cv. Maluka) at the flowering stage were used. Representative plants were dried to determine dry weight of the material.

Plant material was either finely ground using a blender or cut into 5-10 cm lengths (that is, 'ground' or 'cut' treatments.

Test species were wheat (cv. "Owlet"), linseed (cv. unknown) and perennial ryegrass (cv. Victorian). These were germinated in 17 cm diameter pots (20 seeds per pot) in a sandy loam soil in glasshouse conditions. To the pots was added various amounts of green rapeseed material; 3 t/ha, 6 t/ha and 12 t/ha of dry matter equivalent, either previously cut or ground.

Plants were harvested 9 weeks later and divided into roots and tops which were dried at 100° C to provide weights for analysis.

**RESULTS**

1. **The effect of amount of material per pot on the early growth of the test species.**

Early growth of the test species, as measured by dry weight, are presented in Figures E3.1 and E3.2, over. Results are meaned for all test species. Results for both top and root growth are presented.

All results were analysed using Analysis of Variance.
The test species generally grew better as the amount of rapeseed material per pot was increased. Top growth was seen to be more stimulated than root growth which showed no positive response until the highest amount of materials was added to each pot.
Experiment Three.

2. Response of the different species used to the presence of green rapeseed material.

Results are shown below (Table E3.1) are meaned for all rates of rapeseed material added.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tops</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>122.24a</td>
<td>114.31a</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>132.30a</td>
<td>112.55a</td>
</tr>
<tr>
<td>Linseed</td>
<td>105.18b</td>
<td>85.43b</td>
</tr>
</tbody>
</table>

LSD (P<0.01) 15.1 19.44

Means sharing the same letter are not significantly different (P<0.01).

Although top growth of all test species was stimulated by the presence of rapeseed material, linseed was significantly (P<0.01) less stimulated than the two other species. Linseed root growth was seen to be significantly reduced by presence of rapeseed material (P<0.01).

3. The effect of amount of green rapeseed material on early growth of test species.

Figures E3.3 and E3.4 (below) shows the effect of the addition of green rapeseed material to the three test species. With wheat and ryegrass the addition of rapeseed material gave stimulation of growth (P<0.01), while linseed was not stimulated. Linseed growth was significantly less stimulated than the other two test species at all levels of rapeseed material addition (P<0.01). The figures show the differences in dry weight of roots and tops evident among the species in the presence of the rapeseed material. In the case of top growth linseed was unaffected, while root growth was reduced by the rapeseed material (P < 0.05). Analysis of Variance was used to determine LSD's.
4. The effect of cutting or grinding the rapeseed material.

Grinding of the rapeseed material prior to adding to pots stimulated growth more than cutting in 5-10 cm lengths. Table E3.2, below, shows the effect of grinding of green rapeseed material to be more stimulatory to plant growth (measured by dry weight) than cutting into 10 cm lengths.
Table E3.2 The effect of prior treatment of material and amount applied on early growth of test species. Plant dry weight, % v’s control.

<table>
<thead>
<tr>
<th>Test species</th>
<th>Residue treatment</th>
<th>0 t/ha</th>
<th>3 t/ha</th>
<th>6 t/ha</th>
<th>12 t/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>Cut</td>
<td>90.9a</td>
<td>91.66a</td>
<td>127.76b</td>
<td>144.94c</td>
</tr>
<tr>
<td></td>
<td>Ground</td>
<td>109.1a</td>
<td>109.52a</td>
<td>138.54b</td>
<td>165.5c</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>Cut</td>
<td>95.26a</td>
<td>114.29b</td>
<td>112.11b</td>
<td>174.36c</td>
</tr>
<tr>
<td></td>
<td>Ground</td>
<td>104.74a</td>
<td>136.32b</td>
<td>152.93c</td>
<td>168.42c</td>
</tr>
<tr>
<td>Linseed</td>
<td>Cut</td>
<td>105.68a</td>
<td>100.04a</td>
<td>93.14a</td>
<td>105.24a</td>
</tr>
<tr>
<td></td>
<td>Ground</td>
<td>94.32a</td>
<td>108.84a</td>
<td>133.92b</td>
<td>100.26a</td>
</tr>
</tbody>
</table>

Means sharing the same letter are not significantly different (P<0.01).

The addition of rapeseed material stimulated growth of wheat and ryegrass more than linseed. Ground material at 6 t/ha gave a low level of stimulation to linseed, but not at 12 t/ha. No level of cut material was stimulatory to linseed. With both wheat and ryegrass ground material was more stimulatory than cut material.

DISCUSSION

The results show that in the case of wheat and ryegrass, the addition of green rapeseed material stimulated growth. Root growth was stimulated less than top growth. This stimulation may be due to a nutrition affect of adding the green material as nutrient would be supplied from this material to the germinating seedlings.

Linseed, however, was not stimulated by addition of up to 12 t/ha of green rapeseed material. Measurements of root dry weight show that the presence of rapeseed material reduced growth markedly. It is possible that effects of additional nutrients provided to linseed from addition of green material was negated by inhibiting agents also in the rapeseed material. These agents may be more active with linseed than the other test species. Linseed has been reported as being adversely affected by allelopathic compounds and residues in other work (Lovett 1979, Lovett and Duffield 1981, Lovett 1982) and, hence, may be a plant sensitive to allelopathic compounds. Any allelopathic compounds coming from the rapeseed material thus may be more effective on linseed than the other species.
The possible inhibitory agents may have had direct effects on the linseed plants or may have affected the linseed plants' ability to utilise the additional nutrients in the rapeseed material.

It is likely that green rapeseed material would contain quantities of glucosinolates which would be available to the germinating plants from the material applied. Breakdown products from glucosinolates (for example, from autolysis due to endogenous myrosinase) would be more likely to be present, particularly where material was ground prior to application. It is postulated that breakdown products were present and exerted an affect on linseed such that stimulation of growth was not observed as in the other test species.
EXPERIMENT 4

EFFECT OF SINIGRIN AND MYROSINASE ON GERMINATION AND EARLY GROWTH OF INDICATOR PLANTS

INTRODUCTION

It is thought that inhibition of growth of plants by rapeseed residues (or products from rapeseed seed meal in experiments) is due to products from glucosinolate breakdown. *Brassica* spp contain glucosinolate compounds and myrosinase, the enzyme responsible for their breakdown. Thio cyanates, iso-thiocyanates and nitriles are formed upon breakdown, with iso-thiocyanates and nitriles being the most likely under field conditions (Ettlinger and Kjear 1968, Cole 1976, Hanley *et al.* 1983).

Previous experiments have shown seed meal to be inhibitory to wheat, and green material to be inhibitory to wheat, ryegrass and linseed, the latter known to be sensitive to allelochemical activity (Lovett 1979, Lovett and Duffield 1981).

The object of this experiment was to test a common glucosinolate (sinigrin) and myrosinase to determine if these are inhibitory alone, and whether the product of myrosinase breakdown of sinigrin has an effect on germinating seeds. Sinigrin is a common glucosinolate of seeds of *Brassica* spp. and has been chosen as a convenient source of the glucosinolate group.

METHODS

This experiment was conducted during late 1989 at Wagga Wagga in NSW.

Solutions of sinigrin monohydrate were prepared at 0, 10, 100, and 1000 uM concentrations.

Seeds of subterranean clover (cv. "Junee") and annual ryegrass (cv. "Wimmera") were surface sterilised in 1% sodium hypochlorite solution for 5 minutes and placed on two Whatman No 1 filter papers in 9 cm petri dishes.
Myrosinase (extracted from mustard seed) was prepared at a ratio of 1:100 to that of sinigrin. Myrosinase was also prepared at 0, 10, 100, 1000 μM solutions for use in 'myrosinase alone' treatments.

Sinigrin and myrosinase were allowed to incubate together at 37°C for 2 Hrs, following which the 100 and 1000μM solutions were cloudy in colour and produced a pungent odour. A slight white precipitate was present.

Seeds were germinated in dishes (10 per dish) in presence of the three solutions: sinigrin alone, myrosinase alone, and sinigrin + myrosinase at the concentrations mentioned. 5 ml of solution was used per dish and dishes were incubated at 15°C in the dark for 14 days. Measurements were taken of root or radicle length.

**RESULTS AND DISCUSSION**

1. **Sinigrin and myrosinase effect on species.**

Results on germination and early growth are of the test species are presented in Figures E4.1 and E4.2 below. Results are mean of all concentrations used.

All results were analysed using Analysis of Variance techniques.

![Figure E4.1 The effect of sinigrin and/or myrosinase on root length of ryegrass](image)

<table>
<thead>
<tr>
<th>Root length (mm)</th>
<th>CONTROL</th>
<th>SINIGRIN</th>
<th>MYROSINASE</th>
<th>SIN + MYROSINASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>91.17a</td>
<td>89.28a</td>
<td>91.33a</td>
<td>75.75b</td>
</tr>
</tbody>
</table>

Means sharing the same letter are not significantly different (P<0.05).
As can be seen in figures E4.1 and E4.2, above, the presence of either sinigrin or myrosinase alone had no affect on germination and early growth (as measured by root / radicle length) of rye grass or sub clover. However, when these were allowed to incubate together the affect was to inhibit these.

2. Interaction between compound and concentration.

Figures 4.3 and 4.4, below, show the effect of different concentrations of the compounds used in the experiment and their effects on root and radicle length of ryegrass and sub clover. The LSD's were established using Analysis of Variance.
Figures 4.3 and 4.4. show that root or radicle length was severely affected by compounds in the sinigrin + myrosinase solution at levels of 1000 μM. This indicates that products of enzymic breakdown of sinigrin were inhibitory to germination of seeds. If similar compounds were present following breakdown of rapeseed or other *Brassica* species *in vivo* detrimental affects on germination may occur.

Hydrolysis of glucosinolates under neutral pH, use of exogenous myrosinase with liberal water present, generally produces isothiocyanates (Fenwick *et al.* 1982). Breakdown of sinigrin under the
conditions of this experiment would be expected to produce mainly isothiocyanates and thiocyanates. These compounds may be responsible for the germination effects seen.

It is possible that *Brassica* spp. containing levels of glucosinolates including sinigrin, and related compounds, and myrosinases could produce compounds upon senescence, or from residues, similar to those produced in this experiment from sinigrin + myrosinase. These compounds may exert inhibitory affects in soils, perhaps in interaction with other compounds, to restrict germination, and early growth, of plants in these situations. The presence of plants, for example, rapeseed crops or cruciferous weeds in crop or pasture situations, may provide a source of these compounds in the field, these potentially being important in crop rotation considerations.

Other effects are possible from the presence of these compounds including affects on soil fungi and other microflora. These effects in total may assist with weed suppression and disease inhibition, these being beneficial to following crops planted some months later.
EXPERIMENT 5

THE EFFECTS OF BRASSICA SPP. RESIDUES ON TEST SPECIES.

INTRODUCTION

A number of workers has reported rapeseed (or, more recently canola) as leaving residues which can be inhibitory to germination of following plants or crops. Kasting (1973) and Kasting et al. (1974) showed water extracts from rapeseed residues as inhibitory to the germination and early growth of a number of crops, including barley and wheat. Others who have shown Brassica spp. residues to be allelopathic include Jessop and Stewart (1983), Varmer (1983), Mason-Sedun (1986), Jiminez-Orsonio and Glissman (1987), Mason-Sedun et al. (1987) and Purvis et al. (1990), building a significant body of evidence pointing to the ability of these plants residues to have allelopathic effects on germinating plants.

This experiment aimed to examine the ability of canola residues to produce allelopathic effects on a number of indicator species.

METHODS

This experiment was carried out in Armidale, NSW in early 1991.

Residues of rapeseed (canola) were collected at Wagga Wagga from breeder plots at the New South Wales Agriculture and Fisheries Research Institute. Two cultivars were collected, cv. Jumbuck, an older cultivar of high seed glucosinolate content, and cv. Maluka a more recent low glucosinolate cultivar suitable for classification as canola. Both stems and roots were collected within two days of harvest and before any rain had fallen on the residues. Residues were stored in dry conditions in the dark for 8 weeks until prepared for production of leachate.

All residues (approx. 2kg of each cultivar) was hammermilled through a 2.5mm screen. 200g of each cultivar was then incubated with 2.0l water and allowed to stand at 23°C for 24 hrs.
The leachates were filtered through 0.45μm millipore filter to remove microbes and diluted to the required concentrations. Seven dilutions were prepared in a logarithmic series from 100mg/ml to 1μg/ml as well as a sterile distilled water control.

20 seeds of each indicator species were surface sterilised (0.1% sodium hypochlorite for five minutes) and placed in 9cm disposable petri dishes on two Whatman No 1 filter papers. Species used were wheat (cv. Sunco), linseed (cv. unknown), annual ryegrass (cv. Wimmera) and sub. clover (cv. Seaton Park). Dishes were incubated in the dark at 24°C and harvested on day 5. Measurements were made of % germination (all test species), longest seminal root length for wheat and ryegrass, and radicle length for linseed. Sub clover germination was such that although % germination was measured radicle length showed wide variability. As such a 1-10 early growth score was assigned to each dish.

Irrigation of dishes was as follows:

<table>
<thead>
<tr>
<th>Indicator species</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Linseed</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Clover</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

RESULTS

All results presented have been statistically anaysed using Analysis of Variance techniques. LSD's, where shown, are based on this analysis.

1. Germination.

A) Wheat germination.

Germination percentage of wheat was not significantly affected by either of the cultivars of rapeseed used to produce leachates when meaned over all concentrations used, though Maluka residue produced a slight (N.S.) depression in germination as shown in Table E5.1.
Table E5.1 The effect of cultivar of residue leachate on wheat germination

<table>
<thead>
<tr>
<th>% Germination</th>
<th>Control</th>
<th>Jumbuck</th>
<th>Maluka</th>
</tr>
</thead>
<tbody>
<tr>
<td>83.7</td>
<td>82.3</td>
<td>77.4</td>
<td></td>
</tr>
<tr>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

Dilution of the leachate was seen not to affect the germination of wheat. A slight, non-significant, depression was evident at the highest concentration as shown in Table E5.2.

Table E5.2 The effect of residue leachate dilution on germination of wheat

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.65</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>83.15</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>83.75</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>83.15</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>79.4</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>83.8</td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>61.9</td>
</tr>
</tbody>
</table>

Only the most concentrated Jumbuck residue leachate gave a statistically significant ($P < 0.01$) reduction of germination of wheat (Table 5.3)

TABLE 5.3 The effect of cultivar and dilution of residue leachate on germination of wheat.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Cultivar</th>
<th>Jumbuck</th>
<th>Maluka</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>85</td>
<td>81.3</td>
<td></td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>82.5</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>82.5</td>
<td>83.8</td>
<td></td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>80</td>
<td>78.8</td>
<td></td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>88.8</td>
<td>78.8</td>
<td></td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>76.3</td>
<td>47.5 ***</td>
<td></td>
</tr>
</tbody>
</table>

LSD between cultivar, within concentration ($P<0.01$)= 24%.

B) Linseed germination.

As for wheat, % germination of linseed was seen not to be affected significantly by either cultivar of residue, although again Maluka was seen to slightly lower germination (see Table E5.4, over).
The two cultivars of residue produced different results on % germination of linseed (Figure E5.1). Jumbuck residues showed a non significant effect on % germination of linseed. Maluka residue, however, was seen to produce very significant (P<0.005) reduction in % germination at both weak dilution (0.001mg/ml and 0.1mg/ml). At the strongest concentration germination was prevented. Again, the % germination of linseed was generally low and, thus, may have led to more variation in the results.

C) Clover germination.

There was a significant (P<0.05) difference between % germination of clover in the presence of the two cultivars of residue leachate. When meant over all dilutions, leachates of Maluka were seen to be more inhibitory than those from Jumbuck as shown in Table E5.5 (over).
TABLE E5.5 The effect of cultivar of residue leachate on clover % germination.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Jumbuck</th>
<th>Maluka</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD (P&lt;0.05)</td>
<td>4.5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dilution of leachate was highly significant in affecting % germination of clover, as shown in Figure E5.2 below. At dilutions of 0.1mg/ml, and stronger, % germination was greatly reduced. There was no significant interaction between cultivar and dilution, with both cultivars showing similar effects at similar dilutions, consistent with the differences seen between cultivars and between dilutions.

Figure E5.2. The effect of rapeseed residue on % germination of clover

LSD (P<0.01) = 13.4%

Means sharing the same letter are not significantly different (P<0.01).

There was no significant difference between the cultivars of rapeseed leachate on germination % of clover.

Ryegrass germination was seen to be significantly affected by differing dilutions of leachate used as shown in Figure E5.3 over.
Germination was seen to be somewhat depressed at the weakest dilution, and severely depressed at the stronger dilutions (above 1mg/ml). This is similar to the variable effect seen with leachate dilution on linseed germination. No significant interaction between cultivar and dilution was seen with ryegrass germination.

2. EFFECTS ON EARLY GROWTH OF TEST SPECIES.

A) Wheat root length.

| TABLE E5.7 Effect of cultivar of residue on root length of wheat (mm): |
|-----------------|----------------|----------------|
| Control         | Jumbuck        | Maluka         |
| 46.7            | 46.2           | 39.0           |

While not statistically significant there was some reduction in root length where Maluka leachates were used (Table E5.7 above).

There was a significant stimulation (P<0.01) of radicle length at 0.01mg residue/ml of water. Only the strongest dilution (100mg/ml) was inhibitory (P<0.01) (Figure E5.4, over).
While there was no significant interaction between dilution and cultivar of leachate, it was noted that Maluka seemed slightly more inhibitory than Jumbuck.

B) Linseed radicle length.

No significant difference in radicle length was evident between the two cultivars of leachate used. The effect of dilution of the leachates was, however, highly significant (P<0.005), with the strongest dilution (100mg/ml) almost completely inhibiting radicle extension. See Figure 5.5., over.
Radicle length was significantly reduced at very weak dilutions (0.001, 0.01 and 0.1mg/ml), and again at the strongest dilutions (that is 10 and 100mg/ml) Both very weak and strong dilutions were inhibitory while this effect was absent at 1mg/ml.

The interaction between cultivar and dilution was not significant.

C) Clover early growth, measured by growth score (1-10).

The early growth of clover was assessed by a score (between 1 and 10) being assigned to each dish with 10 being best and 1 being poorest growth. A score of 0 was assigned to dishes where germination failed to occur.

Both cultivars of leachate were inhibitory to early growth, with Maluka being more inhibitory than Jumbuck (P< 0.05).

Table 5.8. Effect of cultivar of residue leachate on early growth of clover.

<table>
<thead>
<tr>
<th>Plant growth score (1-10)</th>
<th>Control</th>
<th>Jumbuck</th>
<th>Maluka</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.0</td>
<td>5.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

LSD (P<0.05) = 2.6
As dilution of leachate was strengthened, clover growth was reduced by a highly significant level (P< 0.01) (Figure E5.6).

**Figure E5.6. The effect of rapeseed residue leachate on early growth of clover**

Means sharing the same letter are not significantly different (P<0.01).

The interaction between leachate cultivar and dilution was also significant, indicating different dilution effects between the two cultivars. Maluka leachate was more inhibitory at weaker dilutions, with Jumbuck more variable and less inhibitory at similar dilutions. Figure 5.7, below.

**Figure E5.7. The effect of cultivar of rapeseed residue leachate on early growth of clover**
D) Ryegrass root length.

No significant effect of leachate cultivar was observed, however as with % germination, Maluka showed a slight (N.S.) depression in growth. Again, while not significant, Jumbuck leachate was slightly stimulatory when maned over all dilutions.

Root length was affected by different dilutions of leachate with stimulation of radicle length at intermediate dilutions and severe inhibition at the most concentrated, as shown in Figure E5.8, below.

**Figure E5.8. The effect of rapeseed residue leachate on root length of ryegrass**

Means sharing the same letter are not significantly different (P<0.01).
As shown in Figure E5.9, below, an interaction effect between cultivar and dilution was present.

**Figure E5.9.** The effect of rapeseed residue leachate on root length of ryegrass

![Graph showing the effect of rapeseed residue leachate on root length of ryegrass](image)

LSD (P < 0.01) between dilutions = 18.1mm, between cultivars = 25.1mm

There was a difference between the two cultivars at a number of dilutions such that Maluka was generally less active than Jumbuck (either stimulatory or inhibitory) except at the most concentrated dilutions where it was clearly more inhibitory. At the weakest dilution, where Jumbuck was stimulatory Maluka was inhibitory, and, at stronger dilutions, where Jumbuck was less stimulatory and becoming inhibitory Maluka was more stimulatory. At the most concentrated dilution Maluka was extremely inhibitory with germination prevented, while Jumbuck was less so.

3. DIFFERENCES BETWEEN CULTIVARS OF RESIDUE LEACHATES:

With all indicator species Maluka residue leachate showed depression of germination to a greater extent than Jumbuck leachate. The results recorded were more easily able to be compared by converting the results to germination depression as a % of control. These results are presented in Table E5.10 over:
Table E5.10. Radicle / root length or growth score as % of control

<table>
<thead>
<tr>
<th>Test species</th>
<th>Germination %</th>
<th>Radicle length / Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jumbuck</td>
<td>Maluka</td>
</tr>
<tr>
<td></td>
<td>Jumbuck</td>
<td>Maluka</td>
</tr>
<tr>
<td>Wheat</td>
<td>98</td>
<td>93</td>
</tr>
<tr>
<td>Linseed</td>
<td>85</td>
<td>72</td>
</tr>
<tr>
<td>Clover</td>
<td>71</td>
<td>64</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>86</td>
<td>75</td>
</tr>
</tbody>
</table>

DISCUSSION

1) Concentration of Leachate.

Wheat germination was seen not to be significantly inhibited by concentrating leachate dilution, however, the trend was to reduced germination as concentration was increased. Maluka leachate was more inhibitory than Jumbuck, such that Maluka at 100mg/ml reduced germination to 55% of that of control, while at 100mg/ml Jumbuck leachate reduced germination by only 6% compared to control.

Clover germination showed a constant reduction in germination as leachate dilution was strengthened. Dilutions of leachate of 0.1mg/ml, and stronger, produced significant inhibition. Maluka, again, was the most inhibitory source of leachate with greater activity at every dilution.

Linseed germination showed an interesting pattern. When both cultivars were meaned, a depression of germination at very weak dilutions was evident, then no effect on germination was seen at intermediate dilutions, before severe inhibition at the strong dilutions. When the effect of cultivar was examined in this response, linseed germination was seen to be affected primarily by Maluka leachate. The pattern of germination inhibition occurring at weak (0.001mg/ml, 0.01mg/ml and 0.1mg/ml) dilutions, then no effect at 1mg/ml and 10mg/ml followed by complete prevention of germination at the most concentrated (100mg/ml). These effects were noted with linseed germination as measured by either germination % or radicle length.

Ryegrass germination showed similar patterns to that of linseed. Depression of germination was seen at very weakest dilution (0.001mg/ml), no effect at 0.01 and 0.1mg/ml and inhibition at 1mg/ml and
stronger. Radicle length measurements showed no inhibition at the very weak dilutions, but showed some stimulation at intermediate levels.

This effect of weak and strong dilutions being inhibitory with intermediate levels being ineffective is curious. It may be that very small amounts of compounds present are able to exert some effects on some processes occurring during germination which may be producing subtle changes in the complex germination process with the result of inhibited germination. Similar effects have been noted with very low concentrations of some herbicides (Duke 1985). At higher concentrations these compounds may be present in amounts able to be 'detected' by the plant and excluded or allowing defence mechanisms to operate. At very high concentrations they may simply be present in such large amounts to overcome any defence or may inhibit a large number of growth processes on a large scale with the resultant severe inhibition.

A possible hypothesis for these effects is that very low concentrations are able to enter cell or organelle membranes, for example, mitochondrial membranes (Balke 1985), possibly being mistaken for other closely related compounds. These compounds may then be able to very subtly produce disruption to some crucial function or pathway by substituting for some other closely related compounds (for example, enzyme or hormone). Disruption of membranes followed by interference with essential intracellular functions has been documented (Lorber and Muller 1976, McLure et al. 1978, Lovett 1982, Balke 1985, Moreland and Novitsky 1987) with membrane disruption being a possible common first site of activity leading to other effects which result in the inhibition to germination and growth.
At higher concentrations the plant or plant cell may be triggered to the compounds presence and be able to activate suitable defences with the result that inhibition does not eventuate. At the highest concentrations it may be that too much of the compound is present and gross effects (biochemical and/or physiological) lead to strong inhibition. That is,

very low concentration: entry gained, plant absorbs or 'sees compound as normal', subtle effects, effects a number of pathways via substitution or other minor effects, result is inhibition,

intermediate concentration: plant 'recognises' compound as present, mounts defence mechanisms (either biochemical or physiological), germination proceeds as normal,

very high concentration: compounds present in very high amounts, gross effects lead to failure of germination processes (biochemical or physiological).

If the compounds responsible closely resemble some natural compounds (for example, auxins) a number of hypotheses is possible.

a) At very low concentrations they may be able to substitute for the natural compound. The allelochemical may be 'mistaken' as a natural compound and may then produce germination inhibition via subtle effects on basic reactions or pathways, as discussed above. When concentrations rise to higher levels the plant may able to compensate for their presence by utilising some defensive mechanisms and respond to the presence of the 'stress' with compensatory growth. The frequently seen stimulation at low concentrations of allelochemicals (for example, Lovett and Duffield 1981) may be a reflection of this compensatory response to the presence of stressing compounds to which the plant is able to recognise and respond.

b) The allelochemical may be able to substitute for the natural compound, however, being a slightly different compound is of lower efficiency in carrying out the growth regulatory functions of the natural compound. As such, the plant cannot germinate as well as in the absence of the allelochemical. At intermediate concentrations the allelochemical, although being less efficient at regulating growth, may be present in a sufficient quantity for the normal germination and growth to take place. At very high
levels the allelochemical may simply produce an overdose response with uncontrolled or overstimulated growth leading to collapse.

The allelochemicals present in the leachates are likely to be indole glucosinolate derivatives, for example, indoleacetylithin (IAN) (Cole 1976, McDannell et al. 1987). The breakdown products of indole glucosinolates, including IAN are known to have auxin-like activity (Mahadevan and Stowe 1972, Slominsky and Campbell 1989b), although IAN has been demonstrated as of lower activity than IAA (Reynolds 1989). Thus, if IAN is one of the allelochemicals present in the leachates the above hypothesis may be possible. IAN present in the weaker dilutions of leachates may represent a stress to the germinating plants and may also gain entry to active sites as easily as IAA. Normal production or activity of IAA may be disrupted by the presence of IAN, leading to inhibition of germination. At higher (intermediate) concentrations the IAN may be able to supply a similar effect to normal levels of IAA, or the plant may be able to compensate for the presence of the stress. High concentrations of IAN may simply overwhelm the plant.

It is also possible that a number of compounds is present and may, together, be responsible for the effects seen (Rasmussen and Einhellig 1977, Duke et al. 1983, Mandava 1985). These compounds may operate via a number of mechanisms including those hypothesised above. The mode of action of allelochemicals may be complex and active at a number of sites and on a number of processes (Benoit and Starkey 1968b, Lorber and Muller 1976, Balke 1985, Lovett and Potts 1987). These compounds, if similar to endogenous compounds may have other properties leading to their effectiveness (for example, different solubility of polarity) or be able to substitute for other crucial compounds in the cell (for example, enzymes). As such, their effects may be on a number of pathways including those responsible for production of other necessary compounds in the germinating plant cells.

The differing responses seen with concentration of leachate may be important in vivo since in soil germinating seeds are presented with a myriad of stressors. The presence of even very low levels of allelochemicals may provide enough additional inhibition in association with other stressors or compounds to produce large negative germination results (Stow and Osbourne 1980, Duke et al. 1983, Gussin and Lynch 1983). Einhellig (1987) discusses the role of plant stressors in increasing the effect of allelochemicals.
Some variable results were seen with ryegrass germination at weak dilutions of leachates, with the possibility that this indicator species is of different susceptibility than wheat, linseed and clover in its response to the compounds present. The responses of different germinating species may be expected to vary as their susceptibility to these compounds and other stressors would be different.

Variability in susceptibility to allelochemicals among affected species may help to explain the effect of very weak dilutions of leachate being inhibitory to only some of the test species used in this experiment. Purvis et al. (1985) showed different responses among test species to presence of crop residues, leading to the hypothesis that some weeds may have evolved very different responses to the presence of crop produced compounds. Different responses to allelochemicals would be likely as some plants may have evolved defense mechanisms to some compounds, similar to that hypothesised by Wadleigh and Yu (1988) with insect resistance to natural plant toxins.

2) Cultivar of residue leachate.

In all cases Maluka was more inhibitory than Jumbuck. In only a few cases was this statistically significant, however, a trend was evident, for example, with ryegrass Maluka was more inhibitory than Jumbuck especially at the strong dilutions. It would seem that Maluka in this experiment was a more potent source of inhibitory agents than Jumbuck.

Reasons for this may lie in:

a) Maluka may have a different makeup of endogenous compounds to that of Jumbuck with resulting different possible allelochemical production from these compounds. Differences in glucosinolate profiles in different species or cultivars of Brassicas have been reported by Heany and Fenwick (1980), Truscott, Minchington and Sang (1983), and Jimenez-Osornio and Gliessman (1987). With rapeseed cultivars this may be particularly the case since one of the selection criteria in breeding Maluka has been the reduction of (seed) levels of goitrogenic glucosinolates. Selection may have altered the levels or presence of glucosinolates in the whole plant with this change in compound profile having a resultant change in allelochemical production. In other studies (Fay and Duke 1977, Lovett 1986) it has been seen that later selected cultivars have reduced allelopathic ability. The increased allelopathic ability seen with Maluka in this case may be the result of changes in
glucosinolate profiles to the extent where selection against some glucosinolates may have led to
selection for others with these also being responsible for more active allelochemicals.

b) There may also be differences in the relative amounts of a number of compounds present such that,
for example, Jumbuck may have more of an allelochemical active at very low levels, but less of another
compound active at high concentrations. Similarly, Maluka may be lacking in a compound active at
very low levels but has more of those active at high concentrations. This was observed where
Jumbuck was more suppressive at the very weak dilutions and Maluka was more active when
leachates were used at the most concentrated dilutions.

c) Maluka, being a more recently selected cultivar, with possibly different growth characteristics may
possess a higher proportion of plant parts responsible for the production of allelochemicals. This may
be evident in larger or more productive foliage or a higher amount of seed producing organs (that is,
selection for high yield) with these alterations coinciding with greater production of any
allelochemicals in these plant parts. 3-Indolylmethyl glucosinolate is the dominant glucosinolate in
foliage of *Brassica* spp. and has been implicated as a precursor for active compounds in these plants,
formed upon breakdown (Van Etten 1982, Slominsky and Campbell 1989), and shown as allelopathic
(Reynolds 1989).

d) Maluka has been bred for low seed glucosinolate levels. It may be that this has been due to
Maluka plants not translocating glucosinolates to the seed, rather leaving these compounds in the
foliage of the plant. This may have led to a higher presence in general of glucosinolates in Maluka
residues.
EXPERIMENT 6

THE EFFECT OF SOME GLUCOSINOLATE BREAKDOWN PRODUCTS ON TEST SPECIES.

INTRODUCTION

Glucosinolate breakdown products have been discussed in the Literature Review earlier, with typical products including thiocyanates, isothiocyanates and nitriles, with these depending on the source glucosinolate and conditions at breakdown.

The products likely to be produced upon breakdown of glucosinolates have been described widely. The indole glucosinolates which are prevalent in the vegetative parts of Brassica spp. generally yield indole acetyl nitrile (IAN) (McDannell et al. 1987, Slominsky and Campbell 1989a) or indole acetyl methanol (IAN). Phenyl ethyl glucosinolate, prevalent in Brassica roots (Slominsky and Campbell 1989a), can break down to form phenyl ethyl amine (PEA).

Several workers have suggested that these breakdown products are the active agents in the allelopathic effects seen with Brassica spp. Reynolds (1989) listed a number of these as allelopathic to a number of species.

This experiment aimed to look at some of these possible candidate compounds as active in the allelopathic responses from Brassicas spp.

METHODS

This experiment was conducted at UNE in Armidale NSW in early 1991.

Three commercially available glucosinolate breakdown products were purchased (Sigma, Aldrich), namely indoleacetylnitrile (IAN), indoleacetylmethanol (IAM) and phenylethylamine (PEA). Both IAN and IAM are possible breakdown products of 3-Indolylmethyl glucosinolate (McDannel et al. 1981, Sang et al. 1984, Slominski and Campbell 1989b), depending upon conditions of breakdown. PEA is the common breakdown product of 2-phenylethyl glucosinolate (McLoed et al. 1981).
A dilution series was prepared from the compounds by dissolving quantities of each compound in distilled water. 5ml of methanol were used to dissolve IAN and IAM prior to adding to the required quantity of water. PEA was readily soluble in water. Six concentrations were prepared in a logarithmic series of $0$, $10^{-6} \text{M}$, $10^{-5} \text{M}$, $10^{-4} \text{M}$, $10^{-3} \text{M}$, $10^{-2} \text{M}$ concentrations.

Indicator species were prepared as in Experiment 5, with the same species and experimental procedure used. Measurements were made on day 5 of % germination and longest seminal root length for wheat and ryegrass, and radicle length for linseed. Sub clover radicle length showed wide variability and a 1 - 10 score was assigned to each dish.

Irrigation of dishes was as follows:

<table>
<thead>
<tr>
<th>Indicator species</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Linseed</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Clover</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

RESULTS

All results were statistically analysed using Analysis of Variance techniques, with LSD's, where shown, based on these analyses.

1. GERMINATION

A) Wheat germination.

There was a large difference in % germination between the different compounds used. IAN was clearly more inhibitory to germination of wheat. There was no difference between IAM and PEA in wheat germination when meaned over all concentrations used.

A strong interaction between concentration and compound was evident such that IAN was clearly the most potent germination inhibitor of those tested (Figure. E6.1). IAN was seen to be more inhibitory than IAM. IAN was inhibitory at all concentrations and especially at 1mM or stronger. PEA was inhibitory only at 10mM.
Figure E6.1 The effect of test compounds, and their concentration, on wheat germination

LSD (P<0.01) Between compounds = 16.3, between concentrations = 13.5

B) LINSEED GERMINATION

Linseed germination was inhibited significantly only by IAN. PEA was seen to have no overall effect on % germination while IAM showed a stimulatory effect on % germination (P<0.05) (Table E6.1). Means are of all concentration used with each test compound.

Table E6.1. The effect of test compounds on linseed germination %.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>IAN</th>
<th>IAM</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37.7b</td>
<td>28.3c</td>
<td>44.9a</td>
<td>31.5bc</td>
</tr>
</tbody>
</table>

LSD (P<0.05)= 6.9

Means sharing the same letter are not significantly different (P<0.05).

A significant interaction occurred between compound and concentration as shown in Figure E6.2. IAN, again, was seen to be more active at lower concentrations than the other test compounds. IAN was slightly stimulatory (not significant) at lower concentrations (0.001mM) than IAM. IAM was stimulatory at concentrations of 0.01mM to 1mM (P<0.05). PEA showed a variable result with no stimulation evident at any concentration. Similarly, IAN was more inhibitory at lower concentrations than the other test compounds. IAN was significantly (P<0.05) inhibitory to % germination at 1mM.
IAM was not inhibitory at any tested concentration and PEA were only inhibitory at concentrations of 10mM. Thus, it seems that IAN is a more active compound than IAM and PEA by a factor of 10 to 100 times.

**Figure E6.2 The effect of test compounds, and their concentration on linseed germination.**

% Germination  
0 0.001 0.01 0.1 1 10  
Concentration (mM)

C) CLOVER GERMINATION

All compounds significantly reduced % germination of clover. IAN showed more activity than the other compounds (Figure. 6.3, over). The results in this case were more variable with IAN showing some inhibition at the lowest concentration, then no significant effect before inhibition at 0.1mM and stronger. Again, IAN was significantly more active in general than the other test compounds, giving complete inhibition of germination at concentrations of 1mM and stronger while neither IAM nor PEA showed complete inhibition at even the highest concentration.
D) RYEGRASS GERMINATION

Ryegrass germination was affected in similar fashion as the other test species. The test compounds were all inhibitory to ryegrass germination. IAN again was more inhibitory than the other compounds with significant inhibition at 100µM and stronger. IAM and PEA were not inhibitory until they were applied at 10mM, as shown in Figure E6.4, over.
Germination of all test species was progressively decreased with increased concentration of all test compounds. All three compounds were able to inhibit germination with this increasing as concentration increased. Differences between the compounds were evident such that indoleacetylnitrile (IAN) showed a higher level of activity than indoleacetylmethanol (IAM) and Phenylethylamine (PEA). This can be illustrated by looking at the test species germination effects as compared to germination of the untreated control, as in summary Table E6.1.

Table E6.1 The effect of test compounds on germination of all indicator species.  
(mean of all concentrations) % of control.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IAN</th>
<th>IAM</th>
<th>PEA</th>
<th>LSD (P&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>63.7</td>
<td>88.7</td>
<td>90.9</td>
<td>12.5</td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>75.1</td>
<td>119.1</td>
<td>83.6</td>
<td>18.3</td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>41.6</td>
<td>64.6</td>
<td>68.2</td>
<td>22.7</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>62.8</td>
<td>78.9</td>
<td>81.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Mean</td>
<td>100</td>
<td>60.8</td>
<td>87.8</td>
<td>81.0</td>
<td>17.8</td>
</tr>
</tbody>
</table>
2. EFFECTS ON EARLY GROWTH

A) Wheat growth.

The different concentrations of the compounds showed a pattern of inhibition where as concentration increased so radicle length decreased (Figure E6.5). This was highly significant (P<0.01). Concentrations of 1mM and higher showed significant inhibition of growth as measured by radicle length.

![Figure E6.5 The effect of test compounds, and their concentration on root growth of wheat.](image)

A degree of interaction between concentration and type of compound was evident (P<0.05) as shown above. IAN was seen to be more inhibitory at lower concentrations than the other test compounds. IAN showed a significant level of reduction to root length at a concentration of 0.01mM or higher, while IAM and PEA were not inhibitory until a concentration of 10mM was used.

B) Linseed radicle length.

The three compounds showed different results as measured by radicle length, with IAN again the most inhibitory compound. Again, the effect of concentration of test compound showed some stimulation at the lowest concentration (P<0.01) and general inhibition at higher concentrations (above 0.01mM). The degree of inhibition increased as concentration increased, (Figure E6.6, over).
IAN was again more active than the other compounds at the same concentrations (Figure E6.6). IAN showed a low level of stimulation at the lowest concentration (0.001 mM) (P<0.1) and was strongly inhibitory at all concentrations above this, to the extent of preventing any radicle growth at 10 mM. Neither IAM nor PEA were stimulatory and were only inhibitory at the highest (10 mM) concentrations. PEA was also able to prevent any radicle growth at 10 mM. IAN shows a 10 to 100 times greater activity than the other compounds.

C) Clover early growth, as measured by growth score (1-10).

Clover radicle length showed wide variability and a growth score assessment was used.

There were significant differences between the test compounds used as measured by this means. IAN was seen as the most inhibitory compound in general, with IAM the next most inhibitory and PEA as the least active. The interaction between compound and concentration was such that IAN showed more activity than the other test compounds which were generally not greatly different from each other in activity (Figure E6.8, over). No stimulation at low concentration was noted with any compound.
Figure E6.8 The effect of test compound, and their concentration on the early growth of clover, as measured by growth score.

Growth Score (1-10) LSD (P<0.01) between concentrations = 2.26, between compounds = 2.30

B) Ryegrass early growth, measured by root growth.

All compounds were inhibitory to early growth of ryegrass (P<0.01). IAN was the most active in inhibiting ryegrass early growth, Figure E6.9, over. While concentrations of IAN at 10μM and above were significantly inhibitory IAM and PEA were not effective in influencing early growth below 1mM.
Table E6.3 presents a summary of the effects of the compounds on early growth of the test species. IAN was clearly the most active compounds in inhibiting growth of the test species.

Table E6.3  The effect of test compounds on the early growth of test species. (Mean of all concentrations).

<table>
<thead>
<tr>
<th>Radicle Length / Growth Score - % v's Control</th>
<th>Control</th>
<th>IAN</th>
<th>IAM</th>
<th>PEA</th>
<th>LSD (P&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>30.3</td>
<td>77.5</td>
<td>91.6</td>
<td>20.8</td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>59.1</td>
<td>92.3</td>
<td>79.5</td>
<td>19.1</td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>40.8</td>
<td>54.5</td>
<td>65.0</td>
<td>16.6</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>36.3</td>
<td>73.4</td>
<td>76.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Mean</td>
<td>100</td>
<td>41.6</td>
<td>74.4</td>
<td>78.2</td>
<td>16.1</td>
</tr>
</tbody>
</table>

3. COMPARISON OF TEST COMPOUNDS

IAN was clearly the most inhibitory of the compounds used in this experiment. Tables E6.4 and E6.5 (over) offer a means of comparison where concentrations of the compounds where strong inhibition was evident (that is, inhibition of 2 X LSD (P< 0.01).
Table E6.4. Comparison of effects of test compounds on % germination of test species.

<table>
<thead>
<tr>
<th>Species</th>
<th>IAN</th>
<th>IAM</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>1mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Linseed</td>
<td>0.1mM</td>
<td>10mM</td>
<td>-</td>
</tr>
<tr>
<td>Clover</td>
<td>0.1mM</td>
<td>10mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>1mM</td>
<td>10mM</td>
<td>10mM</td>
</tr>
</tbody>
</table>

Table E6.5 Comparison of effects of test compounds on early growth of test species.

<table>
<thead>
<tr>
<th>Species</th>
<th>IAN</th>
<th>IAM</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>0.01mM</td>
<td>10mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Linseed</td>
<td>0.01mM</td>
<td>10mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Clover</td>
<td>0.1mM</td>
<td>0.1mM</td>
<td>1mM</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>0.01mM</td>
<td>10mM</td>
<td>10mM</td>
</tr>
</tbody>
</table>

IAN showed similar inhibitory activity to the other compounds at concentrations generally up to 100 times lower than the other compounds.

4. COMPARISONS OF THE INDICATOR SPECIES

The four test species were unable to be statistically evaluated for relative susceptibility to the compounds. However, a comparison may be made by considering the germination performance of the species as compared to the Control treatment. This is presented for each compound below: (Tables E6.6 to E6.11).

Table E6.6 The effect of IAN on indicator species % germination (% versus control).

<table>
<thead>
<tr>
<th>Species</th>
<th>Control</th>
<th>1μM</th>
<th>10μM</th>
<th>100μM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>74.3</td>
<td>77.0</td>
<td>65.4</td>
<td>23.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>134.4</td>
<td>120.1</td>
<td>67.2</td>
<td>33.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>62.1</td>
<td>80.0</td>
<td>14.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>111.1</td>
<td>88.0</td>
<td>57.9</td>
<td>20.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table E6.7 The effect of IAN on early growth of indicator species
(root/radicle length/growth score), (percent versus control).

<table>
<thead>
<tr>
<th>Indoleacetylnitrile (IAN) concentration</th>
<th>Species</th>
<th>Control</th>
<th>1μM</th>
<th>10μM</th>
<th>100μM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>76.2</td>
<td>47.4</td>
<td>21.3</td>
<td>12.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>121.7</td>
<td>60.7</td>
<td>45.9</td>
<td>15.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>66.7</td>
<td>66.7</td>
<td>9.1</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>62.1</td>
<td>34.1</td>
<td>9.6</td>
<td>4.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

The results above show wheat and linseed to be less affected at any given concentration than ryegrass or sub clover. At the intermediate or higher concentrations the latter species were more inhibited than the former.

Table E6.8 The effect of IAM on % germination of indicator species, (percent versus control).

<table>
<thead>
<tr>
<th>Indoleacetylmethanol (IAM) Concentration</th>
<th>Species</th>
<th>Control</th>
<th>0.001mM</th>
<th>0.01mM</th>
<th>0.1mM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>103.0</td>
<td>100.0</td>
<td>99.9</td>
<td>100.0</td>
<td>58.0</td>
<td></td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>118.0</td>
<td>134.0</td>
<td>128.0</td>
<td>137.0</td>
<td>88.0</td>
<td></td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>102.0</td>
<td>65.0</td>
<td>43.0</td>
<td>46.0</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>77.0</td>
<td>100.0</td>
<td>100.0</td>
<td>82.0</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

Table E6.9 The effect of IAM on early growth of indicator species (root/radicle length/growth score), (percent versus control).

<table>
<thead>
<tr>
<th>Indoleacetylmethanol (IAM) Concentration</th>
<th>Species</th>
<th>Control</th>
<th>0.001mM</th>
<th>0.01mM</th>
<th>0.1mM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>86.0</td>
<td>92.3</td>
<td>96.0</td>
<td>85.0</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>106.0</td>
<td>115.0</td>
<td>89.0</td>
<td>93.0</td>
<td>26.2</td>
<td></td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>88.0</td>
<td>88.0</td>
<td>30.0</td>
<td>30.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>86.0</td>
<td>106.0</td>
<td>93.0</td>
<td>60.0</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

Table E6.10 The effect of PEA on % germination of indicator species, (percent v’s control).

<table>
<thead>
<tr>
<th>Phenylethylamine (PEA) concentration</th>
<th>Species</th>
<th>Control</th>
<th>0.001mM</th>
<th>0.01mM</th>
<th>0.1mM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>100.0</td>
<td>108.0</td>
<td>99.0</td>
<td>92.0</td>
<td>92.0</td>
<td></td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>67.0</td>
<td>127.0</td>
<td>127.0</td>
<td>83.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>104.0</td>
<td>56.0</td>
<td>88.0</td>
<td>60.0</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>109.0</td>
<td>76.0</td>
<td>92.0</td>
<td>85.8</td>
<td>38.0</td>
<td></td>
</tr>
</tbody>
</table>
Table E6.11 The effect of PEA on early growth of indicator species
(root/radicle length/growth score), (percent versus control)

<table>
<thead>
<tr>
<th>Species</th>
<th>Control</th>
<th>0.001mM</th>
<th>0.01mM</th>
<th>0.1mM</th>
<th>1mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100</td>
<td>100.0</td>
<td>105.0</td>
<td>114.0</td>
<td>102.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Linseed</td>
<td>100</td>
<td>110.0</td>
<td>107.0</td>
<td>110.0</td>
<td>72.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Clover</td>
<td>100</td>
<td>76.0</td>
<td>53.0</td>
<td>62.0</td>
<td>29.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>100</td>
<td>106.0</td>
<td>109.0</td>
<td>110.0</td>
<td>43.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The above tables indicate that, although IAN was similarly active on all species, the results for IAM and PEA show some possible differences between the test species.

With IAM, wheat and linseed seemed less affected than ryegrass with clover the most sensitive species to this compound. Wheat and linseed were, essentially, unaffected at concentrations below 10mM. Ryegrass showed reduced germination at 1mM, and clover was inhibited at concentration of 0.01mM and above.

With PEA wheat was also the least sensitive to this compound, being not affected until the highest concentration was used. Again, linseed and ryegrass showed a lower sensitivity than clover. Clover was inhibited from 0.01mM. Ryegrass and linseed were, essentially, not affected until 1mM where a slight effect was noted. At 10mM strong inhibition occurred.

DISCUSSION

1) GERMINATION EFFECTS

All compounds were seen to affect germination of indicator species, especially at the highest concentrations.

In a number of cases, however, variable results were seen, especially at the lowest concentrations. For example wheat germination was inhibited at 0.001mM concentration of the compounds, with 0.01mM having no effect and all stronger concentrations being markedly inhibitory. This was seen to be occurring mainly with IAN (Figure E6.1).
Linseed germination showed stimulation at low concentrations of all compounds, with an interesting effect with IAN, being the more active compound, being stimulatory at lower concentrations than the other compounds. This may be indicative of a general trend with allelochemicals such that at a given level of activity (the concentration of which may vary with degree of activity of the particular compound) low doses show stimulation while high doses become inhibitory. This pattern has been reported by, for example, Lovett and Duffield (1981).

IAN was a far more active inhibitor of germination and early growth than IAM or PEA which were of generally equivalent activity. As such, IAN showed some stimulation at the lowest concentrations in some instances, but at lower concentrations than the other test compounds (Figures. E6.2 and E6.4 for example).

Hypotheses for this stimulatory affect at low compound concentrations have been advanced, for example:

- some compounds (for example IAN) are structurally similar to natural plant growth regulatory compounds, for example IAA (Anderson and Muir 1969), with low concentrations of these compounds causing stimulation due to the compound having some growth stimulation effect similar to what may occur were additional growth regulator present (Butcher, El Tigani and Ingram 1974, Reynolds 1989). Differences in effects of the test compounds and between the indicator species noted in this work may be due to the test compounds being more or less similar to natural growth regulatory compounds, and indicator species being more or less susceptible to their effects.

- the test species may show a stress response to the presence of low amounts of the compounds, such that some compensatory growth occurs.

B) EARLY GROWTH

In many instances low concentrations of the compounds showed stimulation, similar to that noted in some cases with germination responses. Figure E6.5 shows this where PEA was seen as stimulatory, and Figure E6.6 where linseed growth was stimulated by low concentrations of all compounds. These
observations are similar to those of Lovett and Duffield (1981), and hypotheses have been advanced (above) concerning these effects.

A notable effect seen with some compounds (IAN and PEA in particular) was that the lowest concentrations used (eg. 0.001mM) at times was inhibitory, with higher concentrations (eg. 0.01mM and 0.1mM) being stimulatory. Higher concentrations were then inhibitory. This response was generally not seen with IAN. Figure E6.5 shows 0.001mM of IAM as more inhibitory than the next higher concentrations, and Figure E6.9 shows 0.001mM of this compound as inhibitory with no further inhibition being seen until a concentration of 1mM was used. In other instances a plateauing or slight reduction in the inhibitory pattern of effect was seen at the middle concentrations, for example Figure E6.5 (IAM and PEA), Figure E6.6 (IAM and PEA), Figure E6.7 (all compounds) and Figure E6.9 (IAM and PEA).

These results parallel results seen in Experiment 5 where very low leachate concentrations showed greater inhibition than mid range concentration with some indicator species. Highest concentrations were always seen as inhibitory in both experiments. It is possible that an effect may exist at very low concentrations as illustrated (Figure E6.10) over.

Possible hypotheses for this effect have been canvassed in experiment 5. Since compounds known to be formed from indole glucosinolate breakdown are similar to auxins (Cole 1976, McDannell et al. 1987) and have been demonstrated to have auxin like activity (Mahadevan and Stowe 1972, Slominski and Campbell 1989b, Reynolds 1989), it is likely that very small amounts of compounds similar to those used in this experiment may be causing the subtle changes discussed in experiment 5, leading to inhibitory responses by plants at these concentrations. These effects may be due to the compounds being able to substitute for the natural compound but act in a less efficient manner resulting in poorer growth. The compounds, if similar to natural growth regulatory substances or pathway intermediates, may be able to enter the site of activity of the natural compound but then disrupt the pathway, membrane, or synthetic activity in question. It is possible that compounds which are very similar to important natural substances may be able to cause very subtle changes, disruptions or reaction effects at the micro level which result in more dramatic responses seen at the macro level. Subtle intracellular effects of compounds has been postulated as a basic means of much

C) COMPARISON OF TEST COMPOUNDS

IAN was seen to be 10 to 100 times more active than the other compounds, which were generally of equal activity. This was evidenced by both germination and growth of all indicator species. If IAN is the major breakdown product of indole glucosinolates present in Brassica residues (McDannell et al. 1987,) then it may be that this compound is active in allelopathic responses seen with residues or leachates from these plants (Jessop and Stewart 1983, Mason-Sedun et al. 1986, Jimenez-Osornio and Glissman 1987).

IAN is similar to IAA (Thimann 1964, Skytt-Anderson and Muir 1969, Rekoslavkaya et al. 1988, Slominski and Campbell 1989b), with auxin like activity being reported (Ballin 1962, Kavanagh et al. 1969, Butcher et al. 1974). Results of this work show IAN as a potent inhibitor of germination and early growth, with this possibly being due to similar modes of action. Both IAA and IAN have shown similar inhibitory effects previously (Reynolds 1989).
IAM and PEA were also seen as inhibitory, though less so than IAN. These compounds while having some activity may be less able to enter cells or intra cellular organelles, thus being less able to cause disruption. Similarly, they may simply be less active at inhibiting growth processes.

Further work could be directed at testing IAN and IAA as test compounds, for comparison of activity. Similarly, residue leachates (for example, those used in experiment 5) could be analysed for presence of IAN, IAM and PEA, and also for IAA.

D) DIFFERENCES IN RESPONSE BETWEEN INDICATOR SPECIES

Clover and ryegrass were, generally, the most sensitive species to the test compounds, with wheat and linseed being less affected. This contrasts to reports (Lovett and Sagar, 1978) where linseed has been used as a sensitive indicator plant. It may be expected that where plants have evolved a close associations, for example wild oats and wheat, the plants may be less susceptible to the presence of compounds produced from the other plant (Purvis et al. 1985). This may be expected where ryegrass, a common weed of many crops, may have been expected to be less susceptible to the compounds used, however, this was not seen in this experiment. Ryegrass may not have evolved in association with the compounds used in this work.

It is also interesting that wheat was less inhibited than most of the other indicator species used. Wheat grows well following canola crops in Southern Australia, and it may be that wheat is less sensitive to any allelochemicals produced from this crop, with this helping explain the results seen here. Brassica weeds having grown in association with wheat over generations may also have led to wheat being less sensitive to these compounds.

The differential sensitivities of the indicator species to the compounds used may have implications in cropping systems where these plants are likely to be grown in rotation (either as crops or weeds or pasture) with canola, specially if the compounds are produced from canola residues. It may be that compounds produced from canola residues are present in soil at concentrations low enough to have little or no affect on following species. This would depend on the relative sensitivity of the following plants. Thus, wheat may essentially not be affected by the compounds present at any concentration, while clover may be suppressed by this concentration. If sub. clover is a more sensitive plant to these
compounds, this may have implications where sub. clover based pastures are intended to follow a crop of canola, or intended to be undersown in a wheat crop following canola.

These effects may be accentuated where a variety of factors operate in concert in soil as plants are germinating. This would be expected to happen in vivo where a number of compounds and other stress factors may be present (Einhellig 1987). These may include a number of compounds (for example, allelochemicals) (Duke et al. 1983, Mandava 1985), environmental factors (moisture, nutrition status), disease organisms, insects and physical factors (for example, sowing depth). Many workers have documented interactions with environmental factors and diseases (Glass 1976, Stow and Osbourne 1980, Einhellig and Schon 1982, Schon and Einhellig 1982, Duke et al. 1983, Einhellig et al. 1985). Any compounds added from residue breakdown, for example, IAN, may simply be another stress present when plants (crops, weeds, pastures) are germinating. Any affects identified with the compounds used in this work in vitro may be significantly amplified if these compounds are present in vivo in association with any other influences on germination.

An analysis of canola residue leachate (used in experiment 5) for the 3 compounds discussed (IAN, IAM, PEA) was carried out using High Performance Liquid Chromatography techniques (HPLC) in an attempt to determine the presence or otherwise of these compounds in the leachates. It would be of interest to know if these compounds were present in the canola residue leachates used in Experiment 5, in that this may help explain the inhibition seen with these leachates.

These results are presented in Experiment 7.
EXPERIMENT 7

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF TEST COMPOUNDS AND LEACHATES

INTRODUCTION

The leachates obtained from rapeseed residues used in Experiment 5 and the test compounds prepared for Experiment 6 were retained for analysis. HPLC was used for qualitative analysis of these solutions in order to investigate the presence of the compounds used in Experiment 6 in the leachates used in Experiment 5.

The three compounds used in Experiment 6; Indoleacetylnitrile (IAN), Indoleacetylmethanol (IAM) and Phenylethylamine (PEA) were possible components in the leachates produced from rapeseed residues in experiment 5 since these compounds are likely breakdown products of glucosinolates prevalent in rapeseed plants (Gmelin and Virtan 1962, Josefson 1970, Mahadevan and Stowe 1972, McDannell et al. 1987, Slominski and Campbell 1989b).

METHODS

The analysis of compounds and leachates was carried at the University of New England in Armidale NSW in early 1991.

The solutions used in Experiment 6 and the leachates used in Experiment 5 were retained for analysis. Leachates were filtered through a millipore filter (0.45μM).

HPLC analysis was carried out using a Waters System consisting of an M41 pump, U6K injector and a UV spectrophotometer. Data were recorded using a 74 Waters Data Module. Injection volume was 0.05 ml, with single injection used per sample. A mobile phase of 60:40 phosphate buffer: acetylnitrile was employed at a flow rate of 2.0ml/minute. A wavelength of 221nM was found to be suitable for best detection of the compounds. Identification of unknowns was made by retention time of unknowns compared to the standards.

RESULTS AND DISCUSSION
1. Indoleacetylnitrile (IAN): At concentration of 0.01mM IAN produced a sharp and distinct peak at retention time of 4.76 minutes and 4.78 minutes, (Figure E7.1.)

![Figure E7.1. HPLC Analysis of IAN at 0.01mM concentration.](image)

2. Indoleacetylmethanol (IAM): At 0.01mM IAM produced distinct peaks at times of 2.43 and 2.42 minutes, (Figure E7.2)

![Figure E7.2. HPLC Analysis of IAM at 0.01mM concentration.](image)
3. Phenylethylamine (PEA): At 0.01mM PEA produced much reduced and less distinct peaks compared to the previous compounds. Small peaks were evident at retention times of 2.99 and 3.01 minutes. (Figure E7.3).

![Figure E7.3. HPLC Analysis of PEA at 0.01mM concentration.](image)

4. A composite sample was prepared of all compounds at 0.01mM. This produced three separate peaks corresponding to those seen with the three compounds noted above. Peaks were at 2.44 and 2.42 minutes, corresponding to that for IAM, 3.04 and 3.01 minutes corresponding to that for PEA, and 4.81 and 4.77 minutes corresponding to that for IAN. (Figure E7.4).

![Figure E7.4. HPLC Analysis of combination of compounds at 0.01mM concentration.](image)
When the leachates were analysed peaks could only be detected at leachate dilution of 10mg/ml.

5. Jumbuck residue leachate at 10mg/ml produced considerable peaks early in analysis. This indicates a number of low molecular weight compounds were present as may be expected with a residue leachate, possibly containing many simple organic compounds. Two peaks were detected at 2.51 and 4.71 minutes retention time. These were considered to correspond to IAM and IAN respectively (Figure E7.5). At a dilution of 1mg/ml a peak at 2.67 minutes was seen (Figure 6), this may have also been IAM but varies from earlier observations of this compound where peaks were seen at lower retention times (2.42, 2.43 mins).

![Figure E7.5. HPLC Analysis of Jumbuck Leachate at 10mg/ml dilution.](image)

![Figure E7.6. HPLC Analysis of Jumbuck Leachate at 1mg/ml dilution.](image)

The differences between Figure E7.5 and Figure E7.6 indicate the likelihood of a number of compounds co-eluting (Figure E7.6) leading to a later retention time for IAM in this sample. The results in Figure E7.5 show a block of early eluting compounds, with partially resolved peaks representing the compounds of interest. The partially resolved peak at 2.51 mins in Figure E7.5
may therefore be the same compounds shown as co-eluting with others at retention time of 2.67 mins in Figure E7.6. Sample purification could assist in gaining better definition of compounds.

6. Maluka at 10mg/ml also produced a large number of peaks, forming a block, especially at early retention times with many of these running into each other. Partially resolved peaks at 2.49, 2.96 and 4.74 minutes were identified, (Figure E7.7), which were considered to correspond to IAM, PEA and IAN respectively. When Maluka leachate at 1mg/ml was tested no conclusive results were obtained.

![Figure E7.7. HPLC Analysis of Maluka Leachate at 10mg/ml dilution.](image)

7. A final analysis of the test compounds was undertaken to check the retention times of the known compounds, ie. IAN, IAM and PEA. Figure E7.8 shows the three compounds as producing peaks as per the previous analyses, 2.43 minutes for IAM, 3.04 for PEA and 4.78 for IAN.

![Figure E7.8: HPLC Analysis of Combination of test compounds at 0.01mM concentration.](image)

From the HPLC results Jumbuck residues seem to have produced a similar profile of compounds to that of Maluka, with differences in the relative quantities. Jumbuck leachate was seen to contain
observable amounts of IAM and IAN with a smaller unresolved peak close to that seen for PEA in the Maluka leachate (Figure E7.5, versus Figure E7.6.) Maluka residue leachate was seen to contain quantities of all three compounds IAM, IAN and PEA. It is likely that both leachates contained similar profiles of compounds with Jumbuck showing smaller quantities of some, notably PEA.

Jumbuck leachate at dilution of 10mg/ml produced peaks of IAM of similar size to that produced from IAM at 10μM to 100μM, and IAN of similar size to that produced from the compound at 1μM to 10μM concentrations.

Maluka leachate at the same dilution produced peaks of similar sizes to those from IAM and PEA at 10μM and IAN at 1μM.

Thus, from examination of the HPLC results of the compounds and leachates, some possible conclusions may be drawn:

1. Maluka differs from Jumbuck residues by showing resolved peaks corresponding to the presence of all three test compounds at 1 mg/ml. Jumbuck leachate of this dilution did not show a resolved peak corresponding to the presence of PEA. This compound did not produce a large peak in any analysis and may have been present in Jumbuck residues but at lower levels than that in Maluka such that no clearly resolved peak was produced. The result, however, indicates some difference in compound makeup of the residues between the two cultivars. The glucosinolate content of different cultivars and species of Brassicas varies (Truscott, et al. 1983), and has been discussed as important in different allelopathic responses seen (Jimenez-Osornio and Gleissman 1987). Jumbuck is an older cultivar than Maluka, and breeding for newer cultivars may have altered the relative amounts of compounds present in the residues of the plants. Several possibilities exist to explain these differences as has been hypothesised in experiment 5.

2. An estimate of the levels of compounds present in rapeseed residues is not possible with the degree of resolution of peaks in the HPLC analysis. Comparisons of the sizes of the peaks, however, allows some conclusions to be drawn.
The level of IAN present in the leachates was generally indicated at concentrations approximately 10 times less than that of IAM and PEA.

This may be an important result since, in Experiment 6, IAN was seen to be 10 to 100 times more active than the other compounds in inhibiting germination and growth. Residues may contain levels of the compounds such that approximately similar levels of inhibitory activity from each compound may result from the leachate. In this scenario residues may produce leachates with different levels of individual compounds but the combined levels are adequate to produce similar effects on germinating plants. Differing activities between cultivars or species may be due to different compound profiles rather than the amounts present. A similar total amount of compounds may be present with different activities due to different relative amounts of the compounds within this total.

3. Calculations from the relative amounts of compounds estimated from the HPLC analysis may give indications of content of the compounds in Canola residues on a dry weight basis. From the results, where leachate dilutions of 10mg/ml produced similar peaks to those from compounds at concentrations of 0.01mM, it is possible to extrapolate that residues may contain levels of the test compounds of 0.1mM/Kg dry weight. Truscott et al. (1983) reported levels of indole glucosinolates of 5 to 9mg/20g of cabbage leaf. These levels correspond to 0.1mM/Kg of these compounds which agree substantially with estimates in this work.

A definite, quantitative analysis is a future requirement to further elucidate these findings.
COMPARISON OF EFFECTS OF LEACHATES AND TEST COMPOUNDS ON GERMINATION AND GROWTH OF INDICATOR SPECIES

It is possible to compare the relative effects of leachates and compounds on germination of each test species. This is done by examining the germination data as compared to untreated. Using these comparisons it is possible to examine the relative germination effects of all dilutions of leachates and concentrations of compound. Thus, where similar germination effects were evident it may be indicative of similar levels of compound between leachate and compound. Where a particular dilution of leachate produces a similar effect as that for a particular concentration of a compound, this may indicate similar amounts of this compound in these (possibly different) dilution of leachate and concentration of compound. These data are discussed below, and shown in Figures E7.9 to E7.16.

1. WHEAT GERMINATION AND GROWTH - BOTH LEACHATE AND COMPOUND EFFECTS.

Figure E7.9. Wheat germination as affected by compounds and leachates.
Wheat germination and early growth, shows IAN as the most powerful germination inhibitor of all treatments, Figures E7.9 and E7.10. IAM and PEA were of similar, though weaker, effect. When comparisons of compound and leachate effects are made the leachates are seen to have had similar effects on germination at levels up to 10 times that of PEA and IAM and 100 times or more that of IAN. Thus, a general comparison suggests, for example, that leachate dilution of 10 to 100mg/ml contain levels of PEA and IAM of about 1 to 10mM and .1 to 1mM of IAN. These comparisons agree, substantially, with the levels estimated from HPLC analysis of the leachates and compounds.

HPLC analysis suggested concentrations of compounds in the range of 0.1mM/Kg dry weight for IAN and 1mM to 10mM/Kg dry weight for the other compounds. The comparisons of germination effects, shown above, suggest similar level comparisons may be made on this basis. Thus, with wheat, leachate dilutions of 1mg/ml to 100mg/ml produced results similar to those from .01mM of IAN. This equates to a concentration of IAN of 100µM per Kg dry weight of residue for IAN. Levels of IAM and PEA equate to 10mM per Kg. These agree, generally, with calculations based on HPLC analysis.
2. LINSEED GERMINATION AND GROWTH EFFECTS - BOTH LEACHATE AND COMPOUNDS.

Linseed results of the comparison between compound and leachate are less easily made as the results with leachates were more variable with linseed than other indicator species (Figures E7.11 and E7.12). However, an examination of radicle length results suggests that residues contained levels of approximately 1mM to 10mM / Kg dry weight IAN and 100mM /Kg dry weight IAM and PEA.

Figure E7.11. Linseed germination as affected by compounds and leachates.
3. CLOVER GERMINATION AND EARLY GROWTH - EFFECTS OF BOTH LEACHATE AND COMPOUND.

With both germination and early growth of clover, leachates gave results relative to those from the compounds such that 0.1mg/ml to 1mg/ml of leachate dilution was generally equivalent in effect to .01mM concentration compounds. In this instance there was not the 10 to 100 fold difference between IAN and the other two compounds, with all compounds being of a more equivalent effect, as shown in Figures E7.13 and E7.14, below.

The conversion of leachate dilution to compound equivalent for clover suggests that rapeseed residue contained approximately 10mM / Kg of IAN and a similar concentration of IAM and PEA.
Figure E7.13. Clover germination as affected by compounds and leachates.

Figure E7.14. Clover early growth score as affected by compounds and leachates.
4. RYEGRASS GERMINATION AND EARLY GROWTH - EFFECTS OF LEACHATE AND COMPOUND.

Figure E7.15. Ryegrass germination as affected by compounds and leachates.

Figure E7.16. Ryegrass early growth as affected by compounds and leachates.

Ryegrass results for the comparison between leachates and compound show that leachate dilution of 100mg/ml gave generally similar germination effects to those seen with IAN at .01mM to 1mM; IAM at 1mM to 10mM, and PEA at 1mM. This converts to an estimated concentration in residues based on
activity comparison of 0.1mM to 10mM / Kg of IAN and 10mM/Kg to 100mM/Kg dry weight for both IAM and PEA (Figures E7.15 and E7.16, above).

A summary can be drawn up as follows. From the relative activity comparisons between leachate and compound based on the data from all test species an estimate of possible compound concentrations in residues may be attempted. These are expressed on a dry weight basis.

Wheat results indicate: 100µM/Kg of IAN
10mM/Kg of IAM, PEA

Linseed results indicate: 1mM - 10mM/Kg of IAN
100mM/Kg of IAM, PEA

Clover results indicate: 10mM/Kg of IAN, IAM, PEA

Ryegrass results indicate: 100µM - 10mM/Kg of IAN
10mM - 100mM/Kg of IAM, PEA

A general estimate may be made based on the above comparison derived levels of:
100µM to 1mM/Kg of IAN
10mM to 100mM/Kg of IAM and PEA.

HPLC derived estimate for the compounds were:
100µM/Kg for IAN
1mM to 10mM/Kg for IAM and PEA.

Thus, while not in total agreement, there is a degree of congruence in the various estimates.

The results presented and comparisons of the levels of compounds derived from the HPLC analysis of leachates may have some interesting implications for any allelopathic effects seen with rapeseed residues.

As noted earlier, IAN can be a significant breakdown product of 3-Indolylmethyl glucosinolate, a dominant glucosinolate of rapeseed plant tissue. IAN was seen to be an active compound in this work as a germination inhibitor. However, IAN was seen to be present in low levels compared to other breakdown products in leachates produced from rapeseed residues. IAM and PEA were present at levels (in general) 10 to 100 times the concentration of IAN. IAN was seen to be 10 to 100 times more
active than these compounds when tested as compound solutions. Thus, canola residues seem to have produced levels of breakdown products in different proportions to what may have been expected from the levels of glucosinolates present as precursors for these breakdown products. The relatively low level of IAN compared to IAM and PEA may be due to a number of factors:

3-Indolylmethyl glucosinolate may produce IAN or IAM, (or possibly the thiocyanate, or other subsequent breakdown products), depending upon conditions during breakdown (Mahadevan and Stowe 1972). It is possible that, under conditions of breakdown during production of the leachates IAM production may have been favoured or produced in greater quantities than IAN. Breakdown in vivo may produce different relative quantities, including more IAN, which may lead to more allelopathic activity than that indicated by the leachates used in this work. Alternatively, IAM may be produced in relative quantities similar to that produced by the leachates used here, with IAM playing a significant role in any allelopathic responses seen in vivo. Alternatively, both IAN and IAM may be produced and act together.

PEA was seen to be of similar activity to IAM as a compound and produced in similar quantities in leachates. 2-phenylethyl glucosinolate is the precursor for this product and is thought to be present in only small amounts in above-ground parts of rapeseed but in larger quantities in root systems (Sang et al. 1984). The residues used in production of the leachates in this work contained only minor amounts of root material and this may explain the relative low levels of PEA produced. It is also possible that PEA may play a minor role in any allelopathic responses seen with rapeseed / canola residues in relation to levels produced. Alternatively, this compound may be an important factor in interactions occurring in the soil since PEA may be more prevalent in soil where canola has been grown. It is possible that a number of compounds is produced as canola breaks down, including IAN and/or IAM from above ground parts and PEA from root systems. These may all play roles in any allelopathic responses seen. Interactions between these compounds, or with other compounds present in soil may lead to allelopathic responses (Glass 1976, Wallace and Whitehead 1980, Mandava 1985). Interactions with other stressors may also lead to greater allelopathic effects than may be due to the compounds alone (Einhellig 1987).
A further possibility exists which may help in accounting for the low amounts of IAN detected in leachate samples. IAN may be further broken down into other compounds not detected in the analysis. IAN can form Indoleacetic acid (IAA) (Thiman 1964) via the action of a nitrilase found in *Brassica* spp. (Mahadevan 1964, Skytt-Anderson and Muir, 1966). It is possible that IAN may have been produced from 3-Indolylmethyl glucosinolate in the residues used and subsequently IAA was produced from this. IAA can also inhibit germination, to levels greater than that seen with IAN (Reynolds 1989). It may be that IAA was also present in leachates and may have been active in inhibiting germination as reported.

It is possible that IAA, IAN and many other compounds are produced with residue breakdown in production of leachates in this work and also *in vivo*. IAA may be active in addition to IAN (and other compounds) and this may assist in explaining the strong activity noted with the leachates. IAA may be a significant factor in allelopathy concerning *Brassica* spp. in general and rapeseed in this instance. It is possible that a variety of Indole derivatives are produced from *Brassica* spp. breakdown, including IAA, IAN and IAM and that all these may have activity as allelopathic compounds.